Construction of recombinant myxoma viruses expressing foreign genes from different intergenic sites without associated attenuation

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Two myxoma virus transient dominant selection vectors were constructed and used to generate recombinant viruses expressing single and double foreign gene insertions from intergenic sites. The intergenic insertion sites were located between the myxoma virus genes MJ2 (thymidine kinase) and MJ2a, and MA24 (β-subunit RNA polymerase) and MA27 (fusion protein) located approximately 60 and 113 kb from the left-end of the viral genome, respectively. Recombinant myxoma viruses expressing the lacZ gene from either intergenic insertion site retained wild-type virulence. However, expression of the gus gene reduced the virulence of the recombinant viruses in vivo. Northern blot analysis indicated that the major late mRNAs encoding the viral RNA polymerase subunit and fusion protein are both of discrete size. Insertion of a foreign gene under the control of a synthetic late promoter between the MA24 and MA27 genes results in a specific-sized major late transcript for the inserted foreign gene. The MA27 gene transcripts directed by these recombinant viruses are heterogeneous in size, implying the typical pattern of poxvirus late transcription by random 3’-termination prior to polyadenylation. The transcription studies suggest signals located downstream of the insertion site direct 3’-processing of late transcripts irrespective of the gene immediately upstream.

Introduction

A major goal for the programme being conducted by the Cooperative Research Centre for Biological Control of Vertebrate Pest Populations is the construction of recombinant myxoma virus (MYXV) expressing species specific reproductive vaccines for biological control of wild rabbits in Australia (Holland & Jackson, 1994). We have previously described the construction of the recombinant MYXV Lu13Z, expressing the E. coli lacZ gene, using the intergenic insertion vector pUrTK13 (Jackson & Bults, 1992 b). Transcription of the thymidine kinase (TK) gene early mRNA by Lu13Z virus was abnormal due to interference with anti-sense RNA transcribed from the inserted vaccinia virus (VACV) F16L early promoter. The VACV F16L promoter overlaps and is in the opposite orientation to, the P11 late promoter contained on the VACV DNA fragment inserted into pUrTK13. Disruption of TK expression can severely affect the virulence of poxviruses (Buller et al., 1985; Kochneva et al., 1994). Virulence of MYXV is important for its transmission by arthropod vectors and therefore important for the ability of the virus to persist in the wild (Fenner & Ratcliffe, 1965). Any future construction of recombinant MYXVs as biological control agents will require that the virulence of the viruses is not compromised by the DNA manipulations so that they can compete and persist against wild-type viruses in the field.

In this manuscript we describe the identification and transcriptional analysis of the MYXV genes encoding the β-subunit RNA polymerase (MA24; rpo132) and fusion protein (MA27) and the manipulation of the viral DNA sequence to create an intergenic insertion site for foreign genes. We describe the development of a MYXV vector system which incorporates a synthetic poxvirus late promoter for expression of foreign genes while maintaining the wild-type virulence phenotype. We use two manipulated intergenic insertion sites for the construction of a recombinant MYXV expressing the E. coli lacZ (β-galactosidase) and gus (UidA; β-glucuronidase)
genes from different intergenic locations using the transient dominant selection method.

Methods

**Cells and virus.** MYXV strain Lausanne (Lu) was grown on rabbit RK13 cells using procedures described in Jackson & Bults (1992a). Methods for the generation of recombinant viruses using transient dominant selection of the E. coli gpt gene were essentially as described by Falkner & Moss (1990). Plaques containing recombinant viruses expressing the E. coli lacZ or gus genes were detected by overlaying the infected cell monolayers with 1% MEM without phenol red (GIBCO-BRL; 2 × concentration) containing 0.7% (w/v) agar, 300 μg/ml X-Gal and/or 150 μg/ml salmon-glc (Biosynth AG). Under this medium, plaques containing viruses expressing β-galactosidase stain blue (X-Gal), those expressing β-glucuronidase stain pink (salmon-glc) and viruses expressing both enzymes stain pink with purple centres.

**DNA sequencing and plasmid construction.** The procedures used to determine the DNA sequence of a selected region of the MYXV EcoRI-G1 fragment were as described previously (Jackson & Bults, 1992a). Complementary oligonucleotides for the DNA sequence AGGATCACAATTTTTTTTTTTTGGCATATAAATAG encoding a synthetic late promoter (underlined) (Davison & Moss, 1990) were synthesized and annealed. The MYXV vector pUrTK13 (Jackson & Bults, 1992b) was digested with XbaI and EcoRI to excise the VACV DNA fragment containing both the P11 and P16L promoters, followed by treatment with Klenow fragment DNA polymerase to fill-in the recessed 3'-ends. The annealed oligonucleotides were blunt-end ligated into the prepared plasmid so that the inserted synthetic late promoter was in the correct sense with the intergenic multiple cloning site. These manipulations generated the transient dominant selection vector pUrTK14L.

The vector pGP7.5gpt was constructed by sub-cloning the EcoRI-Apal fragment, containing the VACV P7.5 early/late promoter and E. coli gpt gene, from pGpt07/14 (Boyle & Coupar, 1988) between the respective sites in pGEM-7Zf(−). This vector can be used to construct transient dominant selection vectors by insertion of poxvirus sequences into the remaining multiple cloning site. The 1.1 kb XbaI-HindIII fragment corresponding to the right-end of the MYXV HindIII-C fragment was treated with Klenow fragment DNA polymerase to fill-in the recessed 3'-ends followed by ligation between the EcoRI and NsiI sites (both sites treated with Klenow DNA polymerase) of pGP7.5gpt in the correct orientation is shown in Fig. 1. The DNA sequence (EMBL Z19600 and X94182) determined is of a region located approximately 113 kb from the left-end of the MYXV genome corresponding to the 1-1 kb XbaI-HindIII fragment from the right-end of the HindIII-C fragment plus the adjacent 0.6 kb HindIII-M fragment. The MYXV DNA sequence encodes three open reading frames (ORFs), MA24 (partial), MA27 and MA28, named to correspond to the related ORFs of the VACV Copenhagen strain (Goebel et al., 1990). The protein sequences encoded by these ORFs were compared to the SWISSPROT database using the FASTA program (Pearson & Lipman, 1988) with default parameters. The MA24 gene encodes the second largest (R) subunit of the poxvirus RNA polymerase with amino acid similarity to the Capripoxvirus sheeppox virus (SPPV) HM1 (score: 1106; 86.0% identity over a 242 aa overlap), VACV A24 (rpo132) (score: 1046; 79.8% over 242 aa) and numerous other Orthopoxviruses, eukaryotic and prokaryotic RNA polymerase subunits. The MA27 gene encodes a protein related to the SPPV HM2 (score: 295; 42.4% over 125 aa), VACV A27 (score: 90; 54.5% over 33 aa) and Poxvirusr (ORFV)10K (score: 91; 26.7% over 75 aa), the so-called membrane fusion proteins. The MA28 ORF encodes a protein of unknown function related to the SPPV HM3 (score: 545; 64.3% over 140 aa), VACV A28 (score: 412; 52.4% over 147 aa) and the Amsacta moorei entomopoxvirus G4R ORF (score: 200; 27.1% over 140 aa) (Goebel et al., 1990; Gershon et al., 1989; Nasse et al., 1991; Hall & Moyer, 1991).

The VACV 14 kDa fusion protein is present in the membrane of intracellular mature virions (IMV) where it is...
The VACV fusion protein is involved in cell-to-cell fusion late protein encoded by ORF A17 (Rodriguez et al., 1991). In addition, the MYXV fusion protein may need to be associated with the cellular membranes via interaction with a 21 kDa protein belonging to the genera Orthopoxvirus or Leporipoxvirus are not known to form intracellular ATIs. The shared organization and nucleotide similarity of the genes encoded by this region confirm the close phylogenetic relationship between the genera Leporipoxvirus and Capripoxvirus observed previously with regard to the gene organization in genomic regions containing the inverted terminal repeats (Gershon & Black, 1989b) and TK genes (Gershon & Black, 1989a; Jackson & Bults, 1992a).

Construction of vectors and recombinant viruses

To allow the construction of recombinant MYXVs which retain the wild-type virulence phenotype, transient dominant selection vectors were constructed with intergenic insertion sites containing synthetic late promoters to direct expression of the inserted foreign genes. To correct the aberrant TK gene early transcription observed with viruses constructed using pUrTK13, the VACV P11/P16L promoter fragment was excised from this vector and replaced with a DNA sequence containing a synthetic late promoter to generate pUrTK14L (Fig. 2a).

A second transient dominant selection vector, pMA243, was constructed by the insertion of a synthetic DNA sequence into the end of the MA24 gene to generate an intergenic insertion site (Fig. 2b). To ensure that the function of the RNA polymerase was not altered, the synthetic DNA sequence contains the same coding information for the terminal six codons as the natural MA24 gene. The synthetic DNA also contained nucleotide redundancies to avoid direct duplication of the DNA sequence which could otherwise result in homologous recombination and deletion of the inserted DNA.

Fig. 1. Restriction map of MYXV for the enzymes EcoRI, BamHI, HindIII and Sall. Shown above the sequence are the relative locations of the thymidine kinase (Jackson & Bults, 1992a), β-subunit RNA polymerase and ‘fusion’ protein genes.
Fig. 2. MYXV transient dominant selection vectors showing the nucleotide sequences of the intergenic insertion sites (a) pUrTK14L and (b) pMA243. Relevant restriction sites and transcription initiation sites (#) are indicated.
from the recombinant MYXV genome. A poxvirus early transcription termination signal, TTTTTAT (Yuen & Moss, 1987), was included at the end of the new MA24 gene to maintain transcriptional integrity if the gene was expressed during the early phase of infection.

Recombinant MYXVs, Lu14LlacZ and Lu243lacZ, containing single intergenic insertions of the lacZ gene, were constructed using the plasmids pUrTK14LlacZ and pMA243lacZ, respectively. The recombinant virus Lu14LlacZ was used to create a second virus expressing the gus gene, Lu14LlacZ/243gus, by a subsequent transformation of infected cells with plasmid pMA243gus and selection for transient gpt gene expression. A recombinant virus, Lu243gus, containing an insertion of the gus gene alone was also constructed using pMA243gus.

### Transcription studies

Early and late mRNA was isolated from RK13 cells infected with wild-type Lu or recombinant virus Lu14LlacZ/243gus. The RNA samples were separately hybridized with probes complementary to the mRNAs for the native viral genes adjacent to the insertion sites and to the inserted foreign genes (Fig. 3). Insertion and late expression of the lacZ gene (Fig. 3, lane 8) positioned between the TK (Fig. 3, lane 2) and MJ2a (lanes 3 and 4); lacZ (lanes 5 to 8); gus (lanes 9 to 12); MA27 (lanes 13 to 16); MA24 (lanes 17 to 20); Lane 21 is a shorter exposure of lane 12.

Extended exposure of the autoradiographs did not show hybridization signals to early mRNA species for the MA24 gene (data not shown). This indicates that early transcription of the MYXV MA24 gene (Fig. 3, lane 17), unlike the corresponding CPXV rpo132 gene (Patel & Pickup, 1989), does not occur at appreciable levels. Interestingly, both the MA24 and MA27 late mRNAs appear to contain major discrete-sized species with a background population of heterogeneous late mRNAs. Insertion of the gus gene between MA24 and MA27 using the vector pMA243 resulted in the generation of a major gus gene mRNA of approximately 2.5 to 3 kb (Fig. 3, lanes 12 and 21). Late RNA transcribed by the recombinant virus contains a heterogeneous population of MA27 late mRNAs (Fig. 3, lane 16) typical of poxvirus late gene expression with no evidence of discrete-sized mRNAs. This suggests that insertion of the gus gene has separated the MA27 gene from a downstream signal involved in processing of the 3'-end of late mRNA. With the Lu14LlacZ/243gus virus, the downstream signal was being used to process the late mRNA encoding the inserted gus gene.

Discrete-sized late gene transcripts have also been observed for the cowpox virus rpo132, ATI (Patel & Pickup, 1987, 1989; Antczak et al., 1992) and the VACV 'ATI' genes (Amegadie et al., 1992). The rpo132 and ATI genes of the orthopoxviruses have the same head-to-head arrangement and are located in the same genomic location as the MYXV MA24 and MA27 late genes. A 345 bp cowpox virus DNA fragment (AX-element) located downstream of the ATI gene has been shown to direct post-transcriptional cleavage of the 3'-end of ATI late gene transcripts prior to polyadenylation (Antczak et al., 1992). Comparison of the equivalent AX-element regions of Orthopoxvirus, Capripoxvirus and Leporipoxvirus does not indicate obvious conserved 3'-processing signals due to the highly conserved rpo132 genes (data not shown). Further characterization of the signals involved in 3'-cleavage of late mRNA requires detailed deletion and mutagenesis studies to identify the RNA sequences involved.

The MA28 gene is likely to be transcribed during the late infection phase since it has a TAAATG consensus late promoter (Rosel et al., 1986; Davison & Moss, 1989b) incorporating the methionine initiation codon. Sequences
related to an upstream early promoter (Davison & Moss, 1989a) or downstream early transcription termination signals are not associated with this gene and it is therefore unlikely to be expressed during the early transcription phase.

Virulence studies

Rabbits inoculated with either Lu or Lu14LacZ had more severe clinical signs until death than rabbits inoculated with either Lu243gus or Lu14LacZ/243gus. The reduced severity of clinical signs was associated with a prolonged survival time for rabbits injected with constructs containing the gus gene (Fig. 4). For analysis, the survival times were pooled into two groups, those injected with Lu or Lu14LacZ and those injected with Lu243gus or Lu14LacZ/243gus. Rabbits infected with viruses not expressing the gus gene had an average survival time (AST) of 10 ± 0.7 (SD) days while those infected with virus expressing the gus gene had an AST of 13.5 ± 1.9 days. These values were significantly different (P < 0.02; Student's t-test).

Based on the criteria of Fenner & Marshall (1957) for virulence of MYXV strains the first group of viruses has type I virulence (< 13 days AST, 99.5% mortality) and the viruses expressing the gus gene have a type II virulence (13–16 days AST, 99% mortality).

Recombinant viruses Lu243gus and Lu14LacZ/243gus were independently isolated from fully virulent parental viruses, Lu and Lu14LacZ respectively. It is possible that the plaque purification resulted in the selection of viruses with random mutations. However, recombinant Lu viruses expressing the lacZ gene from intergenic sites have been repeatedly isolated which show no reduction in virulence for domestic rabbits (Opengorth et al., 1992; R. Jackson & P. Kerr, unpublished). This suggests that the genetic background of the cell culture adapted Lu virus is very stable. The observed reduction in virulence of these viruses was most likely due to either the physical presence of the gus gene inserted between MA24 and MA27 or expression of the β-glucuronidase enzyme. It is possible that separation of the MA27 gene from its 3' late RNA processing signals may be detrimental to transcription, stability or translation of the MA27 mRNA. Alternatively, high-level expression of the β-glucuronidase could be detrimental to either virion morphogenesis or infected-cell viability. Either instance could result in a reduction of the number of infectious particles produced, resulting in a delay in the onset of the disease and therefore a reduced relative virulence.

The virus Lu243lacZ was constructed to confirm that the reduced virulence of viruses containing the gus gene was not the result of foreign DNA insertion between MA24 and MA27. The Lu243lacZ virus retains type I virulence with an AST of 10.7 ± 0.7 days (Fig. 4). It would appear that separation of the MA27 from the 3' post-transcriptional cleavage signals had little detrimental effect upon the virulence of this virus, presumably because this did not alter either the overall rate of mRNA synthesis or translation of the 'fusion' protein. However, it is not possible to exclude the possibility that the lacZ gene could contain cryptic 3' late RNA processing signals and therefore the MA27 transcripts may be processed conserving the virulence phenotype.

We have previously shown that a recombinant MYXV expressing the highly immunogenic influenza virus haemagglutinin protein can induce high serum and moderate mucosal antibody titres to the foreign antigen following infection of rabbits (Kerr & Jackson, 1995). Using the vectors described here it should be possible to construct recombinant MYXVs expressing reproductive antigens which retain wild-type phenotype as long as the expressed antigens have innocuous biological activity when expressed by the virus.

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References


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