### ABSTRACT

Vaccinia virus, a member of the poxvirus family, is widely used as a mammalian cell expression vector. Vaccinia virus replicates in the cytoplasm and has its own transcriptional system, making it necessary to use viral promoters. Here, we describe the design, construction, and use of a 40-bp synthetic, vaccinia virus promoter with largely overlapping early and late regulatory elements. Convenient plasmid transfer vectors are depicted for expression of one or two genes under control of strong early/late promoters and allowing for thymidine kinase (TK) or antibiotic selection of recombinant viruses.

### INTRODUCTION

Vaccinia virus, a member of the poxvirus family, has been developed as a vector for the expression of genes in mammalian cells (13). As a research tool, recombinant vaccinia viruses are used to synthesize biologically active proteins and investigate structure-function relationships, to determine the targets of humoral and cell-mediated immunity and to investigate the immune responses needed for protection against specific infectious agents. In addition, vaccinia virus vectors are being tested as live recombinant vaccines and for large-scale production of proteins. Advantages of the vaccinia virus vector system include a large capacity for foreign DNA, cytoplasmic site of expression and wide host range. Since the initial description of vaccinia virus vectors (12,15), considerable new information regarding the molecular biology of vaccinia virus has been obtained (14). Detailed mutagenesis of vaccinia virus early and late (E/L) promoters (7,8) provided a basis for the improvement of expression vectors. A transfer vector containing a strong synthetic late promoter that contains a run of 20 T-residues has been described (6). Nevertheless, early gene expression is important for studies in which the late cytopathic effects of vaccinia virus interfere with in vitro or in vivo assays, protein processing or presentation of antigens to the immune system (2,5). Naturally occurring, nonoverlapping, tandem E/L promoters of moderate strength, known as P7.5 and H5 (4,16), have been used for gene expression. Here, we describe the construction and use in expression vectors of a compact, synthetic, vaccinia virus promoter in which optimized early and late regulatory sequences were largely overlapped.

### MATERIALS AND METHODS

#### Construction of Recombinant Plasmids

Synthetic oligonucleotides were prepared with a Model 394 DNA/RNA Synthesizer (PE Applied Biosystems, Foster City, CA, USA) and ligated to plasmids that had been cut with restriction endonucleases using standard methods.

#### Table 1. Expression of E. coli lacZ by Recombinant Viruses

<table>
<thead>
<tr>
<th>Virusa</th>
<th>Promoter</th>
<th>β-galactosidase Synthesis (units/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vSC56</td>
<td>Synthetic early/late</td>
<td>62 539</td>
</tr>
<tr>
<td>vGK</td>
<td>P7.5 early/late</td>
<td>1271</td>
</tr>
<tr>
<td>vMJ21</td>
<td>P7.5 early</td>
<td>256</td>
</tr>
<tr>
<td>vMJ23</td>
<td>P28 late</td>
<td>490</td>
</tr>
<tr>
<td>vSC8</td>
<td>P11 late</td>
<td>31 157</td>
</tr>
<tr>
<td>vMJ344</td>
<td>Synthetic early</td>
<td>513</td>
</tr>
<tr>
<td>vMJ345</td>
<td>Synthetic early</td>
<td>631</td>
</tr>
<tr>
<td>vMJ441</td>
<td>Synthetic late</td>
<td>52 400</td>
</tr>
<tr>
<td>vMJ454</td>
<td>Synthetic late</td>
<td>37 500</td>
</tr>
</tbody>
</table>

*Viruses vSC8 (3), vMJ21, vMJ23, vMJ344, vMJ345, vMJ441 and vMJ454 (7,8) have been described elsewhere.*
Construction of Recombinant Vaccinia Viruses

Procedures for making recombinant vaccinia viruses have been described in detail (9). Recombinant vaccinia virus vSC56 was constructed as follows. Overlapping complementary oligonucleotides AGCTTAAAAATTGAAATTTATTTTTTTTTTTTGGAATATAAATAAGC and ATTTTTAACTTTAAATAAAAAAAAAACCTTATTATTATTCGAGCT containing the synthetic E/L promoter sequence were annealed and ligated to HindIII and XhoI-cleaved pMJ4 (7) to form the transfer plasmid pSC56. Then, pSC56 was transfected into cells infected with vaccinia virus strain WR, and vSC56 was isolated by standard procedures (3).

Measurement of β-Galactosidase Activity

BS-C-1 cells \((2 \times 10^5)\) in the absence (-) or presence (+) of 40 µg/mL of cytosine arabinoside (araC) were infected with \(2 \times 10^6\) plaque-forming units (pfu) of recombinant vaccinia virus containing the indicated natural (P7.5, P28 or P11) or synthetic promoters regulating lacZ. The cells were harvested at 24 h, lysed and assayed for β-galactosidase (β-gal) activity using o-nitrophenyl β-D-galactopyranoside (7).

RESULTS AND DISCUSSION

Design of a Synthetic E/L Promoter

Early promoters consist of a 15-bp critical region, in which most single nucleotide substitutions have a major effect on expression, separated by an 11-bp spacer from a 7-bp region within which initiation with a purine usually occurs (7). Late promoters have an upstream core sequence of about 20 bp rich in T and A residues, separated by a spacer of about 6 bp from a highly conserved TAAAT element within which transcription initiates (8). We considered that early and late promoter elements might be largely overlapped if care were taken to keep optimal nucleotides in their correct locations. However, such overlapping promoters are unprecedented, and it was not known whether they would be nega-

tively regulated by putative stage-specific repressor proteins. Figure 1 shows optimized elements of an early promoter, a strong synthetic late promoter and the overlapped synthetic E/L promoter. An A residue was inserted into the distal part of the T tract of the E/L promoter so that the modified T tract would simultaneously fulfill the requirements for part of the core and spacer region of an early promoter as well as the core region of a late promoter. Although the majority of late promoters have a G residue following the TAAAT initiation sequence, which forms a methionine initiation codon, substitution of an A has a minor effect on expression (8) and allows the downstream insertion of a complete open reading frame (ORF). Because of the extensive overlapping of
promoter elements, the late and early RNA start sites of the 40-bp synthetic E/L promoter were predicted to be only five nucleotides apart.

**Determination of Promoter Strength**

To evaluate the strength of the synthetic E/L promoter, we made recombinant vaccinia virus vSC56 containing the promoter regulating the *E. coli lacZ* gene. The predicted early and late RNA start sites were verified by primer extension analysis of RNA obtained from BS-C-1 cells infected with vaccinia virus in the presence or absence of cycloheximide, respectively (indicated in Figure 1). As occurs with other late promoters, slippage of RNA polymerase at the TAAAT transcriptional initiation site caused the formation of a 5′ poly(A) leader, which has been predicted to enhance translation. β-gal activity was measured in lysates of BS-C-1 cells that were infected with vSC56 in the presence or absence of araC, an inhibitor of DNA replication, to measure early or total expression, respectively (Table 1). In parallel, cells were infected with previously made recombinant vaccinia viruses that express *lacZ* under control of other vaccinia virus promoters. vSC56 gave the highest activity, greatly exceeding that of a virus utilizing the P7.5 promoter (vGK) and higher than that of a virus with the natural late P11 promoter (vSC8). Furthermore, the activity of vSC56 equaled or exceeded that of viruses using the synthetic late promoter (vMJ441 and vMJ454). Enzyme activity was detected within 2 h after infection with vSC56, and β-gal subsequently appeared as a prominent Coomassie® Brilliant Blue-stained band in sodium dodecyl sulfate (SDS) polyacrylamide gels (data not shown). When early promoter function was determined in the presence of araC, vSC56 again gave the highest value of all tested viruses. The synthetic E/L promoter also provided higher expression of the hepatitis B virus surface antigen and the human immunodeficiency virus gp160 genes than the P7.5 promoter (data not shown).

**Plasmid Transfer Vectors**

The synthetic E/L promoter is only 40-bp-long, making it simple to genetically manipulate. Figure 2 depicts plasmid transfer vectors containing one or two synthetic E/L promoters, unique

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**Figure 2. Plasmid transfer vectors.** The pSC59 and pSC65 transfer plasmids were constructed by ligating synthetic duplex oligonucleotides into pBR322 derivatives. pJS4 and pJS5 are derivatives of pUC18. Unique restriction endonuclease sites for expression cloning are boxed. Other unique restrictions are depicted without a box. PE/L, synthetic E/L promoter; tk<sub>L</sub> and tk<sub>R</sub>, sequences from the left and right parts of the vaccinia virus thymidine kinase gene; lacZ, *E. coli* gene expressing β-gal; gpt, *E. coli* gene encoding guanosine phosphoribosyltransferase; amp<sup>R</sup>, ampicillin-resistance gene. The sequences of the promoter and expression cloning sites are shown at the bottom. Compiled sequences of the plasmids are available upon request.
restriction endonuclease sites for insertion of ORFs, reporter or antibiotic selection markers and flanking sequences for homologous recombination. The plasmid pSC59 contains 3 unique restriction sites next to the synthetic E/L promoter and allows selection of recombinant viruses in thymidine kinase negative cell lines (11). The plasmid pSC65 provides 8 unique restriction endonuclease sites and the lacZ gene, which may be used for positive identification of recombinant viruses and subsequently for identifying infected cells. The plasmid pJS4 contains “back-to-back” synthetic E/L promoters with two sets of 6–10 unique restriction endonuclease sites; pJS5 also contains the E. coli gpt gene for antibiotic selection (10). These transfer vectors and modified versions have been successfully used in our laboratory and those of others to produce recombinant vaccinia viruses following standard protocols (9). The synthetic E/L promoter has been adapted for use with the non-replicating modified vaccinia virus Ankara strain (17) and with a vector providing plaque selection of recombinant viruses (1).

REFERENCES


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