JOURNAL OF VIROLOGY, Sept. 1992, p. 5509–5515
0022-538X/92/095509-07$02.00/0

Vol. 66, No. 9

A Cell-Free Recombination System for Site-Specific Integration of Multigenic Shuttle Plasmids into the Herpes Simplex Virus Type 1 Genome

PHILIP J. GAGE,[1] BRIAN SAUER,[2] MYRON LEVINE,[3] AND JOSEPH C. GLORIOSO[4]*

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620[1]; Experimental Station, E328, Dupont-Merck Pharmaceutical Company, Wilmington, Delaware 19880-0328[2]; Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48109-0618[3]; and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261[4]

Received 23 April 1992/Accepted 19 June 1992

This report describes a novel method for complementation studies of defective herpes simplex virus (HSV) genes. Viral test gene and nonviral reporter gene cassettes were rapidly integrated into the HSV genome in a site-specific and reversible manner by using the P1 phage-based Cre-*lox* recombination system. Shuttle plasmids contained a functional *lox*P recombination site, an expressible form of the bacterial *lacZ* gene, and a copy of the wild-type glycoprotein B (gB) gene or double mutant gB allele containing both a temperature-sensitive (*ts*) mutation and a syncytium (*syn*)-forming mutation. A recipient viral genome, KΔT::*lox*1, was constructed from the HSV type 1 (*syn*) gB-deficient mutant virus, KΔT, by marker transfer of the *lox*P recombination site into the viral thymidine kinase locus. Shuttle plasmids of up to 12.9 kb in length were recombined with high efficiency (11 to 20%) into the KΔT::*lox*1 genome in cell-free, Cre-mediated recombination reactions. Expression of a functional wild-type or double mutant gB polypeptide complemented the nonfunctional polypeptide expressed from the deleted, normal gB locus and allowed production of either wild-type or Syn⁻ plaques on Vero cells. The latter recombinant virus was also *ts* for growth. The ability to express viral genes from plasmids which can be shuttled into and out of the HSV genome in cell-free recombination reactions makes this a powerful method for performing genetic studies of the biologic properties of viral gene products.

Herpes simplex virus type 1 (HSV-1) belongs to a family of animal viruses typified by having large, complex DNA genomes (100 to 250 kb) (for a review, see reference 25). HSV-1 contains approximately 72 genes encoding proteins which may be classified as providing either essential functions for infectious-particle production in cell culture or accessory functions which contribute to viral pathogenesis and extend the virus host cell range during natural infections in vivo. Although the entire HSV genomic sequence is now known (21–23), the biological properties and roles in replication for many of these viral functions have not yet been elucidated.

The production and characterization of HSV mutants have provided the central approach to uncovering the functional roles of individual genes in carrying out different phases of the complex virus life cycle. The principal strategy has been to genetically alter cloned viral DNA segments and then study the affected biologic features of the mutant products either in transient transfection experiments or in infections after marker transfer of the mutant gene into the viral genome by homologous recombination. While transient assays can be useful for the initial characterization of mutant constructs, many experimental approaches require that mutant genes be transferred into the viral genome (3, 7, 9, 11, 12, 29) for analysis of their impact in complex settings such as infection of cell cultures or animals. Genetic studies which rely on marker transfer procedures are usually slowed by the requirement for prior characterization of the mutant construct and by a low yield of recombinants. As a consequence, this approach is rather laborious, particularly for experiments requiring the analysis of large panels of mutant constructs. Moreover, sequences transferred into the viral genome by homologous recombination are not directly recoverable but must be recloned prior to further characterization or manipulation. A more rapid method for introduction of mutant constructs into the HSV genome, which would permit the identification of relevant mutations that result in a particular phenotype, would greatly facilitate the genetic and biochemical characterization of these viral genes and their products.

Work by Sauer et al. (28) demonstrated that plasmid DNA could be efficiently inserted into a herpesvirus genome in an in vitro recombination reaction. By taking advantage of the Cre-*lox* site-specific recombination machinery of bacteriophage P1 (2, 16, 33), a plasmid containing the 34-bp *lox*P recombination site, but completely lacking in homologous herpesvirus sequences, could be specifically inserted at a *lox*P site engineered into the pseudorabies virus genome. The inserted plasmid disrupted the nonessential gIII glycoprotein gene. Recombination was efficient and could be accomplished in a cell-free reaction mixture consisting of only the viral and plasmid DNAs and the Cre recombinase. Transfection of the reaction mixture into suitable eukaryotic cells allowed for the rescue of infectious recombinant virus. Recombination efficiencies of at least 8% were reported, and the inserted plasmids were stably maintained in the viral genome. Furthermore, because the Cre recombination reaction is reversible, the inserted shuttle plasmid could be recovered from the recombinant viral genome by a Cre-

* Corresponding author.

mediated intramolecular recombination reaction and by propagation after transformation of *Escherichia coli*.

In this report, we describe how the Cre-*lox* shuttle vector strategy was adapted for complementation of an HSV-1 mutant with the essential glycoprotein B (gB) gene deleted. Wild-type and mutant copies of the gB gene were efficiently shuttled into and out of a gB$^-$ *lox*P recipient virus genome. Recombinant viruses were identified on the basis of their blue-plaque phenotype derived from expression of a *lacZ* reporter gene present within the inserted shuttle plasmid. Expression of functional gB polypeptides from the inserted plasmids complemented the gB$^-$ defect of the recipient virus. Insertion of a temperature-sensitive (*ts*) and *syn* double mutant gB allele conferred both mutant phenotypes on the recombinant virus. These studies provided evidence that the Cre-*lox* shuttle system will be useful for the genetic analysis of the roles of both viral genes as well as *cis*-acting genomic elements which function during virus replication.

MATERIALS AND METHODS

Cells and virus strains. Vero cells and the D6 cell line were maintained in Eagle's minimum essential medium (MEM) (GIBCO Laboratories, Gaithersburg, Md.) supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), the nonessential amino acids, and 5% newborn calf serum (GIBCO Laboratories). D6 cells, which were previously derived by transforming Vero cells with pKBXX (8) and pSV$_2$neo (31), express HSV gB-1 upon infection and complement the growth of gB$^-$ viruses (8). D6 cells were maintained in culture medium supplemented with 200 μg of the antibiotic G418 (GIBCO Laboratories) per ml. For transfection experiments, the cells were maintained in MEM supplemented with 5% fetal calf serum (GIBCO Laboratories) instead of newborn calf serum.

KΔT is a derivative of HSV-1 (strain KOS) carrying a 969-bp *Bst*EII deletion within the essential gB gene and expresses an internally deleted, nonfunctional gB polypeptide which is secreted into the medium (8). Both KΔT and the complementing cell line, D6, were kindly provided by S. Person. KΔT and KΔT::*lox*1 (described below) were propagated, and their titers on D6 cells were determined at 37°C. Cre-mediated recombinants derived from KΔT::*lox*1 were first isolated on D6 cells and then grown, and their titers on Vero cells were determined at 37°C. Recombinants containing the *ts* form of gB were tested for this phenotype by plaque assays on Vero cells at 34 and 39°C. All recombinant virus stocks were derived from plaque-purified isolates.

For plaque assays or individual plaque isolations, serial dilutions of virus stocks were inoculated onto confluent monolayers of the indicated cell line which were subsequently incubated under methylcellulose (0.5% methylcellulose in MEM supplemented with 10 mM HEPES and 2% newborn calf serum) at 37°C until plaques developed. The cultures were then stained either with crystal violet (1% crystal violet in 50% ethanol) or with Bluo-gal (halogenated indolyl-β-D-galactoside) for β-galactosidase (β-gal) activity (see below), and when appropriate, virus from individual blue plaques was isolated for subsequent purification.

Bacterial strains. Plasmids were isolated and propagated in *E. coli* strains DH5α (15) or GM119 (gift from D. Oxender). Plasmid-transformed bacteria were grown in Luria broth (10 g of tryptone per liter, 5 g of yeast extract per liter, 171 mM NaCl) supplemented with 75 μg of ampicillin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml or on L agar plates containing ampicillin.

Construction of plasmids. All cloning steps and plasmid propagations were performed by standard procedures (15, 17, 27). Enzymes were purchased from GIBCO-BRL, Inc., or Boehringer Mannheim Biochemicals and used as specified by the manufacturers. pLink760 (34), pON1 (32), pKBXX (8), and pBS64 and pBS65 (28) have all been described previously. pUCXI (19) contains the HSV-1 *Bam*HI P fragment containing the complete thymidine kinase (*tk*) locus subcloned from pX1 (18) into pUC9. pJG101 was constructed from pUCX1 by inserting a 354-bp *Nae*I-*Sma*I fragment containing a functional *lox*P site from pBS64 into the unique *Sna*BI site within the coding sequence for the *tk* gene. This 354-bp insertion occurs between residues 110 and 111 of the TK protein and was expected to abrogate TK activity. A 270-bp *Bam*HI-*Pvu*II fragment from pBS65, including a functional *lox*P site, was inserted into pBSM13$^-$ (Stratagene, La Jolla, Calif.) between the *Bam*HI and *Sma*I sites, creating pBS*lox*P. pIEP*lacZ* was constructed by subcloning a 763-bp *Bgl*II fragment from pLink760 into the unique *Bgl*II site of pON1, placing a promoterless cassette containing the bacterial *lacZ* gene under the control of the human cytomegalovirus immediate-early promoter (HCM VIEP). The HCMVIEP-*lacZ* gene cassette from pIEP*lacZ* was inserted at the unique *Bam*HI site of pBS*lox*P as a 4.7-kb *Bam*HI fragment, creating pJG103. Finally, to construct pJG108 (see Fig. 3), pJG103 was partially digested with *Bam*HI, the linear unit-length plasmid fragment was gel purified, and a 3.7-kb HSV-1 *Bam*HI-*Bcl*I fragment containing the entire gB-1 (strain KOS) gene from pKBXX was inserted in the orientation shown (see Fig. 3). pJG102 (see Fig. 3) was constructed in a similar fashion from pBS*lox*P by inserting (i) the *ts syn* gB-1 allele from the HSV-1 mutant *ts*B5 as a 4.9-kb *Kpn*I fragment (0.346 to 0.377 map units) at the pBS*lox*P *Kpn*I site and (ii) the 4.7-kb *Bam*HI HCM VIEP-*lacZ* gene cassette from pIEP*lacZ* at the pBS*lox*P *Bam*HI site.

Viral DNA isolation. Viral DNA was isolated by a modification of standard methods for high-molecular-weight eukaryotic DNA isolation (4, 14). Confluent Vero cell monolayers were infected at a multiplicity of infection of 5. At 24 h postinfection, virions free in the medium and released by freeze-thawing cells were combined and pelleted at 75,000 × *g* for 40 min. The virion pellet was resuspended in lysis solution (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.6% sodium dodecyl sulfate, and 0.25 mg of proteinase K per ml) and incubated at 37°C for 8 to 12 h. DNA was multiply extracted first with phenol-chloroform (1:1) and then with chloroform, precipitated with isopropanol, and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer.

Marker transfer of *lox*P into KΔT. The *lox*P site in pJG101 was marker transferred into the *tk* locus of the KΔT genome by homologous recombination after cotransfection of viral and plasmid DNAs into subconfluent D6 cells as described by Graham and van der Eb (13) and modified by Homa et al. (18). Because marker transfer of the *lox*P fragment would interrupt TK expression, transfection lysates were enriched for TK$^-$ viruses (24, 26) by serial propagation at low multiplicity of infection (<0.001) on D6 cells in MEM supplemented with 100 μg of thymine 1-β-D-arabinofuranoside (araT) (Sigma Chemicals, St. Louis, Mo.) per ml. The enriched TK$^-$ stock was used to inoculate D6 cell monolayers, individual plaques were isolated, and small virus stocks were grown on D6 cells in microtiter plates. Infected cell lysates were tested for the *lox*P insert by DNA dot blot assay with the *Nae*I-*Sma*I *lox*P fragment from pBS64 as probe. A recombinant virus, KΔT::*lox*1, containing the *lox*P insert,

was purified by repeated rounds of limiting dilution and dot blot analysis. The *lox*P insertion into the *tk* locus was confirmed by Southern blot analysis (see Fig. 2) (30). Nytran filters containing the electrophoretically separated *Eco*RI or *Bam*HI fragment from the indicated DNAs were first probed with a *tk* probe (2,416-bp *Eco*RI fragment from pUCXI) (see Fig. 3), stripped to remove the *tk* probe, and rehybridized with a *lox*P probe (350-bp *Nae*I-*Sma*I fragment from pBS64) (see Fig. 3). All probes used for dot blot and Southern analyses were labeled with a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemicals).

In vitro Cre-mediated recombination. *lox*P shuttle plasmids were recombined into the KΔT::*lox*1 genome in cell-free Cre reactions under conditions similar to those described by Abremski and Hoess (1) as modified by Sauer et al. (28). The DNA mixture was incubated at 30°C for 30 to 40 min in the presence of Cre buffer (50 mM Tris-HCl [pH 7.5], 33 mM NaCl, 10 mM $MgCl_2$) and 40 ng of purified Cre protein (NEN Dupont Inc., Wilmington, Del.). For intermolecular recombination, reaction mixtures included KΔT::*lox*1 viral DNA and a 20- to 50-fold excess of shuttle plasmid DNA, and polyvinyl alcohol was added to a final concentration of 1.7% to enhance intermolecular recombination (28, 35). Recombination reactions were terminated by heating samples to 65°C for 10 min, and reaction mixtures were transfected into D6 cells to produce infectious virus. When recovery of the shuttle plasmid from viral DNA was required, purified KΔT::108 viral DNA was incubated with Cre in the absence of polyvinyl alcohol and the recovered plasmid was used to transform DH5α by standard protocols (27).

Purification of Cre-mediated recombinant viruses. Recombinant viruses were identified on the basis of their blue-plaque phenotype and purified by plaque purification and limiting dilution. Transfection lysates were diluted and plated at low multiplicity of infection (<0.01) onto confluent D6 cell monolayers. Plaques were tested for *lacZ* activity with Bluo-gal (GIBCO-BRL), and viruses producing blue plaques were picked as an agarose plug and resuspended in phosphate-buffered saline (PBS). Individual plaque isolates were then cloned by limiting dilution on D6 cells in microtiter plates. Viruses were considered pure when 100% of the plaques stained blue through three successive rounds of limiting dilution.

Assays for *lacZ* activity. Two assays were used to detect *lacZ* activity in developed plaques. For isolation of viable virus, cultures were rinsed with PBS (10 mM sodium phosphate [pH 7.1], 136 mM NaCl), overlaid with 300 mg of Bluo-gal–0.5% low-melting-point agarose (GIBCO-BRL) per ml in MEM supplemented with 10 mM HEPES and 5% fetal calf serum, and incubated at 37°C until color developed. When isolation of viable virus was not required, infected cultures were fixed and stained by a histological method with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Boehringer Mannheim Biochemicals). Infected cultures were rinsed with PBS, fixed for 5 min at room temperature in 2% formaldehyde–0.3% glutaraldehyde in PBS, and incubated in a chromophore solution containing 0.1% X-Gal, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. Incubation continued at 37°C until color developed (usually after 30 to 60 min), and the monolayers were again rinsed with PBS.

RESULTS

Direct isolation of Cre-*lox*-derived recombinant viruses on the basis of β-gal reporter activity. The general strategy for using an HSV-1 shuttle vector system based on the bacteriophage P1 Cre-*lox* site-specific recombination machinery is shown in Fig. 1. The system consists of a recipient virus genome and a shuttle plasmid, each containing a functional *cis*-acting *lox*P recombination site, and the Cre recombinase protein. In a cell-free reaction, Cre-mediated recombination occurs directly between the two *lox*P sites (1) such that the entire plasmid is inserted into the recipient viral genome. Because the Cre reaction is reversible, the shuttle plasmid can also be recovered from the viral genome.

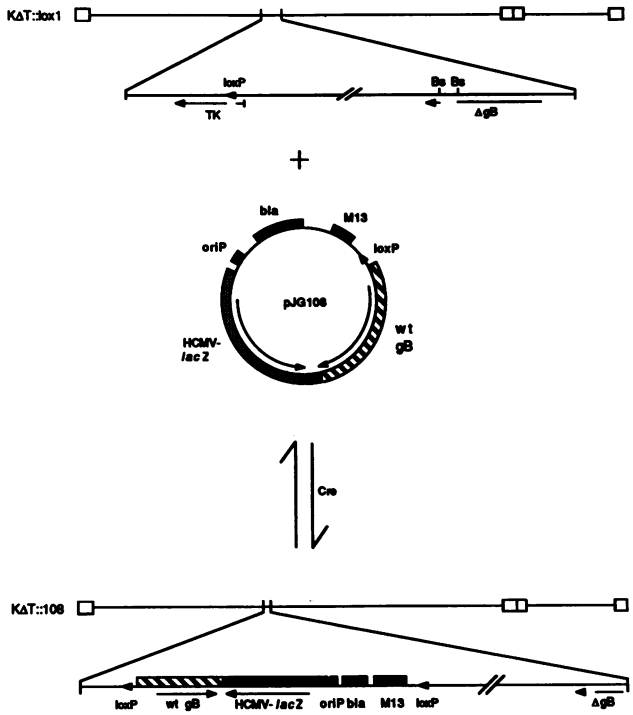

FIG. 1. HSV-based Cre-*lox* site-specific recombination system. The target virus for site-specific recombination, KΔT::*lox*1, was derived from KΔT, and its genome contained (i) a *cis*-acting site-specific recombination site *lox*P from bacteriophage P1 (arrowhead) inserted into the *tk* coding sequence and (ii) a defective gB allele (ΔgB) due to a 969-bp *Bst*EII (Bs) deletion in the gB coding sequence, including the transmembrane domain. In the example shown, the pJG108 shuttle plasmid was recombined into KΔT::*lox*1. pJG108 contained a *lox*P recombination site (arrow), an HCMVIEP-*lacZ* reporter gene cassette for identification of virus recombinants, and the wild-type gB allele from HSV-1 strain KOS. The polarities of both the viral and the plasmid *lox*P sites are shown. pJG108 was inserted into the KΔT::*lox*1 genome at the *tk* *lox*P site in a cell-free Cre-mediated recombination reaction, and infectious virus was produced by transfection of the reaction mixture into D6 cells (8). The recombinant KΔT::108 virus was diploid for the gB gene, containing both the deleted KΔT::*lox*1 allele (ΔgB) and the wild-type KOS allele (wt gB). KΔT::108 also yielded blue plaques in the presence of Bluo-gal or X-Gal because of the HCMVIEP-*lacZ* reporter gene.

A recipient target virus, KΔT::*lox*1, was derived by marker transfer of a functional *lox*P site into the *tk* locus of the gB^- HSV-1 mutant KΔT genome (8). Like KΔT, the KΔT::*lox*1 recipient virus contains a 969-bp *Bst*EII deletion within the essential gB gene and must be propagated on the gB-complementing D6 cell line (8). KΔT::*lox*1 genomic DNA was analyzed by Southern blot (30) to confirm the presence of the *lox*P site within the *tk* coding sequence (Fig. 2).

Shuttle plasmids containing a functional *lox*P site, a re-

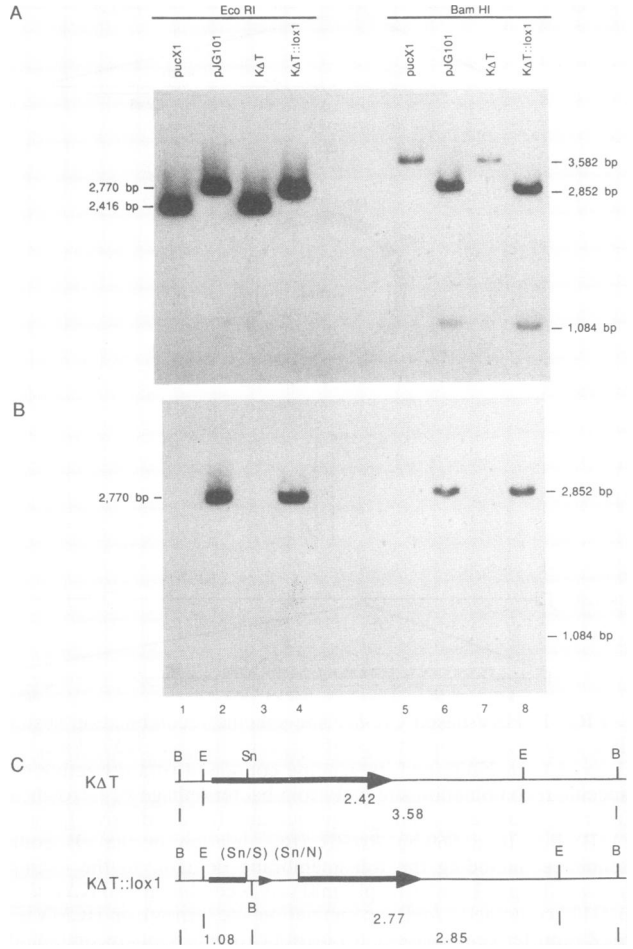

FIG. 2. Southern analysis of KΔT::*lox*1 viral DNA. Plasmid and viral DNAs were digested as indicated, electrophoresed in 1% agarose, and transferred to a Nytran filter (Schleicher & Schuell, Keene, N.H.). The filter was sequentially hybridized with ^{32}P-labeled probes to *tk* (2,416-bp *Eco*RI fragment from pUCX1) (A) and the *lox*P-containing fragment (364-bp *Nae*I-*Sma*I fragment from pBS64) (B). The *tk* probe was quantitatively removed prior to hybridization with the *lox*P probe. (C) Restriction maps of relevant regions. Enzymes used were *Bam*HI (B) and *Eco*RI (E). The positions of the *tk* gene (arrow) and the *tk Sna*BI (Sn) site used to clone the 354-bp *Sma*I (S)-*Nae*I (N) from pBS64 are also shown. Expected restriction fragment sizes in kilobases are also shown.

porter gene consisting of the bacterial *lacZ* gene under the transcriptional control of the strong HCMVIEP-enhancer element, and one of two different alleles of the HSV gB-1 gene were constructed from pBSM13$^-$ (Fig. 3). The reporter gene was included for use as a marker for identifying recombinant viruses containing the inserted shuttle plasmid. pJG108 contained the complete gB gene from the wild-type HSV-1 strain KOS. pJG102 contained the HSV-1 strain *ts*B5 mutant gB gene; the mutant protein product has been shown to be defective at 39°C and to induce polykaryocyte formation, or the syncytial (*syn*) phenotype, in infected cells at 34°C (20).

The pJG102 and pJG108 shuttle plasmids were independently introduced into the KΔT::*lox1* genome in a cell-free Cre reaction. Infectious virus was produced by transfection of the Cre reaction mixtures into the gB-complementing D6 cell line. After 2 days, transfection lysates were harvested

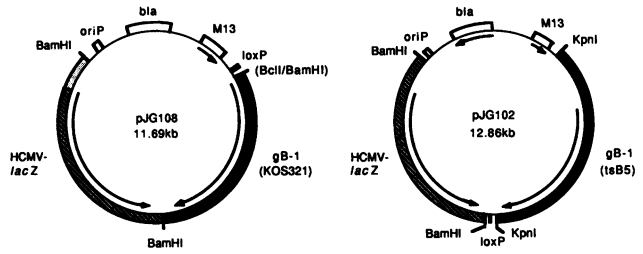

FIG. 3. pJG108 and pJG102 shuttle plasmids. Each plasmid was derived from pBS$^+$ and contains a *lox*P site-specific recombination site, an HCMVIEP-*lacZ* reporter gene, and an HSV-1 gB gene allele as the test gene. The gB genes of pJG108 and pJG102 are derived from HSV-1 strains KOS and *ts*B5, respectively. Restriction sites used to clone the respective fragments are indicated.

and replated on D6 cells and plaques were tested for β-gal activity. Virus from individual blue plaques from each experiment was isolated and subsequently cloned on D6 cells by multiple rounds of limiting dilutions. Viruses were considered pure when 100% of the isolates tested were β-gal$^+$ through three rounds of limiting dilutions. For each virus, insertion of the shuttle plasmid was confirmed by Southern blot analysis (data not shown). Recombinant viruses were named by combining the KΔT:: prefix of the recipient virus with the numeric suffix of the shuttle plasmid to yield KΔT::102 and KΔT::108, respectively.

Cre-mediated recombination is highly efficient. One appealing property of the Cre-*lox* system for introducing DNA into the pseudorabies virus genome was the relatively high recombination efficiency achieved (28). It was therefore of interest to determine the recombination efficiency of the HSV-based system. The presence of the *lacZ* reporter gene on the inserted shuttle plasmid provided a convenient marker for identifying recombinant viruses against a background of nonrecombinant viruses. Since Cre reaction mixtures were transfected into the gB-complementing D6 cells, both the nonrecombinant gB$^-$ KΔT::*lox*1 and the Cre-mediated gB$^+$ recombinants should undergo productive replication with essentially equal efficiencies. Therefore, the percentage of β-gal$^+$ plaques produced on D6 cells from a given transfection lysate provided a reliable estimate of the recombination efficiency of a particular recombination reaction mixture.

DNA from four independent preparations of pJG108 was used in separate Cre reactions with a single preparation of KΔT::*lox*1 viral DNA, and viruses in the resulting transfection lysates were examined for frequencies of recombination. The results are reported as the number of blue plaques per total number of plaques (Table 1). The Cre-mediated

TABLE 1. Cre-mediated recombination efficiency

Plasmid[a]	Dilution[b]	No. of blue plaques	No. of total plaques	% blue plaques[c]
None	−7	0	120	0
pJG108	−5	8	40	20
pJG108	−5	5	36	14
pJG108	−4	26	233	11
pJG108	−5	5	41	12

[a] Cre reactions contained either no shuttle plasmid DNA or DNA from one of four pJG108 plasmid DNA stocks.
[b] Dilution of transfected cell lysate used for blue-plaque assay.
[c] Percent blue-plaque values are (blue plaques/total plaques) × 100.

TABLE 2. Rescue of gB$^-$ phenotype of KΔT::*lox*1 by Cre-mediated insertion of pJG108 or pJG102

Virus	Titer (PFU/ml)		D6/Vero efficiency[a]	Plaque morphology	
	D6	Vero		D6	Vero
KΔT::*lox*1	1.8×10^8	0	0	Syn$^+$	NA[b]
KΔT::108	8.5×10^8	9.1×10^8	1.07	Syn$^+$	Syn$^+$
KΔT::102	1.7×10^7	1.8×10^7	1.06	Syn	Syn

[a] For each virus, efficiency is defined as the titer on Vero cells/the titer on D6 cells.
[b] NA, not applicable.

recombination efficiencies ranged from 11 to 20% and averaged about 15%.

Complementation of the gB$^-$ phenotype of KΔT::*lox*1. Experiments were carried out to confirm that expression of the polypeptide from the shuttle plasmid would complement the defective gB gene at its native viral locus and that the phenotype of the resulting recombinant virus would reflect the genotype of the plasmid copy of the gene in the *tk* locus. KΔT::108 and KΔT::102 both encode functional gB polypeptides on their respective shuttle plasmids, with KΔT::108 encoding the wild-type gB polypeptide and KΔT::102 encoding the mutant gB polypeptide from HSV-1 strain *ts*B5. The *ts*B5 gB polypeptide includes a *ts* mutation within the external domain (10) and a *syn* mutation (Arg to His) at residue 858 within the cytoplasmic domain (5, 6, 10). Therefore, KΔT::108 and KΔT::102 were used to demonstrate (i) that complementation of the gB$^-$ defect of the parental KΔT::*lox*1 virus after insertion of the pJG108 or pJG102 shuttle plasmid occurred and (ii) that the *ts*B5 mutant gB polypeptide encoded by pJG102 conferred both mutant phenotypes (temperature sensitivity and syncytial plaque morphology) on KΔT::102.

In an initial experiment, both the KΔT::108 and the KΔT::102 viruses were shown to replicate normally on Vero cells in plaque assays (data not shown). Therefore, titers of KΔT::108 and KΔT::102 stocks prepared on Vero cells and a KΔT::*lox*1 stock grown on D6 cells were determined on both D6 and Vero cells. As expected, KΔT::*lox*1 developed a titer on the gB-complementing D6 cell line but not on Vero cells (Table 2). In contrast, KΔT::108 and KΔT::102 each grew equally well on either cell line. The plaque morphology of each virus was also examined. KΔT::102 produced the mutant *syn* plaque morphology on both cell lines (Table 2). Therefore, expression of functional gB polypeptides from the inserted shuttle plasmids complemented the deletion within the gB gene at its native viral locus and expression of the allele from pJG102 conferred the *syn* phenotype on KΔT::102. The KΔT::102 recombinant viruses also demonstrated temperature-dependent virus growth. The ratio of KΔT::102 recombinant virus produced at 39°C compared with that produced at 34°C was approximately 5×10^{-4}, an index of temperature sensitivity similar to that of the parent virus *ts*B5. KΔT::108 grew equally well at both 34 and 39°C.

Efficient Cre-mediated recovery of intact shuttle plasmids from KΔT::108 viral DNA. Sauer et al. (28) demonstrated that inserted shuttle plasmid DNA could be excised from the pseudorabies virus genome in a Cre-dependent manner but did not examine the integrity of the recovered shuttle plasmid. If the Cre-*lox* methodology is to be used as a true shuttle vector system, the inserted shuttle plasmids recovered from the viral genome must be intact. The shuttle plasmid was therefore recovered from extracted KΔT::108 viral DNA and compared with the original pJG108 plasmid stock by restriction enzyme analysis. The plasmid was released from purified KΔT::108 DNA in an intramolecular Cre reaction and propagated after transformation of DH5α. Small batches of plasmid DNA (17) were prepared from 10 isolated colonies, and their *Pst*I restriction patterns were compared with that of pJG108. All 10 miniprep DNAs showed restriction fragment patterns that were identical to that of pJG108 (Fig. 4), demonstrating that large shuttle plasmids could be recovered intact from the viral genome by Cre-mediated recombination.

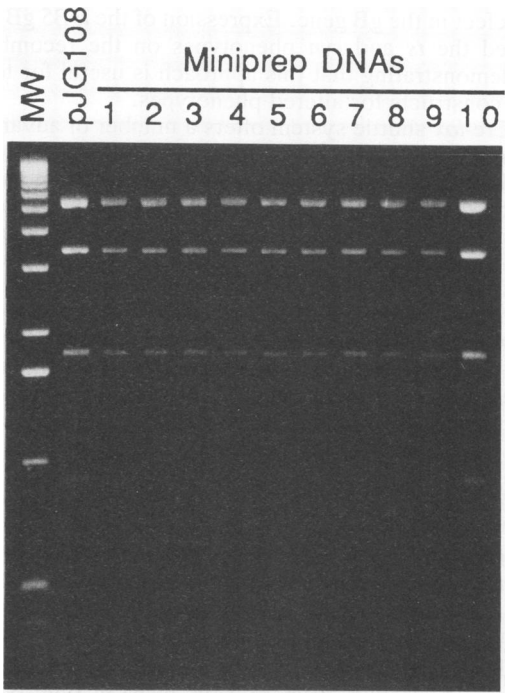

FIG. 4. Cre-mediated rescue of intact shuttle plasmid DNA from KΔT::108 viral DNA. KΔT::108 viral DNA was reacted with Cre recombinase and transformed into DH5α to propagate the rescued shuttle plasmid. Miniprep DNAs were isolated from 10 independent colonies, restricted with *Pst*I, and electrophoresed in 1% agarose. All 10 miniprep DNAs are identical to the pJG108 control.

DISCUSSION

In this report, we describe the adaptation of the Cre-*lox* site-specific recombination system of bacteriophage P1 for use with HSV-1 and investigate the potential usefulness of this approach for the genetic analysis of an essential viral gene. Site-specific recombination mediated by the Cre recombinase protein occurred between two *lox*P sites, one on the shuttle plasmid and the other on the recipient virus genome. A constitutively expressed *lacZ* reporter gene cassette on the shuttle plasmids allowed easy identification of recombinant viruses on the basis of their blue-plaque phenotype. Because Cre-mediated recombination was reversible, the plasmids containing cloned copies of the essential gB gene could be recovered for further analysis by using a second Cre reaction. Recovered shuttle plasmids had a restriction fragment pattern identical to that of the original plasmid. Expression of functional gB polypeptides from the inserted shuttle plasmids at the *tk* locus complemented a

lethal defect in the gB gene. Expression of the *ts*B5 gB allele conferred the *ts* and *syn* phenotypes on the recombinant virus, demonstrating that this approach is useful for testing mutant constructs for altered phenotypes.

The Cre-*lox* shuttle system offers a number of advantages for detailed genetic analyses compared with a strategy based on marker transfer. (i) Since recombination occurs through the 34-bp *cis*-acting *lox*P site, no homologous viral flanking sequences are required to facilitate recombination, minimizing cloning steps that may be needed for marker transfer. Moreover, this site-specific recombinant event avoids potential problems related to rearrangement of the integrated DNA which sometimes occurs in marker transfer experiments. This is particularly important in experiments involving the insertion of large DNA fragments. Although the Cre-*lox* approach does introduce plasmid DNA sequences along with the test gene(s), the function of either the reporter or the gB gene was not affected by the presence of these nonviral sequences. (ii) The recombination frequencies observed for the HSV Cre-*lox* system were even higher than those reported for the pseudorabies virus-based system and are as much as 1 order of magnitude (10 to 20% versus $\leq$2%) greater than those typically observed in marker transfer experiments. The constructs contained both a complete test gene and a reporter gene such that recombinants can be readily detected and purified on the basis of reporter gene expression. Also, in these studies, the expected complementation of the deleted gB allele by the plasmid-encoded wild-type and *ts*B5 gB proteins could have been used as a means of selecting for recombinants by transfection of the Cre-mediated recombination reaction mixtures directly into noncomplementing Vero cells. (iii) Unlike DNA introduced by marker transfer, genes introduced on shuttle plasmids are directly recoverable from the viral genome in a cell-free Cre reaction without additional cloning steps and are thus readily available for further analysis. This is particularly important for studies requiring the generation and testing of a large panel of mutants. For example, randomly mutagenized genes can be introduced into the viral genome and tested for a particular phenotype such as syncytial plaque morphology, and then only those mutant constructs demonstrating the desired phenotype may be recovered from plaque-purified viral DNA for analysis by DNA sequencing. Thus, the ability to easily recover inserted shuttle plasmids from the viral genome should make a prior detailed analysis of genetic constructs unnecessary. (iv) Finally, the Cre-*lox*-based recombination system should be useful for viral systems for which marker transfer procedures are currently difficult or unavailable. These include the cytomegaloviruses, Epstein-Barr virus, and varicella-zoster virus.

ACKNOWLEDGMENTS

We thank Neal DeLuca, William Goins, Ramesh Ramakrishnan, and Stan Person for suggestions, discussions of the data, and critical reading of the manuscript.

This work was supported by U.S. Public Health Service grants GM34534, AI26937, and AI18228 from the National Institutes of Health.

REFERENCES

1. **Abremski, K., and R. Hoess.** 1984. Bacteriophage P1 site-specific recombination: purification and properties of the Cre recombinase. J. Biol. Chem. **259:**1509–1514.
2. **Abremski, K., R. Hoess, and N. Sternberg.** 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. Cell **32:** 1301–1311.
3. **Arsenakis, M., L. Foa Tomasi, V. Speziali, B. Roizman, and G. Campadelli-Fiume.** 1986. Expression and regulation of glycoprotein C gene of herpes simplex virus 1 resident in a clonal L-cell line. J. Virol. **58:**367–376.
4. **Blin, N., and D. W. Stafford.** 1976. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. **3:**2303–2308.
5. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137:**185–190.
6. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:**2596–2604.
7. **Cai, W., S. Person, C. Debroy, and B. Gu.** 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1: an analysis of linker insertion mutants. J. Mol. Biol. **201:**575–588.
8. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61:**714–721.
9. **Costa, R. H., K. G. Draper, G. Devi-Rao, R. L. Thompson, and E. K. Wagner.** 1985. Virus-induced modification of the host is required for expression of the bacterial chloramphenicol acetyltransferase gene controlled by a late herpes simplex virus promoter (VP5). J. Virol. **56:**19–30.
10. **DeLuca, N. A., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology **122:**411–423.
11. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. **56:**558–570.
12. **Dennis, D., and J. R. Smiley.** 1984. Transactivation of a late herpes simplex virus promoter. Mol. Cell. Biol. **4:**544–551.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
14. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. **36:**32–38.
15. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
16. **Hoess, R., and K. Abremski.** 1985. Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system. J. Mol. Biol. **181:**351–362.
17. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
18. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes virus late (γ_2) gene lie within bases -34 to $+124$ relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6:**3652–3666.
19. **Krikos, A.** Unpublished results.
20. **Manservigi, R., P. G. Spear, and A. Buchan.** 1977. Cell fusion induced by herpes simplex virus is prompted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74:** 3913–3917.
21. **McGeoch, D. J., M. A. Dalrymple, A. J. Davidson, A. Dolan, M. C. Frame, D. M. McNab, L. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region of the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
22. **McGeoch, D. J., A. Dolan, S. Donald, and D. H. K. Brauer.** 1986. Complete DNA sequence of the short repeat region of herpes simplex virus type 1. Nucleic Acids Res. **14:**1727–1745.
23. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. **181:**1–13.
24. **Post, L. E., and B. Roizman.** 1981. A generalized technique for

deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227–232.
25. **Roizman, B.** 1982. The family herpesviridae: general description, taxonomy, and classification, p. 1–23. *In* B. Roizman (ed.), The herpes viruses 1. Plenum Press, New York.
26. **Roizman, B., and F. J. Jenkins.** 1985. Genetic engineering of novel genomes of large DNA viruses. Science **229:**1208–1214.
27. **Sambrook, T., E. F. Fritsch, and T. Maniatis.** 1982. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
28. **Sauer, B., M. Whealy, A. Robbins, and L. Enquist.** 1987. Site-specific insertion of DNA into a pseudorabies virus vector. Proc. Natl. Acad. Sci. USA **84:**9108–9112.
29. **Silver, S., and B. Roizman.** 1985. γ2-thymidine kinase chimeras are identically transcribed but regulated as γ2 genes in herpes simplex virus genomes and as β genes in cell genomes. Mol. Cell. Biol. **5:**518–528.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
31. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. J. Mol. Appl. Genet. **1:**327–341.
32. **Spaete, R. R., and E. S. Mocarski.** 1985. Regulation of cytomegalovirus gene expression: α and β promoters are *trans* activated by viral functions in permissive human fibroblasts. J. Virol. **56:**135–143.
33. **Sternberg, N., and D. Hamilton.** 1981. Bacteriophage P1 site-specific recombination. 1. Recombination between *loxP* sites. J. Mol. Biol. **150:**467–486.
34. **Stinski, M. F., and T. J. Roehr.** 1985. Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. J. Virol. **55:**431–441.
35. **Tanford, C.** 1961. Physical biochemistry of macromolecules. Wiley, New York.