A myxoma virus intergenic transient dominant selection vector

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The purpose of this study was to construct an intergenic transfer vector which can be used for the generation of recombinant myxoma viruses (MVs) expressing a foreign gene insert. Recombinant MVs expressing the *Escherichia coli* lacZ gene were constructed *in vitro* by transfection of MV-infected rabbit cells with a transfer expression vector, and isolated under growth conditions selecting for transient expression of the *E. coli* gpt gene. The effect of inserting foreign DNA sequences between the viral thymidine kinase gene and open reading frame MF8a upon the transcription of these genes was investigated.

The myxoma virus (MV) belongs to the family Poxviridae, genus leporipoxvirus (Fenner, 1976). The MV is the aetiological agent of myxomatosis, a usually fatal disease of the European rabbit (*Oryctolagus cuniculus*). In 1950, the virus was deliberately released in Australia as a biological control agent for the wild European rabbit. Following its initial release, devastating epizootics occurred with a greater than 99% mortality rate. However, myxomatosis fell short of total eradication of the rabbit. The evolution of attenuated viral strains and the development of host resistance are thought to be responsible for the now diminished effectiveness of the disease (Fenner & Ratcliffe, 1965; Fenner, 1983).

The long-term goal of this project is the construction of recombinant MVs expressing rabbit-specific immunocontraceptives. Such recombinant viruses could be used to enhance myxomatosis in the biological control of the wild European rabbit in Australia. To be successful, recombination protocols need to be developed that avoid disruption of native viral gene expression which can result in the attenuation of the recombinant virus.

A variety of techniques has been developed for the construction of recombinant poxviruses. The recombination techniques are usually designed to introduce a new phenotypic marker to distinguish rare plaques containing recombinant viruses from the much larger population of plaques containing parental virus. These recombination protocols include thymidine kinase (TK)-negative phenotypic selection (Mackett *et al.*, 1982), β-galactosidase expression with visual colour selection (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986) and expression of a dominant selective marker for neomycin resistance (Franke *et al.*, 1985).

The insertion and expression of the *Escherichia coli* xanthine–guanine phosphoribosyltransferase (XGPRT, gpt) gene has been used as a dominant selectable marker for mycophenolic acid (MPA) resistance in the isolation of recombinant vaccinia viruses (VV) (Boyle & Coupar, 1988; Falkner & Moss, 1988). Falkner & Moss (1990) have further developed this isolation method by selecting for VV recombinants transiently expressing the gpt gene. The authors refer to their recombination protocol as transient dominant selection (TDS).

Recombinant leporipoxviruses expressing a variety of *E. coli* genes have been reported (Macaulay & McFadden, 1989; Upton *et al.*, 1990, 1991; Opgenorth *et al.*, 1992). The transfer vectors used in these studies were not designed to be used as versatile vectors for the expression of alternative foreign gene inserts. Additionally, the vectors were constructed such that recombinant viruses were generated by insertional inactivation of viral genes. Insertional inactivation of some poxvirus genes has been shown to be associated with attenuation *in vivo* (Buller *et al.*, 1985, 1988; Upton *et al.*, 1990, 1991; Opgenorth *et al.*, 1992). We have constructed a MV intergenic transfer expression vector which could be used to insert any gene of interest into the genome of MV. Recombinant MVs expressing the *E. coli* lacZ gene, as a convenient marker, were constructed employing the TDS method.

Plaque formation of the Lausanne (Lu) strain of MV (Bouvier, 1954; Russell & Robbins, 1989), is completely inhibited on rabbit SIRC cells (ATCC CCL60) with MPA (1 μg/ml) in the medium. The MPA inhibition of Lu plaque formation is fully reversible upon the addition of guanine (25 μg/ml) to the medium (data not shown). The cell monolayers did not require pretreatment with
MPA nor the addition of HAT supplement for MPA inhibition of virus plaque formation. This is in contrast to the results of Boyle & Coupar (1988) who observed that the addition of HAT supplement was required to allow expression of MPA inhibition of VV plaque formation on CV-1 cells.

The MV TK gene is located approximately 58 kb from the left end of the virus genome. The TK gene and adjacent open reading frames (ORFs) have been cloned in a restriction fragment contained in pUrTK1 and their DNA sequences fully determined (Jackson & Buls, 1992). The putative early promoter motif for ORF MF8a overlaps the TK gene stop codon by one nucleotide. A natural Dral restriction endonuclease site is located within the TK gene stop codon, overlapping the TK gene early transcriptional termination signals. This Dral site was selected as an intergenic insertion point for foreign DNA sequences in the construction of recombinant MVs.

The MV intergenic transfer vector pUrTK13 (Fig. 1) was constructed by DNA manipulation of pUrTK1. The DNA sequences inserted within the intergenic region between the TK gene and ORF MF8a are shown in Fig. 2. The vector pUrTK13 essentially contains the insertion into the Dral site, a ScaI linker (Stratagene, no. 901076) to reconstitute the TK gene stop codon, and an XbaI–HindIII fragment derived from pBCB08 (Coupar et al., 1986) containing a multiple cloning site (MCS) and 109 bp of VV DNA encoding the P11 late promoter. The short DNA sequence between the HindIII site and the remnant Dral site is derived from a previous construction (R. Jackson, unpublished results). The VV P7.5 early/late promoter and the E. coli gpt gene were excised from pGpt07/14 (Boyle & Coupar, 1988) on an EcoRI–ApaI fragment, which is devoid of simian virus 40 DNA sequences, and inserted into the NheI site located in pGem3 nucleotide position 2625. The restriction sites of the plasmid vector pGem3, which are duplicated in the MCS of pUrTK13, were removed by digestion with the respective enzymes, then the ends of the DNA fragments were modified using T4 DNA polymerase and religated. The E. coli lacZ gene cassette was excised from pGH101 (Herman et al., 1986) on a BamHI fragment and ligated into the BamHI site present within the MCS of pUrTK13, generating pUrTK13–β-gal. The lacZ gene contained in pUrTK13–β-gal is positioned in-frame with the initiation codon for the VV gene encoding an 11K protein.

Recombinant MVs were prepared by infection of confluent monolayers of SIRC cells, in 60 mm diameter dishes, with MV strain Lu at an m.o.i. of 0.2 p.f.u. per cell. At 1 h post-infection, 5 μg of pUrTK13–β-gal was used to transfect the infected cells using lipofectin reagent (Gibco-BRL) as described by the manufacturer.
apparent the infected monolayers were overlaid with MEM containing 1% (w/v) agar and 300 µg/ml X-gal to visualize plaques containing recombinant MVs expressing β-galactosidase.

A full description of the homologous recombination events involved in the TDS protocol can be found in Falkner & Moss (1990). Briefly, a single crossover event results in the integration of the entire TDS vector into the homologous region of the MV genome. The resulting recombinant virus contains the DNA insertion flanked by the direct duplication of the viral sequences involved in the crossover. As the gpt gene is flanked by directly repeated sequences it is readily deleted by subsequent recombination events. However, while these intermediate recombinants are grown under selection, viruses expressing the gpt gene can be selectively isolated. Upon removal of selection, viruses that are deleted for the gpt gene are able to grow. Depending upon the position of the second recombination event with respect to the foreign DNA insertion, either the desired recombinant or parental DNA segment is retained. In the transformation experiment described above, 25% of the progeny virus expressed β-galactosidase. One recombinant, Lu13Z, was chosen at random, plaque purified and used for further analysis.

Intracellular viral particles were prepared from virus-infected RK13 cells (ATCC CCL37) as described by Russell & Robbins (1989). Viral genomic DNA was prepared by suspension and lysis of the viral particles in 4 M-guanidinium thiocyanate, 1% (w/v) N-lauroylsarcosine, 50 mM-2-mercaptoethanol, 20 mM-sodium acetate (pH 5.5) at room temperature for 10 min. The DNA solution was extracted twice with phenol–chloroform–isoamyl alcohol (50:50:1), twice with chloroform and the aqueous phase dialysed against 10 mM-Tris-HCl pH 7.5, 1 mM-EDTA. Southern blots of restriction-digested Lu and Lu13Z DNA were probed with [α-32P]ATP-labelled probes: the EcoRI–SalI MV fragment of pUrTK1, the BamHI lacZ fragment of pGH101, the plasmid vector pGem3 and the EcoRI fragment from pGpt07/14 containing the P7.5 promoter and gpt gene (Fig. 3). The hybridization pattern of Lu13Z DNA indicated that the P11 promoter and lacZ gene were correctly inserted within the MV 4·1 kb BamHI PI fragment, between the TK gene and ORF MF8a. Recombinant Lu13Z DNA failed to hybridize to the P7.5 promoter, gpt gene or pGem3.

Viral early and late RNAs were prepared from infected RK13 cells as described previously (Jackson & Bults, 1992). Polyadenylated RNA was isolated from total RNA preparations by oligo(dT) chromatography using an mRNA purification kit (Pharmacia) as described by the manufacturer. Northern blot analyses of Lu and Lu13Z mRNAs for lacZ and MF8a gene expression using as probes the [α-32P]ATP-labelled lacZ BamHI fragment from pGH101 and the 32P 5′ end-labelled oligonucleotide MF8a-1 (CTCTTTGATGCC-GATCCCCGCTCAGAG) are shown in Fig. 4.

As expected the lacZ gene is expressed by Lu13Z as a late gene under the control of the P11 promoter. Insertion of foreign DNA sequences at this intergenic site has no observable effect upon the expression of MF8a mRNA.
This was confirmed by primer extension analysis using the primer MF8a-1, as described previously (Jackson & Bults, 1992). Major primer extension products of equivalent autoradiographic intensity and size were generated using Lu and Lul3Z mRNA as substrates (data not shown). The major MF8a mRNA transcription initiation site corresponds to a guanosine residue located 15 (± 1) nucleotides downstream of the ORF MF8a early promoter motif. Numerous minor extension products were also generated, corresponding to initiation sites at −2 to +2 relative to the major transcription initiation site. The position of the MF8a transcription initiation sites are shown in Fig. 2.

Northern blot analyses of Lu13Z early poly(A)+ RNA using the 32P 5′ end-labelled oligonucleotide probes TK-1 (ACGTTAACCTAGACCGATTAGTCCGTGCT), TK-2 (ATGTACTGTTACAAGGA) and TK-4 (AGGCATCGTTCTAGACTTCTA) are shown in Fig. 5. The sense-specific TK gene probe, TK-1, hybridized to a smear of transcripts, including three major RNA species of approximately 400, 700 and 1400 nucleotides. Primer extension analyses of Lu and Lu13Z TK gene transcripts using the TK-1 primer (data not shown) identified extension products corresponding to transcription initiation sites identical to those previously described (Jackson & Bults, 1992). There was no observable difference between the transcriptional efficiency of the TK gene early promoter in Lu- and Lu13Z-infected cells. The TK-2 probe is specific for anti-sense TK gene RNA and hybridized to a faint smear of transcripts with sizes ranging between 400 and 600 nucleotides present in the Lu13Z RNA preparation. The TK-4 probe is specific for sense TK gene transcripts extending beyond the transcription termination signals of recombinant MVs constructed using pUrTK13. Only the 700 nucleotide Lu13Z TK mRNA hybridized to TK-4 probe, indicating that it is the only full-length transcript contiguous with the TK gene.

The VV 109 bp XbaI–EcoRI fragment encoding the P11 late promoter for ORF F17R also encodes 13 codons of ORF F16L (Goebel et al., 1990). Tsao et al. (1988), using the same promoter fragment, reported a weak promoter activity reading in the opposite direction to the P11 late promoter; however, they did not distinguish between early and late promoter activity. Golini & Kates (1984) reported an early transcriptional activity initiating near the EcoRI site and coding for a 25K protein. This RNA species most likely represents the ORF F16L early mRNA. A potential early promoter motif, ACATAATGBAAATCTA, which shares 13 of 16 nucleotides of the VV early promoter consensus sequence (Davison & Moss, 1989), is located 30 nucleotides upstream of the ORF F16L initiation codon. It is probable that a weak ORF F16L early promoter activity results in the synthesis of the MV TK anti-sense RNA resulting in aberrant synthesis of Lul3Z TK mRNA. It should be possible to alleviate this abnormal TK RNA synthesis by replacing the P11 promoter of pUrTK13 with a synthetic late promoter motif, such as that used by Davison & Moss (1990).

The TDS procedure has been shown to be a useful technique for the construction of recombinant MVs. Using DNA manipulation techniques similar to those described here, it should be possible to construct a suite of MV TDS expression vectors using alternative intergenic insertion sites. Recombinant MVs expressing multiple foreign gene insertions could be constructed by sequential transfection and transient selection of recombinant viruses expressing the gpt gene.

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References


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