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Construction and Characterization of Vaccinia Direct Ligation Vectors

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Poxvirus vectors are extensively used as expression vehicles for protein and antigen expression in eukaryotic cells. Customarily, the foreign DNA is introduced into the poxvirus genome by homologous recombination. An alternative method using direct ligation vectors has been used to efficiently construct chimeric genomes in situations not readily amenable for homologous recombination. We describe the construction and characterization of a new set of direct ligation vectors designed to be universally applicable for the generation of chimeric vaccinia genomes. These vectors contain the pair of unique restriction sites *Not*I and *Apa*I to eliminate religation of poxvirus arms and fix the orientation of the insert DNA behind strongly expressing constitutive vaccinia promoters. The insertion cassette has been placed at the beginning of the thymidine kinase gene in vaccinia to use drug selection in the isolation of recombinants. These viruses provide a set of universally applicable direct ligation poxvirus cloning vectors, extending the utility of poxvirus vectors for construction and expression of complex libraries.

INTRODUCTION

Their ease of cloning and propagation in a variety of host cells has led to the widespread use of poxvirus vectors for expression of foreign protein and as delivery vehicles for vaccine antigens (Moss, 1991). Generally, the target protein coding sequence is cloned behind a vaccinia promoter flanked by sequences homologous to a nonessential region in the poxvirus and the plasmid intermediate is recombined into the viral genome by homologous recombination. This methodology works efficiently for relatively small inserts tolerated by prokaryotic hosts. The method becomes less viable in cases requiring large inserts as the frequency of homologous recombination is low and decreases with insert size; in cases requiring construction of labor-intensive plasmid intermediates such as in expression library production; and in cases where the propagation of DNA is not tolerated in bacteria. In two laboratories, a direct ligation protocol obviating the need for homologous recombination has been developed to generate poxvirus chimeric genomes. The DNA from the genome was digested, ligated to insert DNA *in vitro,* and transfected into cells infected with a helper virus (Merchlinsky and Moss, 1992; Scheiflinger *et al.,* 1992). In one protocol, the genome was digested at the unique *Not*I site and a DNA insert containing elements for selection or detection of the chimeric genomes was ligated to the genomic arms (Scheiflinger *et al.,* 1992). Alternatively, the vaccinia WR genome was modified by removing the *Not*I site in the *Hin*dIII F fragment and reintroducing a *Not*I site proximal to the thymidine kinase gene such that insertion of a sequence at this locus disrupts the thymidine kinase gene, allowing isolation of chimeric genomes via use of drug selection (Merchlinsky and Moss, 1992).

The direct ligation vector v*Not*I/tk allowed one to efficiently clone and propagate DNA inserts at least 26 kb in length (Merchlinsky and Moss, 1992). Although large DNA fragments were efficiently cloned into the genome, proteins encoded by the DNA insert will be expressed only at the low level corresponding to the thymidine kinase gene, a relatively weakly expressed early class gene in vaccinia, and the DNA will be inserted in both orientations at the *Not*I site. Our aim was to modify the genome of v*Not*I/tk so as to acquire direct ligation vectors that will be more universally useful. First, we decided to change the insertion site by placing the sites for two unique restriction enzymes at the beginning of the thymidine kinase gene. This allows one to fix the orientation of the insert DNA and eliminates the production of contaminating wild-type genomes after religation of viral arms. Second, to generate a direct ligation vector that would express high levels of protein we decided to precede the thymidine kinase gene with a strong constitutive vaccinia virus promoter.

MATERIALS AND METHODS

Plasmid construction

Pairs of oligonucleotides were constructed that, when annealed, contained the 7.5k gene promoter (MM436: GGCCAAAAATTGAAAAACTAGATCTATTTATTGCA-

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CGCGGCCGCCATGGGCCC, MM437: GGCCGGGCCCATGGCGGCCGCGTGCAATAAATAGATCTAGTTTTTCAATTTTT) or the synthetic EL promoter (MM438: GGCCAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAAGCGGCCGCCATGGGCCC, MM439: GGCCGGGCCCATGGCGGCCGCTTTATATTCCAAAAAAAAAAAATAAAATTTCAATTTTT) and restriction sites for *Not*I and *Apa*I. The double-stranded oligonucleotides were annealed by ramping from 94° to 20° over 2 h and ligated (New England Biolabs) into the *Not*I site present in pJ*Not*I/tk, a plasmid containing the *Hin*dIII J fragment from v*Not*I/tk, resulting in plasmids p7.5/tk and pEL/tk.

A polymerase chain reaction (PCR) was performed on pBI221 (Clontech), a plasmid containing the *Escherichia coli gusA* gene encoding for β-glucuronidase (β-glu), using primers MM440 (GGGAAAGGGGCGGCCGCCATGTTACGTCCTGTAGAAACC) and MM441 (GGGAAAGGGGGGCCCTCATTGTTTGCCTCCCTGCTG), or MM440 and MM442 (GGGAAAGGGGCGGCCGCCTCATTGTTTGCCTCCCTGCTG), and the resulting fragment was cloned into pCRII (TA cloning kit, Invitrogen). The plasmids were excised with *Not*I (MM440/MM442 product) and cloned into pJ*Not*/tk digested with *Not*I yielding pJ*Not*/tk-GUS, or excised with *Not*I and *Apa*I (MM440/MM441 product) and inserted into pEL/tk and p7.5/tk previously digested with *Apa*I and *Not*I, yielding p7.5/tk-GUS and pEL/tk-GUS.

Pairs of oligonucleotides were constructed that, when annealed, contained the 7.5k gene promoter and the nucleotide sequence encoding for a cytotoxic T-cell epitope for ovalbumin (Moore *et al.,* 1988) (SIINFEKL) (75ova: GGCCAAAAATTGAAAAACTAGATCTATTTATTGCACCAT - GAGTATAATCAACTTTGAAAAACTGTAGTGA, 75ovarv: GGCCTCACTACAGTTTTTCAAAGTTGATTAATACTCATG - GTGCAATAAATAGATCTAGTTTTTCAATTTTT) or the EL promoter and the peptide SIINFEKL (ELova: GGCCAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAACCATGAGTATAATCAACTTTGAAAAACTGTAGTGA, ELovarv: GGCCTCACTACAGTTTTTCAAAGTTGATTATACTCATGGTTTATATTCCAAAAAAAAAAAATAAAATTTCAATTTTT). The double-stranded oligonucleotides were annealed by ramping from 94° to 20° over 2 h and ligated into the *Not*I site present in pJ*Not*I/tk, a plasmid containing the *Hin*dIII J fragment from v*Not*I/tk, resulting in plasmids p7.5/tk-ova and pEL/tk-ova.

Generation of recombinant viruses

Cells and viruses were maintained and manipulated as previously described (Earl and Moss, 1991). Recombinant viruses were made using homologous recombination by infecting CV-1 cells at a m.o.i. of 0.05 and, 2 h later, transfecting DNA into the infected cells using Lipofectamine (Life Technologies Inc.) as suggested by the manufacturer. After 72 h the cells were harvested and isolated plaques were selected by passage in Hutk$^-$ cells in the presence of bromodeoxyuridine (Earl and Moss, 1991) or HAT supplemented medium (Weir *et al.,* 1982) as described in the text.

Vaccinia virus was generated from viral DNA by rescue with fowlpox virus (Scheiflinger *et al.,* 1992). Vaccinia virus was isolated from infected HeLa cells by banding and sedimentation in sucrose (Earl and Moss, 1991). The purified virions were treated with proteinase K (Boehringer-Mannheim), gently extracted with buffer-saturated phenol, phenol:chloroform (50:50), and chloroform before precipitation with 2.5 vol of ethanol in 0.3 M sodium acetate, and resuspended in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA (Earl and Moss, 1991). Confluent wells of BSC-1 cells from a 12-well dish were infected with fowlpox virus and, after a 2-h incubation at 37°C, were transfected with 0.6 μg full-length vaccinia DNA using Lipofectamine as suggested by the manufacturer. After incubation (usually 72 h), the cells were harvested, lysed by three freeze–thaw cycles, and screened by plaque assay on BSC-1 cells (Earl and Moss, 1991).

Generation of recombinant viruses by direct ligation

The 1.1-kB *Eco*RI/*Eco*RV restriction endonuclease fragment containing ovalbumin from pHbeta-Ova-neo (Pulaski *et al.,* 1996) was inserted into the *Eco*RI and *Eco*RV sites of pBluescript KS+ (Stratagene), generating pBS.ova. The DNA product from a PCR on pBS.ova using primers VV0LZ5 (GCAGGTGCGGCCGCCGTGGATCCCCCGGGCTGCAGG) and VVTLZ3 (GTACCGGGCCCACAAAAACAAAATTAGTTAGTTAGGCCCCCCCTCGA) was digested with *Apa*I and *Not*I (Life Technologies Inc.), gel purified from low-melting-point agarose (Bio-Rad) using β-Agarase (Life Technologies, Inc.) following the recommendations of the manufacturer, and cloned into pBluescript KS+ that had been digested with *Not*I and *Apa*I, generating pBS.VVova. A DNA fragment encoding ovalbumin was excised from pBS.VVova by digestion of this plasmid with *Apa*I and *Not*I and purified after electrophoresis through a low-melting-point agarose gel using β-Agarase. One microgram of purified vEL/tk DNA was digested with *Apa*I and *Not*I and centrifuged through a Centricon 100 concentrator (Amicon) to remove the small intervening fragment. The vEL/tk DNA arms and the DNA fragment encoding ovalbumin were ligated overnight at room temperature, at a 4:1 (insert: virus) molar ratio, in 30 μl with 5 units T4 DNA ligase. The ligation product was transfected using Lipofectamine into a well of confluent BSC-1 cells from a 12-well plate 2 h after infection with fowlpox virus at 1 PFU/cell. Three days later the cells were harvested and isolated plaques were selected by passage in Hutk cells in the presence of bromodeoxyuridine (Earl and Moss, 1991).

Analysis of viral DNA genomes

BSC-1 cells were infected at approximately 10 PFU per cell by vaccinia WR, vEL/tk, v7.5/tk, or v*Not*I/tk. After 24

h the cells were harvested and resuspended in cell suspension buffer (Bio-Rad Genomic DNA Plug Kit) at 1 × 10^7 cells/ml. An equal volume of 2% CleanCut agarose (Bio-Rad) preincubated at 50° was added and the cell suspension was formed into 100-μl plugs. After hardening at 4° the plugs were treated as previously described to digest protein (Merchlinsky and Moss, 1989). The plugs were equilibrated in the appropriate restriction enzyme buffer and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 16 h at room temperature and incubated with restriction enzyme buffer, 100 ng/ml bovine serum albumin, and 50 units *Not*I or *Apa*I for 2 h at 37° (*Not*I) or room temperature (*Apa*I) prior to electrophoresis.

Determination of β-glucuronidase activity

A well of BSC-1 cells from a 12-well plate was infected at a m.o.i. of 1 with v*Not*I/tk-GUS, v7.5/tk-GUS, and vEL/tk-GUS; the cells were harvested 20 h postinfection, resuspended in 0.5 ml PBS, and disrupted by three cycles of freeze–thawing. The extract was clarified by a short microfuge spin (1 min, 14,000 rpm) and the supernatant was analyzed for β-glu units as described by Miller (1972) as adapted for 96-well plates. The A_{405} values were determined on a microplate reader (Dynatech MR3000), and β-glu activity was determined by comparison to β-glu (Clontech) standards analyzed in the same assay.

Analysis of cytoxic T-cell response

Confluent monolayers of MC57G cells in wells of a 6-well plate were infected at a m.o.i. of 1 with vEL/tk, v7.5/tk-ova, vEL/tk-ova, vEL/tk-ovaFL clone 1, and vEL/tk-ovaFL clone 2 (vEL/tk-ovaFL are virus clones of full-length ovalbumin generated by direct ligation). At 16 h postinfection cells were harvested and labeled with 100 μCi ^{51}Cr (Dupont) for 1 h at 37°, and 10^4 cells were added to wells of a 96-well round-bottom plate in quadruplicate. A sample of uninfected MC57G cells incubated with 1 μM purified ova 257–264 peptide was also incubated with ^{51}Cr as a positive control and untreated MC57G cells were used as a negative control. T cells specific for ova 257–264 were added to target cells at ratios of 2:1 and 10:1. Cells were incubated at 37° for 4 h, supernatants were harvested, and ^{51}Cr release was determined. Spontaneous release was derived by incubating target cells with medium alone and maximal release was determined by incubating target cells with 5% Triton X-100. Percentage specific lysis was calculated using the formula % specific lysis = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. In each case the mean of quadruplicate wells was used in the formula.

RESULTS

Construction of direct ligation vectors

The vaccinia WR genome is approximately 190 kb in length and rich in A and T residues. The complete sequence of the vaccinia WR genome was provided by the Bernard Moss laboratory (P. Earl, personal communication) and a restriction enzyme search of the genome using MacVector (IBI) revealed a lack of restriction sites for *Apa*I, *Asc*I, *Bsp*120I, *Fse*I, *Rsr*II, *Sfi*I, *Srf*I, and *Sgf*I. The ready availability of highly active and pure preparations of the enzyme as well as the generation of a staggered end on digestion led us to choose to use *Apa*I as the second site in conjunction with the *Not*I site already present in v*Not*/tk.

Vaccinia virus-based expression vectors are most useful when the foreign protein is expressed constitutively. The expression of foreign proteins during the early stage of viral replication is essential for cytotoxic T-cell response (Bennick and Yewdell, 1990) and high levels of total protein expression have been observed using promoters active during the late stage of viral replication. We decided to incorporate the promoters corresponding to the constitutively expressed 7.5k gene (Mackett *et al.,* 1984) and a constitutively expressed synthetic promoter EL noted for high-level expression (Chakrabarti and Moss, unpublished observation).

A useful feature of v*Not*I/tk that must be retained in any new vector is the ability to discriminate for recombinant viral genomes using selection against an active thymidine kinase gene. The introduction of the *Apa*I site within the coding sequence for the tk gene necessitates an increase in the total number of amino acids to accommodate the restriction enzyme site. A comparison of the amino acid sequence for thymidine kinase genes from a variety of animal and viral species showed the region of greatest heterogeneity was at the N terminus of the protein, suggesting that this region of the protein could tolerate a modest increase in the number of amino acids.

The recombination-independent cloning vectors were constructed by making plasmid intermediates containing the modified thymidine kinase (tk) gene and replacing the tk sequence in the v*Not*I/tk genome by homologous recombination. Two sets of oligonucleotide pairs were constructed that, when annealed, contained the promoter for the 7.5k gene or the synthetic EL sequence and restriction sites for *Not*I and *Apa*I. The modified thymidine kinase genes were constructed by annealing the double-stranded oligonucleotides and ligating the product into the *Not*I site present at the beginning of the thymidine kinase gene in pJ*Not*I/tk, a plasmid containing the *Hin*dIII J fragment from v*Not*I/tk. The oligonucleotide pairs annealed to and eliminated the *Not*I site in pJ*Not*I/tk, generating a new *Not*I site closely followed by an *Apa*I site after the promoter and flanking the nucleotides coding for the initial methionine in the thymidine kinase gene,

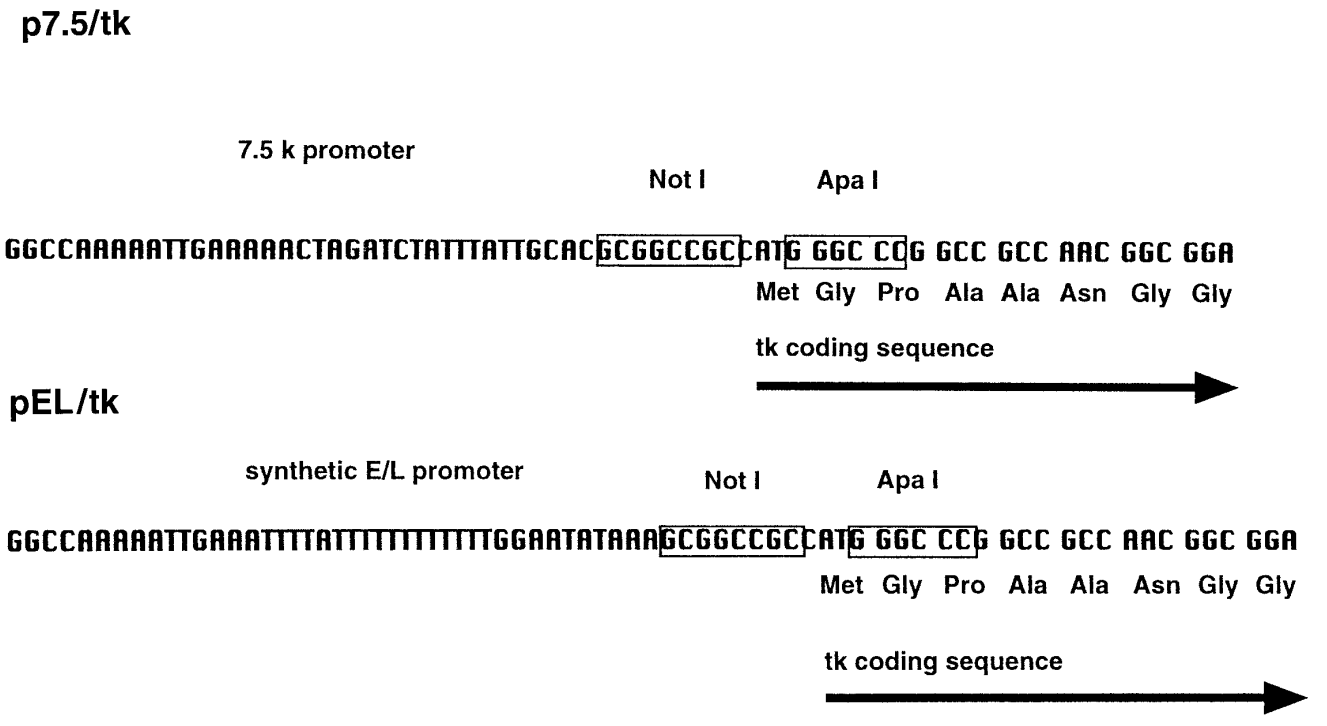

FIG. 1. Nucleotide sequences of p7.5/tk and pEL/tk. The nucleotide sequence of the promoter and beginning of the thymidine kinase gene for v7.5/tk and vEL/tk.

resulting in plasmids p7.5/tk and pEL/tk (Fig. 1). The acquisition of the *Apa*I site was verified by restriction enzyme analysis of plasmid DNA and the nucleotide sequence of the thymidine kinase gene promoter was determined and found to be as depicted in Fig. 1.

The recombinant viruses derived from p7.5/tk and pEL/tk were isolated using a strategy relying on positive drug selection in the presence of HAT (hypoxanthine, aminopterin, thymidine) (Weir *et al.*, 1982). The viruses vp*Not*I, a virus that contains a copy of pBR322 inserted at the *Not*I site of v*Not*I/tk (Merchlinsky and Moss, 1992), and v*Not*I/lacZ/tk, a virus with a copy of the lacZ gene interrupting the thymidine kinase in v*Not*I$^-$ (Merchlinsky and Moss, 1992), are thymidine kinase-negative (tk$^-$) viruses that are identical to v*Not*I/tk except for the inserted DNA at the beginning of the tk gene. The plasmids p7.5/tk and pEL/tk were recombined with vp*Not*I and v*Not*I/lacZ/tk helper viruses in CV-1 cells and the infected monolayers were harvested and passaged in the presence of HAT medium on Hutk$^-$ cells. Individual plaques were passaged and isolated an additional three rounds on Hutk$^-$ cells before expansion and analysis.

Analysis of the structure of the viral genomes

The growth of v7.5/tk and vEL/tk virus in HAT supplemented medium implies these viruses, in contrast to vp*Not* and v*Not*/lacZ/tk, contain an active tk gene. However, an active tk gene could arise from multiple crossovers that delete the 7.5k or EL promoter sequences, generating a virus with the normal tk promoter. The v7.5/tk and vEL/tk genomes should contain a unique site for both *Not*I and *Apa*I within the *Hin*dIII J fragment. The genomic structure of the isolated virus stocks was analyzed by restriction enzyme digestion of DNA in agarose plugs derived from virus-infected cells using *Not*I or *Apa*I and electrophoresis of the products through 1% agarose (Fig. 2). Uncut vaccinia WR (lane 2) migrates at a size of 190 kb as compared with multimers of bacteriophage λ (lane 1). After digestion with *Not*I, vaccinia WR is cleaved into two fragments approximately 150 and 40 kb in length (seventh lane from left), whereas v*Not*/tk, vEL/tk, and v7.5/tk were cleaved into fragments of about 110 and 80 kb. When the same samples were digested with *Apa*I, only one fragment the size of the uncut genome was observed for both vaccinia WR and v*Not*/tk while vEL/tk and v7.5/tk gave the same-sized fragments observed after digestion with *Not*I. Therefore, both v7.5/tk and vEL/tk contain a unique site for both *Apa*I and *Not*I, the sites are at the same locus as the *Not*I site in v*Not*/tk, and the sites are in a more central location in the genome than the *Hin*dIII F fragment which contains the *Not*I site in vaccinia WR. The background of cellular DNA fragments was more pronounced in the *Apa*I digestion, which has a 6-bp recognition site, than in the *Not*I digest.

The *Not*I and *Apa*I restriction sites in vEL/tk and v7.5/tk were shown to be present within the vaccinia *Hin*dIII J fragment by Southern blot analysis of DNA from cells infected with the viruses (data not shown). Also, the nu-

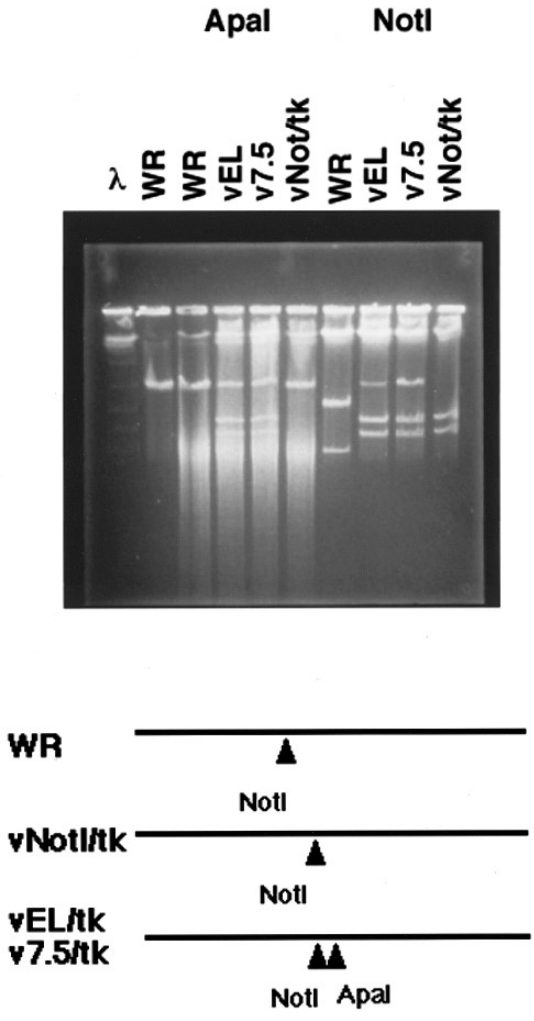

FIG. 2. Restriction enzyme analysis of virus genomes using CHEF gel. BSC-1 cells were infected at a m.o.i. of approximately 10 PFU/cell by vaccinia WR, vEL/tk, v7.5/tk, or v*Not*I/tk. After 24 h the cells were harvested and formed into agarose plugs. The plugs were equilibrated in the appropriate restriction enzyme buffer and 1 mM PMSF for 16 h at room temperature; incubated with restriction enzyme buffer, 100 ng/ml bovine serum albumin, and 50 units *Not*I or *Apa*I for 2 h at 37° (*Not*I) or room temperature (*Apa*I); and electrophoresed in a 1.0% agarose gel on a Bio-Rad CHEFII apparatus for 15 h at 6 V/cm with a switching time of 15 s. The leftmost sample contains λ DNA, the second sample contains undigested vaccinia DNA, and the remainder of the samples contain the DNA samples described above each well digested with *Apa*I or *Not*I, where vEL refers to vEL/tk and v7.5 refers to v7.5/tk. The lower portion of the figure is a schematic map showing the location of the *Not*I and *Apa*I sites in each virus.

cleotide sequence of the promoter region in v7.5/tk and pEL/tk was determined from DNA fragments derived from PCRs using primers flanking the promoter for the thymidine kinase gene and shown to match the sequence displayed in Fig. 1.

Quantitation of promoter activity

The v7.5/tk and vEL/tk vectors have been designed to constitutively express elevated levels of insert protein in comparison to v*Not*I/tk. The level of RNA synthesis was measured by infecting confluent BSC-1 cells in the presence and absence of cytosine arabinoside (AraC) at a m.o.i. of 5, harvesting the cells, isolating the RNA using Trizol (Life Technologies), and analyzing the level of thymidine kinase RNA synthesis by primer extension (Weir and Narayanan, 1990). Incubation with AraC blocks viral DNA replication, allowing one to identify the class of viral promoter. The early class of viral promoters is active prior to DNA replication and will be unaffected by AraC in the infection. Late promoters are expressed only after the onset of DNA replication and their activity is abrogated in the presence of AraC. Perusal of the products on a denaturing polyacrylamide gel demonstrated that significantly more (estimated to be at least 10-fold) tk RNA primer extension products were synthesized in vEL/tk infections as compared with v*Not*/tk infections. In cells infected with v*Not*/tk a single RNA start site insensitive to AraC incubation was observed, whereas in vEL/tk infections two distinct start sites, one resistant to AraC and corresponding to the appropriate early start site (Davison and Moss, 1989a) and one sensitive to AraC and corresponding to the appropriate late start of RNA (Davison and Moss, 1989b), were observed (data not shown). The pattern of RNA species derived from infection with v7.5/tk was similar to that observed for vEL/tk, with the absolute levels of RNA expression intermediate to that observed for vEL/tk and v*Not*/tk.

To verify the levels of expression for genes inserted into the viral vectors the *E. coli gusA* gene encoding for β-glu was cloned into v*Not*I/tk, v7.5/tk, and vEL/tk viral vectors and the relative promoter strength was measured. The DNA fragment encoding for the β-glu gene was inserted into plasmids containing each promoter, generating pJ*Not*/tk-GUS, p7.5/tk-GUS, and pEL/tk-GUS. The correct orientation of the insert β-glu gene in pJ*Not*/tk was verified by restriction enzyme analysis. The plasmids containing β-glu were recombined with v*Not*I/tk (Earl and Moss, 1991) and recombinant virus was identified by incubation of the monlayers with 5-bromo-4-chloro-3-indolylglucuronide (X-glu) (Carroll and Moss, 1995). The recombinant viruses were passaged for three rounds through Hutk$^-$ cells and expanded to generate the viral stocks v*Not*I/tk-GUS, v7.5/tk-GUS, and vEL/tk-GUS. The structures of the recombinant viruses were verified by Southern blot analysis (data not shown).

The level of expression of β-glu by v*Not*I/tk-GUS, v7.5/tk-GUS, and vEL/tk-GUS was measured from infected confluent monolayers of BSC-1 cells in the presence or absence of AraC (Fig. 3). The level of β-glu expression for v7.5/tk-GUS and vEL/tk was much higher than that observed for v*Not*I/tk-GUS and highest (approximately 20-fold higher) in vEL/tk-GUS. Expression of β-glu was observed for all three viruses in the presence of cytosine arabinoside, indicating that each promoter is a member of the early class of viral promoters. The level of β-glu

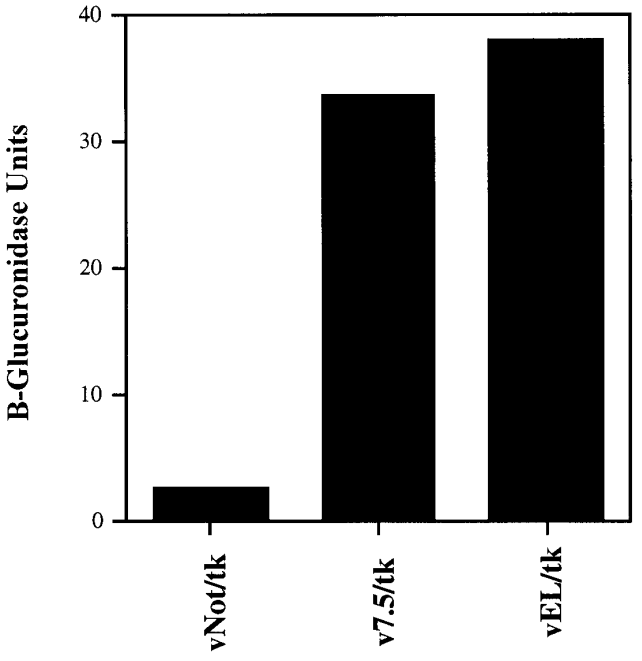

FIG. 3. Promoter strength of recombinant viruses. The units of β-glucuronidase activity were determined as described by Miller (1972) as adapted for 96-well plates. The A_{405} values were determined on a microplate reader (Dynatech MR3000) and the β-glucuronidase activity was determined by comparison with β-glucuronidase (Clontech) standards analyzed in the same assay.

in v*Not*I/tk-GUS was unchanged in the presence or absence of AraC, indicating that this promoter is active only early during infection, whereas the β-glu levels in v7.5/tk-GUS and vEL/tk-GUS were lower in the presence of AraC, indicating these promoters are active both early and late during infection.

Bromodeoxyuridine sensitivity of virus vectors

The v7.5/tk and vEL/tk vectors were initially isolated by growth in the presence of HAT supplemented medium and are designed to contain an active tk gene to allow selection for viruses with inserts via passage in Hutk$^-$ cells in the presence of bromodeoxyuridine (Earl and Moss, 1991). Both vectors were tested by plaque assay in Hutk$^-$ cells using drug selection. Incubation without drug or with HAT supplement at a concentration sufficient to interfere with plaque formation for vp*Not* or v*Not*/lacZ/tk, (data not shown) gave an equivalent number of like-sized plaques. Surprisingly, an equal number of plaques, albeit much smaller in size, were observed for vEL/tk with incubation in 25 μM bromodeoxyuridine, a concentration sufficient to interfere with the ability of vaccinia WR to plaque on Hutk$^-$ cells (data not shown). Addition of 125 μM bromodeoxyuridine was sufficient to inhibit plaque formation for vEL/tk and v7.5/tk (data not shown). The higher concentration of bromodeoxyuridine did not interfere with the growth of tk$^-$ viruses such as v*Not*I/lacZ/tk (data not shown) or affect the viability of the Hutk$^-$ cell line.

Construction of recombinant virus by direct ligation

Direct ligation vectors will be useful for the generation of complex expression libraries only if the production of infectious virus from the naked DNA is facile and efficient. Previously, helper virus activity was supplied in cells transfected with DNA ligation products by coinfection with conditionally lethal temperature-sensitive virus (Merchlinsky and Moss, 1992) or fowlpox (Scheiflinger *et al.,* 1992). Since high levels of replicating wild-type virus interfere with the ability to package viral DNA and vaccinia virus can recombine with the input DNA, only conditionally defective vaccinia virus can be used as helper (Merchlinsky and Moss, 1992). Fowlpox should be a superior helper virus as it is used at 37°, will not revert to a highly replicating strain, and, because it does not recombine with vaccinia DNA or productively infect primate cell lines, can be used at a higher m.o.i. than vaccinia. To determine if fowlpox can serve as an efficient helper virus a series of wells from a 12-well plate containing BSC-1 cells were infected with varying m.o.i. of fowlpox and transfected with full-length vaccinia WR DNA; the cells were harvested after 24, 48, or 72 h and the virus titer was determined. Transfection of DNA or fowlpox infection alone resulted in no plaques. The yield of vaccinia virus, as measured by plaque assay, increased with later harvest and was proportional to the m.o.i. of the fowlpox infection.

A 1.1-kb fragment of the ovalbumin cDNA (Pulaski *et al.,* 1996) was used as a model insert to study the generation of functional recombinant virus by direct ligation. The ovalbumin insert was modified as described under Materials and Methods to include a *Not*I site at its 5′ end, translation stop codons, a vaccinia transcription stop signal, and an *Apa*I site at its 3′ end. This insert was digested with *Not*I and *Apa*I and ligated with purified vEL/tk DNA arms that had been digested with *Not*I and *Apa*I. The ligation mix was transfected into fowlpox-infected BSC-1 cells, the infected cells were harvested, and after 3 days, the cell extract was passaged on Hutk$^-$ cells in the presence or absence of 125 μM bromodeoxyuridine. The titer obtained without drug selection was 2.7×10^3 PFU, and that with drug selection, 2.8×10^3 PFU. Individual plaques were picked from Hutk$^-$ cells in the presence and absence of bromodeoxyuridine and tested for the presence of the ovalbumin insert by dot-blot hybridization with an ovalbumin cDNA probe. All 15 plaques picked in the presence of bromodeoxyuridine and all 10 plaques picked in its absence contained the ovalbumin insert. These viruses were named vEL/tk-ovaFL. Two individual clones were expanded further and tested for the ability to sensitize host cells to lysis by ova 257–264-specific cytotoxic T lymphocytes (CTLs). The results of

TABLE 1

CML Assay on Recombinant Vaccinia Virus-Infected Cells

MC57G cells	Effector:target ratio 2:1	10:1
	Percentage specific lysis	
Untreated	−1.3	−1.3
ova 257–264 peptide, 1 μM	54	83
vEL/tk	−0.5	0
v7.5/tk-ova, homologous recombination	50	78
vEL/tk-ova, homologous recombination	47	71
vEL/tk-ovaFL, direct ligation clone 1	48	70
vEL/tk-ovaFL, direct ligation clone 2	46	74

Note. Virally infected MC57G cells were generated as described under Materials and Methods. One sample of MC57G cells was treated with ova 257–264 peptide (1 μM); another sample of cells was left untreated. Cells were incubated with two different ratios of ova-specific cytotoxic T lymphocytes for 4 h at 37° and percentage specific lysis was determined as described under Materials and Methods.

this experiment are shown in Table 1. As controls, vaccinia recombinants for an ova 257–264 minigene, v7.5/tk-ova and vEL/tk-ova, were generated by homologous recombination. These ova peptide recombinant viruses were tested in concert with the vEL/tk-ovaFL clones for the ability to sensitize host cells to lysis by ova-specific CTLs. As shown in Table 1, infection with either full-length or minigene ovalbumin vaccinia recombinants was as efficient as pulsing with 1 μM purified ova 257–264 peptide for sensitization of target cells to lysis by ova-specific CTLs.

DISCUSSION

Large DNA viruses are particularly useful expression vectors for the study of cellular processes as they can express many different proteins in their native form in a variety of cell lines. The gene of interest is normally cloned in a plasmid under the control of a promoter flanked by sequences homologous to a nonessential region in the virus and the cassette is introduced into the genome via homologous recombination. A panopoly of vectors for expression, selection, and detection have been devised to accommodate a variety of cloning and expression strategies. However, homologous recombination is an ineffective means of making a recombinant virus in situations requiring the generation of complex libraries or when the insert DNA is large. An alternative strategy for the construction of recombinant genomes relying on direct ligation of viral DNA "arms" to an insert and the subsequent rescue of infectious virus has been explored for the genomes of poxvirus (Merchlinsky and Moss, 1992; Pfleiderer *et al.,* 1995; Scheiflinger *et al.,* 1992), herpesvirus (Rixon and McLauchlan, 1990), and baculovirus (Ernst *et al.,* 1994).

Poxviruses are ubiquitous vectors for studies in eukaryotic cells as they are easily constructed and engineered to express foreign proteins at high levels. The wide host range of the virus allows one to faithfully express proteins in a variety of cell types. Direct cloning strategies have been devised to extend the scope of applications for poxvirus viral chimeras in which the recombinant genomes are constructed *in vitro* by direct ligation of DNA fragments to vaccinia "arms" and transfection of the DNA mixture into cells infected with a helper virus (Merchlinsky and Moss, 1992; Scheiflinger *et al.,* 1992). This approach has been used for high-level expression of foreign proteins (Pfleiderer *et al.,* 1995) and to efficiently clone fragments as long as 26 kb (Merchlinsky and Moss, 1992).

Use of the thymidine kinase gene as the insertion site for foreign DNA in the viral vectors allows implementation of selection protocols for distinguishing recombinants from helper or wild-type genomes. The level of tk expression in v7.5/tk and vEL/tk should be much higher than in vaccinia WR or v*Not*/tk. However, the *Apa*I site at the beginning of the tk gene in v7.5/tk and vEL/tk was formed from v*Not*/tk by adding extra nucleotides at the *Not*I site. The additional nucleotides increase the amino acid sequence at the N terminus of the wild-type tk gene from Met–Asn–Gly to Met–Gly–Pro–Ala–Ala–Asn–Gly in v7.5/tk and vEL/tk. Modifications in the expression level and N-terminal amino acid sequence of the thymidine kinase enzyme may increase (more protein) or decrease (different sequence) the sensitivity of the virus to bromodeoxyuridine. Plaques, albeit smaller, were observed with v7.5/tk and vEL/tk infection at a concentration of bromodeoxyuridine sufficient to completely suppress plaque formation for wild-type vaccinia WR. Plaque formation was suppressed at fivefold higher concentrations of bromodeoxyuridine, a level of drug that does not interfere with the viability of the cells or impede the ability of tk^- virus to form plaques. The explanation for the altered sensitivity to bromodeoxyuridine awaits further characterization of the protein as the altered thymidine kinase enzyme may have a different reaction rate for formation of the monophosphate form of the bromodeoxyuridine or a reduced ability to bind bromodeoxyuridine.

The application of v7.5/tk and vEL/tk to protocols for construction of complex expression libraries requires efficient production of recombinants and strong selection to eliminate or minimize wild-type and contaminants. The use of two restriction sites allows one to design cloning strategies for the oriented cloning of DNA fragments such as products of PCR (Pfleiderer *et al.,* 1995) and increases the frequency of the desired recombinant as wild-type genomes are no longer generated by ligation of vaccinia arms. When v7.5/tk or vEL/tk DNA previously digested with *Not*I and *Apa*I was transfected into cells infected with fowlpox the virus titer was 100-fold lower than for intact uncut DNA (data not shown). Also, all plaques isolated in the presence and absence of bromo-

deoxyuridine (15 with bromodeoxyuridine and 10 without) during the isolation of vEL/tk-ovaFL contained the ovalbumin insert. The efficiency of infectious virus formation increased with the amount of fowlpox helper up to at least a m.o.i. of 1. Also, transfection of large DNA fragments varies with the type and preparation of lipid (M. Carroll, personal communication) and we are presently assaying different lipid mixtures and cell types as well as investigating other parameters to find optimum conditions for the direct ligation protocol. The v7.5/tk and vEL/tk vectors provide a set of universally applicable direct ligation cloning vectors for poxviruses.

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