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Cloning and Expression of Foreign Genes in Vaccinia Virus, Using a Host Range Selection System

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A simple selection system has been developed for the cloning and expression of open reading frames in vaccinia virus. The selection system is based on a conditional lethal (host range) mutant of vaccinia virus. A deletion mutant of the vaccinia virus WR strain was generated by insertion of the neomycin resistance gene from transposon Tn5 and selection with the antibiotic G418. This deletion recombinant, vP293, lacked approximately 21.7 kilobases of DNA beginning 3.8 kilobases from the left end of the genome. vP293 was capable of plaquing on primary chicken embryo fibroblasts and two monkey cell lines (BSC-40 and Vero) but was defective in replication in the human cell line MRC-5. Insertion of the host range gene K1L into vP293 restored the ability to grow on MRC-5 cells. A series of plasmids were constructed which in addition to the K1L gene contained a vaccinia virus early-late promoter, H6, followed by a unique polylinker sequence, translational initiation and termination signals, and an early transcription termination signal. These plasmids, pHES1 through 4, allowed for rapid single-step cloning and expression of any open reading frame when recombined in vivo with vP293 and scored for growth on MRC-5 cells.

A search for additional markers to further poxvirus genetics led McClain (23) to study the *u* mutants of Gemmell and Fenner (11). These studies (23) provided the first description of host range mutants of poxviruses. These initial observations were quite helpful in the early studies of poxvirus genetics (8, 24, 35). The host range mutants were interpreted to be defective in some control function required for virus replication (8). Subsequent genomic analysis of these rabbitpox virus mutants (18, 25) demonstrated extensive terminal deletions (up to 30 kilobases [kb]) of DNA.

Host range mutants of vaccinia virus have also been described (4, 5, 10, 15, 22, 39). Nitrous acid mutagenesis of the Copenhagen strain of vaccinia virus allowed Drillien et al. (4) to isolate a host range mutant defective in replication in most human cells. Genomic analysis of this mutant revealed an extensive deletion of approximately 18 kb toward the left terminus (4). Additional analysis by marker transfer studies mapped the genetic function responsible for host range to a 5.2-kb *Eco*RI fragment (12) and finally to an 855-base-pair open reading frame overlapping the *Hin*dIII M and K fragments (13). With reference to previously published results describing overlapping and unique deletions in the left end of the vaccinia virus genome (30), this host range gene is located between 24 and 25.2 kb from the left end of the vaccinia virus L-variant genome. This host range gene, transcribed leftward from *Hin*dIII K into *Hin*dIII M, is described herein as the K1L gene according to the nomenclature recommended by Rosel et al. (33).

We have previously described spontaneous and engineered deletions in the left end of the WR strain of vaccinia virus (27, 30). None of these deletions extended rightward beyond the unique *Bgl*II site in *Hin*dIII-M located 24.1 kb from the left terminus of the L-variant genome (30). None of these deletion mutants demonstrated host range restriction on human cells, consistent with the mapping of the K1L host range gene.

In this communication we report the generation of vP293, a vaccinia virus in which 21.7 kb of DNA, including the K1L gene, was deleted. This virus was defective for growth on the human cell line MRC-5. Further, we report the development of a simple selection system for cloning and expressing open reading frames in a vaccinia virus recombinant based on manipulation of the host range function.

MATERIALS AND METHODS

Cells and virus. The WR strain of vaccinia virus was utilized. Its origin and conditions of cultivation have been previously described (27). Primary chicken embryo fibroblasts (CEF), monkey cell lines (BSC40 and Vero), and the human cell line MRC-5 were cultivated in Eagle minimal essential medium (MEM) containing 5% (BSC40 and Vero cells) or 10% (MRC-5 and CEF cells) fetal bovine serum.

Cloning reagents. Plasmids were constructed, screened, and grown by standard procedures (21, 31, 32). Synthetic *Sma*I linkers were obtained from Collaborative Research, Bedford, Mass. Restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, Md.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; New England BioLabs, Beverly, Mass.; and International Biotechnologies, Inc., New Haven, Conn. The Klenow fragment of *Escherichia coli* DNA polymerase was obtained from Boehringer Mannheim, and phage T4 DNA ligase was obtained from New England BioLabs. The reagents were used as specified by the various suppliers.

Cloning of the neomycin phosphotransferase gene under the control of a vaccinia virus promoter. A fragment containing the gene for neomycin phosphotransferase from transposon Tn5 (1) was isolated from pSV2–neo (37) (ATCC 37149) and put under the control of an early vaccinia virus promoter (designated here as Pi). The Pi promoter had been localized by analysis of early vaccinia virus transcription to a *Sau*3A subclone of the *Ava*I H (*Xho*I G) fragment of the L-variant WR vaccinia virus strain (A. Piccini, unpublished data). This promoter element has been shown to express foreign genes in vaccinia virus recombinants at early times after infection (42). The map location of the promoter is approximately 1.1

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kb from the left end of the *Ava*I H fragment (approximately 12.5 kb from the left end of the vaccinia virus genome) and about 9.1 kb left of the *Hin*dIII C-N fragment junction. More precisely, the Pi promoter DNA sequence corresponds to the region immediately upstream from an open reading frame coding for a 5-kilodalton glycine-rich protein recently reported (17). A 1.5-kb *Sma*I ended Pi promoter-*neo* gene cassette containing 0.5 kb of vaccinia virus DNA including the Pi promoter followed by 1 kb of Tn*5* sequences from the *Bgl*II site through the *Sma*I site (1), was used to construct vaccinia virus recombinant vP293. Recombinant virus was selected by the addition of 300 μg of G418 per ml (9, 37).

Transfection conditions. Procedures for in vivo recombination and in situ hybridization of nitrocellulose filters were as previously described (29), with the following modifications. Plasmid DNA was introduced into vaccinia virus-infected cells by electroporation. Subconfluent monolayers of Vero or MRC-5 cells were infected with rescuing virus for 1 h. The cells were harvested with trypsin, washed with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (14), and electroporated in the presence of 25 μg of plasmid DNA in HEPES-buffered saline. Virus-infected cells were electroporated with a Bio-Rad gene pulser equipped with a Bio-Rad gene pulser capacitance extender. The cell suspension (0.8 ml) was placed on ice for 10 min in a Bio-Rad gene pulser cuvette, pulsed at 200 V (capacitance, 960 μF), and placed on ice for another 10 min. The cells were then diluted in 8 ml of MEM–5% fetal bovine serum, plated (4 ml per dish) in 60-mm dishes containing corresponding Vero or MRC-5 cell monolayers, and incubated at 37°C overnight. At 24 h postinfection the virus was harvested by three freeze-thaw cycles and plated to screen for recombinants.

β-Galactosidase screening. Cell monolayers were infected with virus diluted in Eagle MEM–2% fetal bovine serum. After a 1-h adsorption period, the monolayer was overlaid with Eagle MEM containing 10% newborn calf serum (Flow Laboratories, McLean, Va.), 1% Noble agar, penicillin (1 U/ml), and streptomycin (1 mg/ml) and incubated at 37°C in 5% CO_2. After 3 days the dishes were stained simultaneously with neutral red to visualize colorless plaques and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim Biochemicals) to visualize blue plaques. An agar overlay of equal volume was added over the original overlay. It was prepared by combining equal parts of 2% Noble agar, 0.16% neutral red, and 2× MEM, 4% newborn calf serum, antibiotics, and X-gal (800 μg/ml). X-gal was dissolved in dimethylformamide at 250 mg/ml before it was added to 2× MEM.

Synthetic oligodeoxyribonucleotide. Oligonucleotides were prepared on a Biosearch 8750 or Applied Biosystems 380B DNA synthesizer. All syntheses were performed at the 0.2-μmol scale by using the manufacturers' synthesis cycles for β-cyanoethyl phosphoramidite chemistry. The oligonucleotides were routinely deprotected at 55°C for 12 to 18 h in concentrated NH_4OH and dried in a Speedvac. The DNA was suspended in deionized, distilled H_2O, adjusted to 2.5 M sodium acetate–10 mM $MgCl_2$, and precipitated at −20°C with 2 volumes of ethanol. After centrifugation, the dried oligonucleotides were dissolved in 10 mM Tris hydrochloride (pH 8.0) containing 1 mM EDTA, quantified by A_{260}, and stored at −20°C.

pHES1 was constructed by the following procedure: pMP528HRH was cut with *Xho*I and *Xma*I, and the annealed pair of oligonucleotides HRL1 5′(TCGACCATGGGATC CCCGGGTACCGAGCTCTCGAGTAAATAAATAATTTTT AT)3′ and HRL2 5′(CCGGATAAAAATTATTTATTTA CTCGAGAGCTCGGTACCCGGGGATCCCATGG)3′ was cloned into this site. pHES2, pHES3, and pHES4 were constructed with similar pairs of annealed oligonucleotides. pHES2 was constructed with the oligonucleotides HRL3 5′(TCGACCATGGGGATCCCCGGGTACCGAGCTCTCGA GTAAATAAATAATTTTTAT)3′ and HRL4 5′(CCGGATA AAAATTATTTATTTACTCGAGAGCTCGGTACCCGGG GATCCCCATGG)3′, pHES3 was constructed with the oligonucleotides HRL5 5′(TCGACCATGGGGGATCCCCGG GTACCGAGCTCTCGAGTAAATAAATAATTTTTAT)3′ andHRL65′(CCGGATAAAAATTATTTATTTACTC GAGAGCTCGGTACCCGGGGATCCCCCATGG)3′, and pHES4 was constructed with the oligonucleotides HRL7 5′(TCGAGGATCCCGGGTACCGAGCTCTAAATAAATA ATTTTTAT)3′ and HRL8 5′(CCGGATAAAAATTATT TATTTAGAGCTCGGTACCCGGGATCC)3′.

RESULTS

Construction of the vaccinia virus deletion recombinant vP293. Previous results from our laboratory describing spontaneous and engineered deletions in the left end of the genome of the WR strain of vaccinia virus (27, 30) demonstrated extensive overlapping deletions proximal to the left terminus, but no deletions were obtained extending rightward beyond the unique *Bgl*II site in *Hin*dIII M located approximately 24.1 kb from the left terminus. These data suggested that essential genetic functions might reside to the right of this locus. This interpretation was consistent with the location of a host range gene described by Drillien's group (4, 12, 13). To extend the previously described overlapping deletions (30) rightward from this locus, we considered engineering deletions by using a selectable marker and screening on a permissive cell to allow for deletion of genes involved in host range. The cloning strategy to achieve this is outlined in Fig. 1. An *Eco*RI-*Sal*I fragment equivalent to the left terminal 3.8 kb of the vaccinia virus genome was isolated from pAG5 (30) and ligated into *Eco*RI-*Sal*I-digested pUC13. The resulting plasmid pMP5 was digested with *Hin*dIII and *Sal*I and ligated with a 3.8-kb *Hin*dIII-*Sal*I fragment containing vaccinia virus sequences corresponding to the right end of the vaccinia virus *Hin*dIII fragment K. The resulting plasmid pMP528 thus contained vaccinia virus sequences at 0 to 3.8 kb and 25.5 to 29.3 kb. The intervening vaccinia virus sequences between the *Sal*I sites at 3.8 and 25.5 kb toward the left end of the genome were effectively deleted. The unique *Sal*I site in pMP528 was changed to a *Sma*I site by the addition of commercially available synthetic linkers producing pMP528L. A 1.5-kb *Sma*I fragment containing the gene for neomycin phosphotransferase from transposon Tn*5* (1) under the control of an early vaccinia virus promoter (designated here as Pi) from the *Ava*I-H region of vaccinia virus was cloned into the *Sma*I site of pMP528L, generating pMP528PiN. pMP528PiN was transfected into primary CEF coinfected with the rescuing vaccinia virus, VTK^-79. Recombinant virus was selected and grown on primary CEF in the presence of G418 (9). In the recombinant vaccinia virus vP293, 21.7 kb of vaccinia virus had been deleted, as predicted, and the virus contained the foreign gene encoding Neo^r. The genomic configurations were confirmed by Southern blot hybridization analysis (data not shown). The restriction map of the left terminus of the rescuing virus VTK^-79 and of the recombinant virus vP293 expressing the Neo^r gene and selected on primary CEF in the presence of G418 are indicated in Fig. 1B and C.

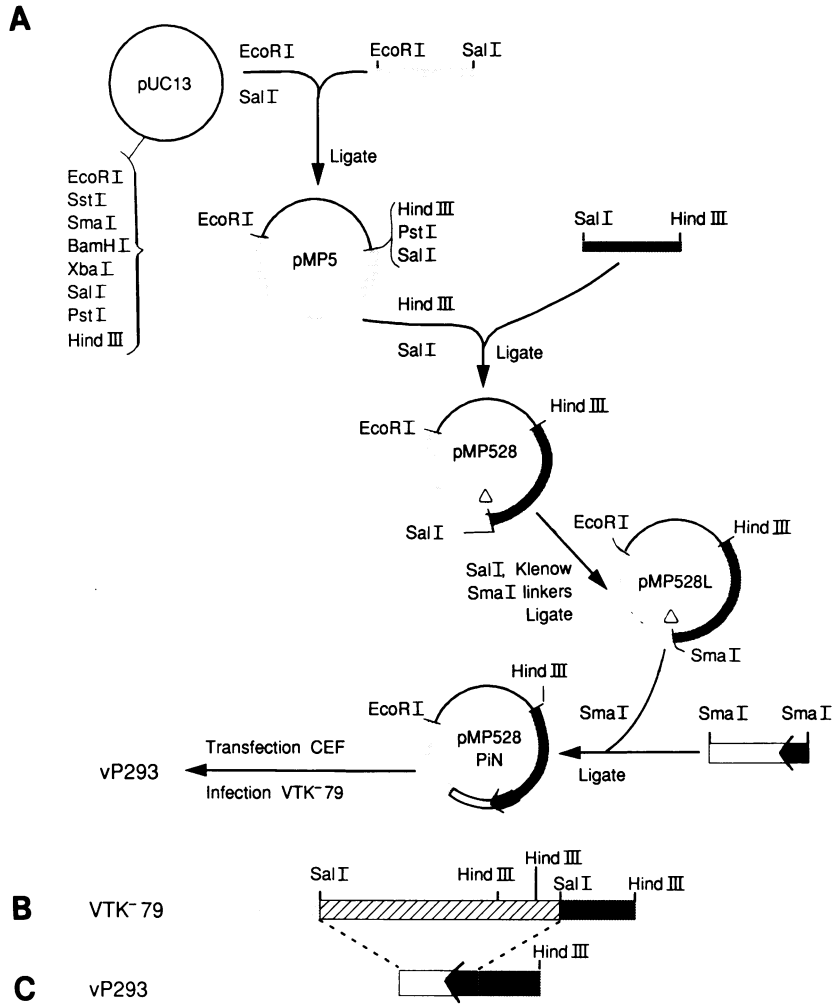

FIG. 1. Construction of plasmid pMP528PiN and generation of vP293. (A) An *Eco*RI-*Sal*I fragment consisting of a 3.8-kb vaccinia virus sequence from pAG5 (30) (left arm, light hatching) was ligated into pUC13 that had been cut with *Eco*RI and *Sal*I, generating pMP5. A *Sal*I-*Hin*dIII fragment consisting of 3.8-kb vaccinia virus sequences from *Hin*dIII-K (right arm, dark hatching) was ligated with pMP5 that had been cut with *Hin*dIII and *Sal*I, generating pMP528. The site of the vaccinia virus deletion between the left and right vaccinia virus arms is indicated by a triangle. pMP528 was cut with *Sal*I and made blunt ended with the Klenow fragment of *E. coli* polymerase; *Sma*I linkers were added, producing pMP528L. pMP528L was cut with *Sma*I and ligated with a *Sma*I-ended cassette containing the neomycin resistance gene (open block) under the control of the early vaccinia virus Pi promoter (dark arrow). The resulting plasmid, pMP528PiN, was transfected into CEF cells infected with VTK⁻79, generating the vaccinia virus recombinant vP293. (B) Map of the left end of VTK⁻79 through *Hin*dIII-K. Only the relevant *Sal*I sites are indicated. The diagonally striped block indicates the vaccinia virus sequences to be deleted. Left and right arms are as indicated above. (C) Map of the left end of vP293 through *Hin*dIII-K. Symbols are as defined above.

In the absence of the antibiotic G418, vP293 produced large plaques on primary CEF and produced plaques well on BSC40 or Vero cells, although vP293 plaques were detectably smaller than the parent VTK⁻79 plaques on Vero cells. Significantly, vP293 gave no measurable replication and failed to form plaques on the human cell line MRC-5. These results are qualitatively consistent with the host range deletion mutant described by Drillien et al. (4).

Reconstitution of vP293 with the host range gene, K1L. Gillard et al. (13) have shown that with their deletion mutant of the Copenhagen strain of vaccinia virus, reconstitution with the host range gene K1L was necessary and sufficient to restore the ability for growth on human cells. This suggested that this host range gene, when reconstituted into the deletion mutant vP293 of the WR strain of vaccinia virus, would also allow the virus to replicate on human cells.

The cloning of the host range gene K1L into plasmid pMP528L and its insertion into vP293 are outlined in Fig. 2. The right vaccinia virus arm of pMP528L (Fig. 1A and 2A) was shortened to eliminate unwanted restriction sites and to facilitate future cloning steps. pMP528L was cut by *Eco*RV-*Hin*dIII, made blunt ended with the Klenow fragment of the *E. coli* polymerase, and self-ligated. The right arm of the resulting plasmid pMP528E was reduced in length to 0.4 kb (Fig. 2A).

An 891-base-pair vaccinia virus *Bgl*II (partial digest)-*Hpa*I fragment containing the entire coding sequence and promoter from the K1L gene (13) was cloned into the polylinker region of pUC8 for the sole purpose of flanking the gene with convenient restriction sites (Fig. 2A). The resulting plasmid, pUC8HR, was digested with *Hin*dIII (partial digest) and *Sma*I to isolate the K1L-containing fragment. The *Hin*dIII

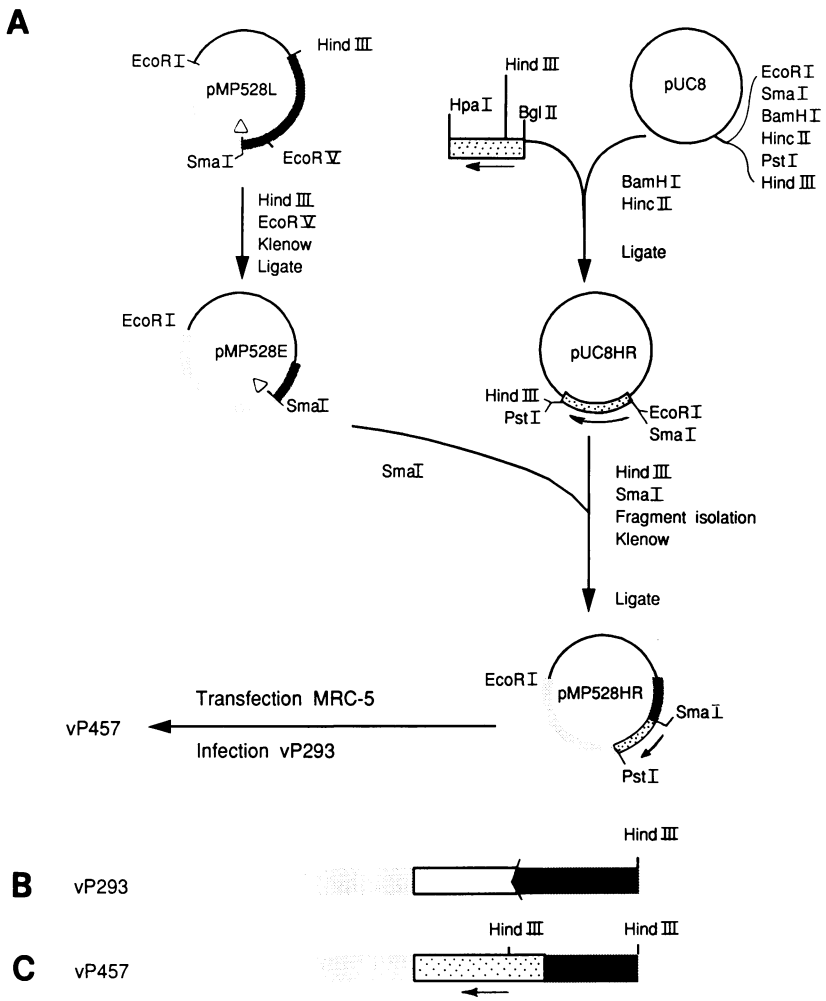

FIG. 2. Construction of plasmid pMP528HR and generation of vaccinia virus recombinant vP457. (A) The right arm of the vaccinia virus deletion plasmid pMP528L (dark hatch) was shortened by digestion with *Hin*dIII and *Eco*RV and then made blunt ended with the Klenow fragment of *E. coli* polymerase. The plasmid was self ligated, generating pMP528E. Regions are indicated by light hatching (left arm), dark hatching (right arm), and triangles (sites of deletion). An *Hpa*I-*Bgl*II fragment containing the K1L gene and its promoter (stippling; direction of gene indicated by arrow) was ligated into pUC8 that had been cut with *Bam*HI and *Hin*cII, generating pUC8HR. A *Hin*dIII (partial digest)-*Sma*I fragment containing the K1L gene was isolated, treated with the Klenow fragment of *E. coli* polymerase, and ligated with pMP528E that had been cut with *Sma*I. The resulting plasmid, pMP528HR, was transfected into MRC-5 cells infected with vP293, generating vaccinia virus recombinant vP457. (B) Map of the left end of vP293 through *Hin*dIII-K. The open block indicates the neomycin resistance gene; the dark arrow indicates the Pi promoter. (C) Map of the left end of vP457 through *Hin*dIII-K. Symbols are as defined above.

end was filled in with the Klenow fragment of the *E. coli* DNA polymerase, and the fragment was cloned into the *Sma*I site of pMP528E. A plasmid, pMP528HR, with the orientation of the K1L gene reading leftward (Fig. 2A) was isolated by standard procedures. pMP528HR contains the host range gene reintroduced into the 21.7-kb deletion in its native right to left orientation with respect to flanking vaccinia virus arms.

The donor plasmid pMP528HR was transfected into either Vero or MRC-5 cells, each coinfected with vP293. Progeny was harvested after an overnight infection and plated on either Vero or MRC-5 cells. The numbers of plaques obtained on Vero cells were 10 to 100 times greater than the numbers of plaques obtained on MRC-5 cells. Isolated plaques of uniform size were picked from MRC-5, and both large and small plaques were picked from Vero cell cultures. These plaque isolates were replated on Vero cells, and after 3 days the resulting plaques were lifted onto nitrocellulose filter disks and prepared for in situ hybridization (26). All of the plaques originating from MRC-5 cells and all of the larger plaques, but not the smaller plaques derived from Vero cells, gave positive hybridization signals when probed with a ^{32}P-labeled probe to the K1L coding sequences. This is consistent with restoration of host range functions contained in the K1L coding sequence. An isolate obtained from MRC-5 cells was further purified and designated vP457. In vP457 the K1L gene has been restored and is situated within the deletion in its native orientation, reading from right to left. The K1L sequences have replaced the Pi promoter-neomycin phosphotransferase gene cassette present in vP293 (Fig. 2B and C). Compared with the genome of the L variant vaccinia virus (27, 30), vP457 contains a 291-base-pair deletion to the right of the K1L gene and a 20.2-kb deletion to the left of the K1L gene. The ability of vP293 and vP457 to form plaques on Vero or MRC-5 cells is shown in Fig. 3. Note that the plaquing efficiency of vP457 is approx-

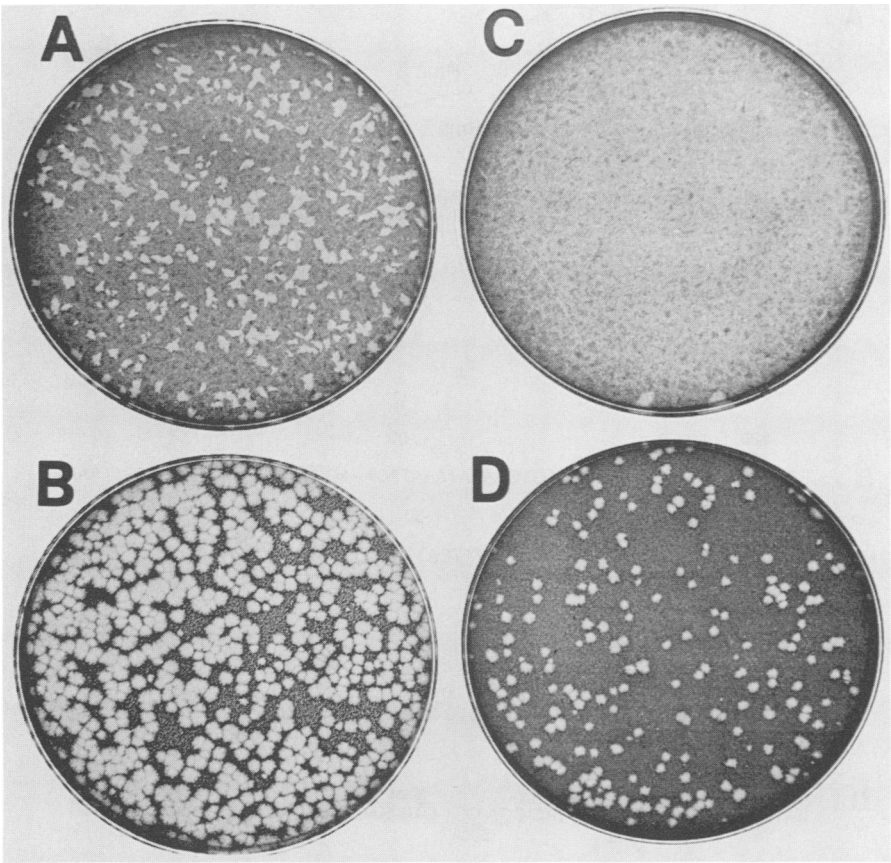

FIG. 3. Plaquing proficiency of vaccinia virus mutants vP293 and vP457 on Vero or MRC-5 cells. Virus was plated on Vero or MRC-5 cells as described in Materials and Methods. Plaques were visualized after 3 days under an agar overlay by staining with neutral red. vP457 was plated on MRC-5 (10^5) (A) or Vero (10^6) (B) cells. vP293 was plated on MRC-5 (10^1) (C) or Vero (10^4) (D) cells.

imately 10 times as high on Vero cells (Fig. 3B, plated at a 10^{-6} dilution) as on MRC-5 cells (Fig. 3A, plated at a 10^{-5} dilution). Under the conditions used here, a similar plaquing differential was typically seen with the wild-type L or S variant viruses (27) plated on Vero and MRC-5 cells (data not shown). No plaques were formed by vP293 on MRC-5 cells (Fig. 3C, plated at a 10^{-1} dilution), even when plated at 1,000 times the concentration used with Vero cells (Fig. 3D, plated at a 10^{-4} dilution).

Construction of plasmids pMP528HRH and pHES1-4. The above results suggested that the conditional lethal phenotype of vP293 could be exploited for constructing donor plasmids into which additional open reading frames could be cloned and expressed. Introduction of these exogenous open reading frames into a plasmid containing the K1L host range gene and recombination into vP293 would yield a simple method for generating vaccinia virus recombinants by virtue of host range restriction. To this end a series of plasmids, pMP528HRH and pHES1 through 4, was constructed.

First, a vaccinia virus promoter was added upstream from the K1L gene in pMP528HR. This early-late promoter was previously identified and localized in *Hin*dIII-H by transcriptional mapping and DNA sequence analysis (A. Piccini and R. Weinberg, unpublished data) and has been utilized to express foreign genes in recombinant fowlpox virus vectors (40, 41). This promoter, H6, maps upstream from the H6 open reading frame and is identical to the sequence published by Rosel et al. (33). Double-stranded DNA corresponding to positions −124 to −1 (with position −102 changed from A to G to remove a potential initiation codon) and followed by *Xho*I, *Kpn*I, and *Sma*I restriction sites was synthesized chemically and cloned into the *Sma*I site of pMP528HR, producing pMP528HRH (Fig. 4A and B). Thus, pMP528HRH contained the H6 promoter upstream from the K1L gene, which was expressed under the control of the K1L endogenous promoter. Both were in a right-to-left orientation with respect to vaccinia virus arms (genome) (Fig. 4). The H6 promoter in pMP528HRH was immediately upstream of unique *Xho*I, *Kpn*I, and *Sma*I restriction sites.

To increase further the utility of the system, plasmids pHES1 through 4 were derived (Fig. 4C through F) from pMP528HRH. In each case pMP528HRH was cut with *Xho*I and *Xma*I, an isoschizomer of *Sma*I, and ligated with the appropriate pair of annealed oligonucleotides. In addition to the elements contained in pMP528HRH, each of plasmids pHES1 through 3 contained a translation initiation codon downstream from the H6 promoter followed by unique multiple restriction sites, translational termination signals, and a specific vaccinia virus early transcription termination signal sequence (44). In each of plasmids pHES1 through 3, the translation initiation codon was in a different reading frame relative to the polylinker region that follows (Fig. 4). Therefore, any DNA sequence that contained an open reading frame could potentially be expressed when cloned into one of these plasmids and recombined into vaccinia virus. A fourth plasmid, pHES4, was also derived from pMP528HRH. This plasmid did not contain a translation initiation codon but did contain unique multiple restriction

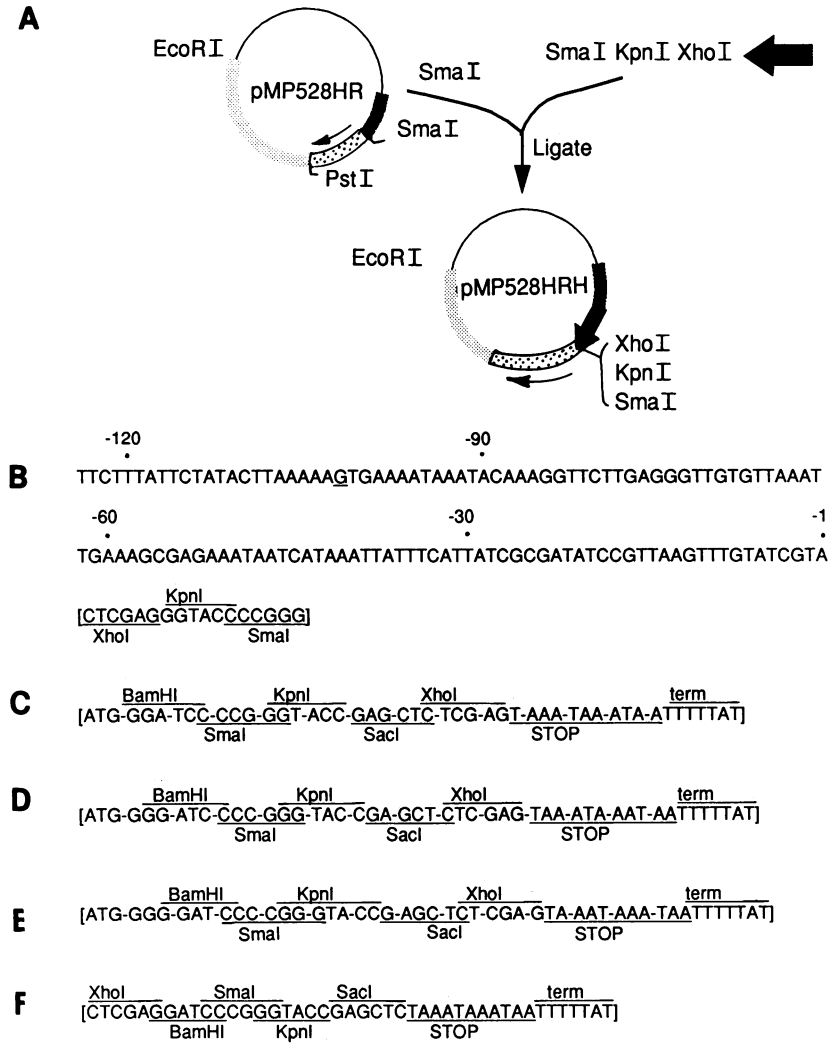

FIG. 4. Construction of plasmids pMP528HRH and pHES1 through 4. (A) pMP528HR (light hatching, left arm; dark hatching, right arm; stippling, K1L gene) was cut with *Sma*I and ligated with a blunt end fragment containing the synthetic H6 promoter positions −124 through −1 (dark arrow), followed by *Xho*I, *Kpn*I, and *Sma*I restriction sites. The resulting plasmid is pMP528HRH. (B) Sequence of synthetic H6 promoter (positions −124 through −1) and downstream restriction sites present in pMP528HRH. Altered base as described in the text at position −102 is underlined. The bracketed sequence is replaced in plasmids pHES1 through 4 (C through F, respectively, below). (C through F) Replacement of bracketed sequence from B (above) in pHES1 through 4. Note that pMP528HRH and pHES4 do not contain an ATG downstream from the H6 promoter, whereas pHES1 through 3 each contain an ATG followed by a frame shift. Restriction sites, stop codons, and early transcriptional termination signals as indicated.

sites, translational termination sequences, and an early transcription termination signal sequence. A DNA sequence that contains an open reading frame and an initiation codon can potentially be expressed when cloned into pHES4 and recombined into vaccinia virus. The pertinent DNA sequence elements, restriction sites, and transcriptional and translational signals of pMP528HRH and pHES1 through 4 are depicted in Fig. 4.

Incorporation of the bacterial *lacZ* gene into vaccinia virus and selection of the recombinant viruses on the basis of host range restriction. To analyze the utility of the pHES1 through 4 and vP293 host range selection system, we chose the *E. coli lacZ* gene encoding β-galactosidase. A *Bam*HI fragment containing codons 8 through the end of the *lacZ* gene was obtained from pMC1871 (36). This *lacZ Bam*HI fragment was cloned into the unique *Bam*HI site of plasmids pHES1 through 4 in the correct orientation. In vivo recombination between the resulting plasmids pHESLZ1, pHESLZ2, pHESLZ3, and pHESLZ4 transfected into Vero cells coinfected with the host range mutant vP293 was performed as described in Materials and Methods. After 24 h, progeny virus was plated on either Vero or MRC-5 cells. When progeny from transfections with pHESLZ1 through 4 were plated on Vero cells and expression of β-galactosidase was assayed in the presence of X-gal, no blue plaques were observed in cells transfected with pHESLZ1, 2, or 4. Significantly, approximately 20% of the plaques generated with plasmid pHESLZ3 gave blue plaques in the presence of X-gal (data not shown). When progeny from transfections with pHESLZ1 through 4 were plated on Vero cells and recombinant viruses were screened by in situ hybridization, 12 to 22% of the plaques gave positive hybridization signals to *lacZ* (Table 1). When analyzed by in situ DNA hybridization (26), every plaque on MRC-5 demonstrated the presence

TABLE 1. Analysis of recombinant *lacZ* vaccinia virus generated with plasmids pHESLZ1 through 4 and VP293 vaccinia virus[a]

Cell line	Stain	Donor plasmid	No. of plaques			% Positive plaques
			Total	Hybridization positive	X-gal positive	
Vero	Neutral red	pHESLZ1	1,056	153		14.5
		pHESLZ2	637	141		22
		pHESLZ3	793	95		12
		pHESLZ4	1,344	269		20
MRC-5	Neutral red	pHESLZ1	60	60		100
		pHESLZ2	56	56		100
		pHESLZ3	ND	ND		
		pHESLZ4	71	71		100
MRC-5	X-gal	pHESLZ1	60		0	0
		pHESLZ2	55		0	0
		pHESLZ3	59		59	100
		pHESLZ4	70		0	0

[a] Donor plasmids pHESLZ1 through 4 were transfected individually into Vero cells infected with vP293 as described in Materials and Methods. After 24 h, progeny were harvested by three freeze-thaw cycles and plated on Vero or MRC-5 cells. Monolayers stained with neutral red were lifted after 3 days onto nitrocellulose filters and prepared for in situ hybridization (26) with a ^{32}P-labeled *lacZ* gene probe. Other MRC-5 dishes were exposed to X-gal, and blue color development was scored after 8 h. ND, Not done.

of the *lacZ* gene (Table 1). β-Galactosidase activity, however, was seen only in plaques on MRC-5 which were derived from pHESLZ3 (Table 1). Only the plasmid construct pHESLZ3 had the *lacZ* gene in frame with the translational initiation codon.

DISCUSSION

Since the initial demonstration of marker rescue in vaccinia virus by Sam and Dumbell (34) and Nakano et al. (26), vaccinia virus has been used extensively for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into live infectious vaccinia virus involves in vivo recombination between vaccinia virus DNA sequences flanking a foreign genetic element in a chimeric donor plasmid and homologous sequences present in the rescuing vaccinia virus (32). Unperturbed, successful recombination occurs at a frequency of approximately 0.1%. Our initial screening strategy involved in situ hybridization of recombinants on replica filters with a radiolabeled probe homologous to the inserted sequences (26, 29).

A number of modifications have been reported to increase the efficiency of recombination itself or to facilitate the identification of recombinants. Among these modifications are the following: use of single-stranded donor DNA (43); identification of recombinants expressing unique enzymatic functions, such as [^{125}I]iododeoxycytidine incorporation into DNA via expression of the herpes simplex virus thymidine kinase (29); use of chromogenic substrates for (co)expression of foreign genes along with β-galactosidase (3, 28); selection for thymidine kinase expression (positive or negative) (20, 29, 30); antibody-based reactions to visualize recombinant plaques (19); use of conditional lethal temperature-sensitive or drug mutants (7, 16); selection of recombinants by using the neomycin resistance gene from Tn*5* and the antibiotic G418 (9); and selection pressures with mycophenolic acid and the *E. coli gpt* gene (2, 6).

We have described in this report a selection system for constructing vaccinia virus recombinants by using a conditional lethal host range mutant. The deletion recombinant mutant vP293 fails to plaque on the human cell line MRC-5. vP293, however, can be readily cultivated and plaqued on nonhuman cells such as Vero, BSC40, and primary CEF cells. The host range function can be restored to vP293 by restoration of the K1L host range gene. Such a recombinant can now plaque on MRC-5. We have designed a number of plasmids, pMP528HRH and pHES1 through 4, which in addition to the K1L host range gene contain another vaccinia virus promoter, unique multicloning restriction sites, appropriate translational start and stop codons, and an early transcription termination signal. Insertion of a foreign open reading frame into these plasmids followed by in vivo recombination will simultaneously restore the host range function (K1L gene) and introduce the foreign open reading frame into the rescuing virus, vP293. The recombinant viruses can now be identified by their ability to plaque on MRC-5 cells. Plasmids pHES1 through 3 contain initiation codons followed by multicloning restriction sites in the three reading frames. Any exogenous open reading frame can be expressed after insertion in one of these plasmids. pHES4, which lacks a translation initiation codon, is designed for expression of exogenous open reading frames that contain their own ATGs.

Advantages of this system include the absence of any non-vaccinia virus exogenous gene in the final recombinant other than the genetic element of interest, no genetic reversion of the virus (since vP293 is a deletion mutant of K1L), and the rapid one-step identification of recombinants. This single-step procedure can also be used for rapid screening of expression of the foreign genetic element, for example, for epitope mapping.

Additional plasmids containing the K1L host range gene have been constructed (unpublished data) where the H6 early-late promoter has been replaced with either a strictly early or a strictly late vaccinia virus promoter. This will allow study of the subtleties of temporal regulation of expression of foreign genetic elements.

It has recently been reported (38) that cowpox virus contains a gene that, when introduced into vaccinia virus, allows vaccinia virus to replicate productively on the normally growth-restrictive CHO cells (5, 15). Other members of the poxvirus family such as fowlpox and swinepox are quite restricted for growth to avians and swine, respectively. These observations suggest that poxviruses have a family of host range functions that determine their relative range of replication competence. The functions of such genes are currently unknown. Information from studying this family of host range functions might be useful in elucidating evolutionary aspects of the poxvirus family.

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