Partial transcriptional mapping of the fowlpox virus genome and analysis of the *EcoRI* L fragment

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Several fowlpox virus (FPV) DNA fragments were selected by differential hybridization using cDNA of transcripts that were strongly transcribed early and/or late after infection of QT-35 cells. The *EcoRI* L fragment contained three strongly transcribed FPV genes: L1L, a late 1452 bp partial (amino end) ORF; L2R, an early/late 522 bp ORF; and L3R, a late 948 bp ORF. The protein products of L1L, L2R and L3R shared homology with the products of vaccinia virus (VV) genes H4L (RAP94), H5R (Ag35) and H6R (topoisomerase), respectively, suggesting a conservation of gene structure and order between VV and FPV. The 5' upstream non-coding sequences of L1L and L3R were A+T rich and the sequence 5' TAAATG 3' overlapped the predicted translation start codon. Primer extension analysis of the L2R transcript mapped the transcriptional start sites of early and late mRNAs 14 nt downstream of a VV early promoter-like critical region sequence, AAAATTGAAA-AAAAAA. A VV-like TAAAT late transcriptional element was present 20 nt upstream of the L2R ATG translational start codon. A plasmid with the putative early L2R promoter cloned upstream of the Newcastle disease virus haemagglutinin-neuraminidase (HN) cDNA as a reporter gene was at least 6-fold more effective in generating HN mRNA than plasmids containing the P7.5 or P11 VV promoters in transient expression assays in FPV-infected CEF cells treated with cytosine arabinoside. The L2R promoter was also able to express an amount of HN mRNA equal to that expressed by the VV promoters late in infection.

Introduction

There is interest in fowlpox virus (FPV) because of its potential as a vector for recombinant vaccines for poultry (Boyle & Coupar, 1988). FPV is a member of the genus *Avipoxivirus* in the family *Poxviridae*. This large complex avian virus has an A + T-rich dsDNA genome of between 260 and 310 kb (Coupar et al., 1990; Mockett et al., 1992; Zantinge et al., 1995). The FPV genome is about 70 to 100 kb larger than the vaccinia virus (VV) genome, with 20 to 25% homology (Mockett et al., 1992). Since the cytoplasmic replication of FPV does not allow for interaction with nuclear host cell transcription factors, the virus carries within its core all the enzymes and transcription factors required for the immediate transcription of early genes. Late gene expression is presumably dependent on early gene expression and occurs after the initiation of viral DNA replication (Moss, 1990). Promoter requirements for early, late (Davison & Moss, 1989a, b) and intermediate (Baldick et al., 1992; Baldick & Moss, 1993) gene expression have been characterized for VV, but not for other poxviruses. The conservation of many molecular features between FPV and VV has enabled the construction of recombinant fowlpox viruses (rFPV). The majority of rFPVs have utilized strong VV promoters to express the foreign genes, although the efficiency of VV promoters in rFPVs is generally lower than that of FPV early/late promoters (Boyle, 1992).

In this study, the entire FPV genome was screened to identify strong FPV promoters expressed both early and late post-infection. Several restriction enzyme fragments carrying DNA sequences which appeared to be strongly transcribed either early, late or throughout the virus infection cycle were identified by a preliminary analysis of overall transcription of the FPV genome in QT-35 cells. In particular, a 6.5 kb *EcoRI* fragment hybridized to a small number of very strongly expressed transcripts. Analysis of this fragment led to the identification of one strongly expressed early/late gene designated L2R and two late genes designated L1L and L3R.
Methods

Virus and cell cultures. FPV (Chick-N-Pox vaccine strain, Salsbury Laboratories, Canada) was cultivated in chick embryo fibroblast (CEF) cells, or in QT-35, a quail cell line (Cho, 1983).

Virus purification and isolation of FPV genomic DNA. FPV was purified from infected CEF cells by a method from Prideaux & Boyle (1987). DNA was isolated from purified virus using a procedure adapted from Mackett & Archard (1979). Briefly, purified virus was lysed at 4°C for 30 min in 50 mm-Tris-HCl pH 7.8, 1 mm-EDTA, 27% (v/v) sucrose, 1% (v/v) sodium n-lauryl sarcosinate and 100 mm-2-mercaptoethanol. Proteinase K (to 300 μg/ml) was then added and incubated at 37°C for 2 h. DNA was extracted with phenol/chloroform (Sambrook et al., 1989), ethanol precipitated and resuspended in TE (10 mm-Tris-HCl, 1 mm-EDTA pH 7.8).

Virus infection, AraC treatment and isolation of total RNA. QT-35 cells were infected with FPV at an m.o.i. of 5. To prevent late gene transcription (Prideaux & Boyle, 1987) cytosine arabinoside (AraC; Sigma) was added to cells at 50 μg/ml for 1 h prior to infection and up to 18 h post-infection (p.i.). RNA was isolated by the guanidinium thiocyanate single step method (Chomczynski & Sacchi, 1987). Preparation of radiolabelled cDNA probes. The procedure for radiolabelling cDNA was adapted from Friemert et al. (1989). Oligo(dT)-primed first-strand cDNA was synthesized from 5 μg of total cellular RNA in a 20 μl reaction volume containing 50 mm-Tris-HCl pH 8.3, 50 mm-MgCl₂, 10 mm-DTT, 4 mm-sodium pyrophosphate, 0.5 mm-SPERMIDINE-HCl, 1 mm each of dATP, dGTP and dTTP, 0.5 mm-dCTP, 100 μg/ml oligo(dT)₁₂-₁₈, 40 U ribonuclease inhibitor, 10 μg/μl 32P-dCTP (3000 Ci/mmol) and 20–30 U AMV reverse transcriptase (BoCain). The cDNA synthesis reaction was performed at 42°C for 2 h and was stopped by the addition of 2 μl of 0.2 m-EDTA. The RNA template was hydrolysed with 0.3 m-NaOH for 30 min at 60°C. The solution was neutralized by the addition of 1/10 volume of 1 m-Tris-HCl pH 7.5 and 3/10 volume of 1 m-HCl. The single-stranded cDNA was precipitated with two volumes of 4 m-ammonium acetate and two volumes of ethanol and incubated at −70°C for 30 min or at −20°C overnight. The DNA was recovered by centrifugation and dissolved in 30 μl TE.

High specific activity (1 × 10⁹-5 × 10¹⁰ c.p.m./μg) second strand DNA was synthesized from 10–15 ng of single-stranded cDNA using the PCR amplification system (Perkin-Elmer). PCR was performed in a 20 μl reaction volume containing 50 μm-Tris-HCl pH 8.3, 50 μm-KCl, 10 μm-MgCl₂, 100 μm-2-mercaptoethanol. Proteinase K (to 500 μg/ml) was then added and incubated at 4°C for 30 min in 50 mM-Tris- HCl pH 7.8, 1 mM-EDTA, 27% (v/v) sucrose, 1% (v/v) sodium n-lauryl sarcosinate and 100 mM-2-mercaptoethanol. Proteinase K (to 300 μg/ml) was then added and incubated at 37°C for 2 h. DNA was extracted with phenol/chloroform (Sambrook et al., 1989), ethanol precipitated and resuspended in TE (10 mM-Tris-HCl, 1 mM-EDTA pH 7.8).

Virus mapping and Northern blot analysis of highly transcribed fragments. Restriction enzyme fragments were isolated from agarose gel slices using the Prep-A-Gene kit (Bio-Rad) and labelled with [α-3²P]dCTP (Amersham). Fragments were then digested with HindIII, which was first digested with HindIII, then digested with EcoRI and XhoI fragments were subcloned into pGEM-T(+) and pGEM-7Z(+) DNA. DNA sequencing and analysis. Plasmid pE68 containing the 6.5 kb EcoRI fragment was purified using Qiagen columns. The 1.9 kb StyI fragment and the three XhoI fragments of pE68 were subcloned into pGEM-T(+) and pGEM-7Z(+) (Sambrook et al., 1989). The 1.84 kb XhoI fragment was further digested with BglII and the 1.84 kb XhoI-BglII, 0.07 kb BglII-BglII and 0.37 kb BglII-XhoI fragments were subcloned into the XhoI and BamHII sites of pGEM-7Z(+) by DNA sequencing. The resulting 170 bp fragment was removed by digestion with HindIII and DraI. The 170 bp fragment was then cloned into pBR322 (Nagy et al., 1990) which was first digested with BamHI, blunt-ended with Klenow and then ligated with HindIII. The junction region between the L2R promoter and the HN gene from the multiple cloning site in the plasmid and DraI. All sequencing was performed in both the forward and reverse direction and the sequenced fragments were completely overlapped.

DNA sequences were analysed with the computer programs CLONE MANAGER and PC Gene. BLASTX homology searches were used to analyse both DNA and protein sequences, using the Experimental GENINFO (R) BLAST Network Service (BLASTER). Sequences were also analysed with CLUSTAL alignments.

Slot blot analysis of HN transcripts. Total RNA was isolated from FPV-infected and plasmid-transfected (pL2RHN, pP7.5HN, pP11HN) CEF cells at 18 h post-transfection (25 h p.i.) and from uninfected, FPV-infected, NDV-infected and FPV-infected pl8RHN (no promoter)-transfected CEF cells. Total RNA was also isolated from uninfected CEF cells transfected with pL2RHN, pP7.5HN, pP11HN or p18RHN. Samples were dissolved in 25 μl RNase-free TE and the amount of RNA was quantified by A₂₆₀/₂₈₀ readings. Ten μg of total RNA was used for Northern blot hybridization and 5-25 μg/slot of total RNA from each sample was used for slot blot analysis on nylon filters. HN RNA transcripts were detected with a 2.4 kb [α-3²P]dCTP-labelled BamHII–KpnI fragment containing the entire NDV HN gene. Hybridizations were replicated three times and each replicate was...
applied to the slot blot apparatus in duplicate or triplicate. The relative levels of expression from each of the promoters were measured by scanning densitometry (Shimadzu Cs-9000 Dual Wavelength Flying-Spot-Scanner) of the resultant autoradiogram. The results from three separate replicate experiments were averaged.

**Results**

**cDNA analysis of FPV transcription**

Labelled cDNA probes derived from RNA isolated at 8 h p.i. (early mRNA) and 18 h p.i. (late or early/late mRNA) from both FPV-infected and mock-infected QT-35 cells and at 18 h p.i. from infected AraC-treated cells, were hybridized to Southern blots containing BglII-, EcoRI- and StyI-digested genomic FPV DNA. A Southern blot of FPV DNA digested with BglII, EcoRI and StyI was also hybridized with genomic FPV DNA to serve as a reference blot. No hybridization was detected between FPV DNA and cDNA transcripts derived from RNA from mock-infected cells. Several FPV EcoRI, BglII and StyI fragments hybridized strongly to cDNA prepared from early mRNA (early and AraC-treated) and late mRNA isolated from FPV-infected QT-35 cells (Fig. 1). DNA fragments ranging from 2.2–6.5 kb in size which hybridized strongly to cDNA are summarized in Table 1. Larger strongly hybridized DNA fragments were also detected for all enzymes but are not listed in Table 1 or further analysed because they could contain many FPV genes and would not allow for precise mapping.

**Mapping of strongly transcribed DNA fragments**

The position of some of the EcoRI fragments hybridizing strongly to labelled cDNA derived from FPV-infected cell RNA are indicated on the physical map of the FPV genome (Fig. 2). BglII and StyI fragments which hybridized strongly to FPV cDNA were mapped to specific EcoRI fragments by Southern blot hybridization. BglII fragments approximately 2.2, 3.1, 3.6 and 4.9 kb in size and StyI fragments approximately 2.7 and 3.3 kb in size were isolated from agarose gels, radioactively labelled and hybridized to Southern blots containing cloned (3.5 to 12.2 kb) FPV EcoRI fragments. The results of these hybridizations are summarized in Table 1. Some of the BglII and StyI probes hybridized to EcoRI fragments previously identified in the cDNA analysis. For example, E68 which contained the 6.5 kb EcoRI L

![Fig. 1. Identification of highly transcribed FPV DNA restriction enzyme fragments. FPV DNA was digested with EcoRI (a), BglII (b) or StyI (c) and analysed by 1% agarose gel electrophoresis. Southern blots were probed with 32P-labelled cDNA derived from RNA isolated either early in FPV infection (lanes E), AraC-treated FPV-infected cells (lanes A) or late in FPV infection (lanes L). These hybridization profiles were compared with reference profiles of ethidium bromide-stained gels (lanes R). Sizes of fragments were estimated by comparison with a gel profile of the 1 kb ladder in lane M.](image-url)
Table 1. Summary of the selection of highly transcribed DNA sequences

The tk gene DNA probe represents a 3.8 kb HindIII fragment located within the cloned EcoRI 5.35 kb \( U_\delta \) fragment. Fragment designations are from Zantinge et al. (1995).

<table>
<thead>
<tr>
<th>Band</th>
<th>Fragment (size in kb)</th>
<th>Source of cDNA</th>
<th>EcoRI clone hybridized</th>
<th>EcoRI Fgt (kb)</th>
<th>Size of transcript (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-3.6</td>
<td>BgII a, b (3.6)</td>
<td>early/late</td>
<td>E68</td>
<td>L (6.5)</td>
<td>2.9 2.9</td>
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<td>S-3.3</td>
<td>StyI f (3.3)</td>
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<td></td>
<td></td>
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<tr>
<td>E-6.5</td>
<td>EcoRI L (6.5)</td>
<td>early/late</td>
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<td></td>
<td>0.6 0.6</td>
</tr>
<tr>
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<td>early</td>
<td>E108</td>
<td>J (8.0)</td>
<td>2.8 2.8</td>
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<tr>
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<td>BgII e, f (3.1)</td>
<td>early/late</td>
<td>E116</td>
<td>o (2.4)</td>
<td>2.5 2.3</td>
</tr>
<tr>
<td>E-2.4</td>
<td>EcoRI o (2.4)</td>
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<td>1.1 1.1</td>
</tr>
<tr>
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<td>late</td>
<td>E125</td>
<td>Y (4.7)</td>
<td>2.4 1.1</td>
</tr>
<tr>
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<td>BgII e, f (3.1)</td>
<td>early/late</td>
<td>E172</td>
<td>F (9.0)</td>
<td>2.7 1.2</td>
</tr>
<tr>
<td>B-4.9</td>
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<td>E202</td>
<td>C (12.2)</td>
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<tr>
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<td>E229</td>
<td>H (8.7)</td>
<td>2.6 1.2</td>
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<tr>
<td>B-4.9</td>
<td>BgII O, P, Q (4.9)</td>
<td>late</td>
<td>E294</td>
<td>T (5.35)</td>
<td>2.7 1.0</td>
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<tr>
<td>S-2.7</td>
<td>StyI m, n (2.7)</td>
<td>early/late</td>
<td>E346</td>
<td>d (3.9)</td>
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Fig. 2. EcoRI physical map of FPV DNA (upper panel) and the location of the EcoRI fragments (filled blocks) used as probes in Northern blots containing total RNA isolated from FPV-infected QT-35 cells (lower panel). The clone designation is indicated above each blot. RNA was isolated from cells in the presence, representing early RNA (lanes E), or in the absence of AraC at 18 h p.i. representing late RNA (lanes L). Sizes of RNA transcripts for E68 in kb are indicated to the left.
Transcriptional analysis of FPV EcoRI L

Fragment, hybridized to strongly transcribed 3.6 kb BglII and 3.3 kb StyI fragments as well as to the 6.5 kb EcoRI fragment. The results from other Southern blot hybridizations were more complex. For example, the 4.9 kb BglII DNA fragments hybridized to three different FPV EcoRI clones, E202, E125 and E308, each containing cloned EcoRI fragments 12.2 (C), 9.0 (G) and 4.7 (Y) kb in size, respectively. The 4.9 kb BglII DNA band, according to the BglII restriction enzyme profile (Zantinge et al., 1995), represented three co-migrating BglII fragments (O, P and Q) and therefore DNA from this band could hybridize with up to three different EcoRI fragments.

Northern blot analysis

DNA from cloned EcoRI fragments E68, E108, E116, E125, E172, E202, E229, E308 and E346 was hybridized to Northern blots of total RNA isolated from FPV-infected QT-35 cells at 18 h p.i. in the presence or absence of AraC (early and late transcripts, respectively; Fig. 2, Table 1). A 3.8 kb HindIII fragment containing the FPV tk gene (Boyle & Coupar, 1986) was included as a probe for comparison because the tk gene has a weak early/late promoter. A randomly chosen clone, E294 (5.35 kb EcoRI T fragment), was also included as a fragment identified by the cDNA analysis as not containing strongly transcribed genes. Clones E68, E108, E172, E202, E229, E308 and E346, relative to the tk and E294 probes, hybridized strongly to RNA transcripts expressed early and/or late during replication. Clones E116 and E125 did not hybridize to RNA transcripts as strongly but hybridization was stronger than for the tk-based probe. The tk probe hybridized only very weakly to a 1.1 kb early/late and weakly to a 2.7 kb late transcript. Of these the 1.1 kb transcript is the tk transcript. The 5.35 kb EcoRI T fragment in E294 was not strongly transcribed as it hybridized only weakly to a single 2.7 kb transcript. No hybridization was observed with total RNA isolated from mock-infected cells (data not shown). The fact that several EcoRI fragments, selected from the initial cDNA analysis, hybridized strongly to RNA transcripts (Fig. 2) showed that the cDNA screening was effective in identifying fragments with strongly expressed genes.

Clone E68, containing the 6.5 kb EcoRI L fragment,
was chosen for further study because it hybridized to the most abundantly transcribed early/late transcript (0-6 kb in size) as well as to two strong late transcripts (1.1 and 2.9 kb in size). Plasmid pE68 was digested with these enzymes to determine the positions of sites. Seven labelled restriction enzyme fragments (identified 1

Fig. 4. DNA sequence and deduced amino acid sequence of the FPV gene ORFs designated L1L, L2R and L3R. The amino acid sequence for the L1L ORF is placed above the nucleotide sequence and is in a leftward orientation. The amino acid sequence encoded by ORFs L2R and L3R are shown below the nucleotide sequence and in a rightward direction as shown by the corresponding arrows at the initiator ATGs. Putative promoter regions and termination sequences are shown in bold. Putative early promoter sequences are

Insert. Digestion of EcoRI L with XbaI resulted in five fragments 0-19, 0-63, 1-84, 1-90 and 2.00 kb in size and with StyI generated 0-22, 1-10, 1-90 and 3-28 kb fragments. The resultant physical map of EcoRI L for BglIII, HindIII, StyI and XbaI is shown in Fig. 3, upper panel. To map the transcripts within EcoRI L, subclones of L were tested and putative early promoters are double underlined. The locations identified by primer extension mapping to the 5' ends of the early (E) and late (L) mRNAs are shown with asterisks (*) above the sequence. BglIII, StyI and XbaI sites are labelled.

In Fig. 4, the DNA sequence and deduced amino acid sequence of the FPV gene ORFs designated L1L, L2R and L3R. The amino acid sequence for the L1L ORF is placed above the nucleotide sequence and is in a leftward orientation. The amino acid sequence encoded by ORFs L2R and L3R are shown below the nucleotide sequence and in a rightward direction as shown by the corresponding arrows at the initiator ATGs. Putative promoter regions and termination sequences are shown in bold. Putative early promoter sequences are
Transcriptional analysis of FPV EcoRI L

Sequence analysis of the EcoRI L fragment

Three XbaI fragments and part of the 1.9 kb StyI fragment which hybridized to the abundant 0.6 kb and 1.1 kb early/late transcripts were sequenced. Three putative ORFs were identified and designated L1L, L2R and L3R (where the L represents the EcoRI L fragment, the number indicates the position of the ORF from left to right and the L or R refers to the orientation of the ORF as leftward or rightward; Figs 3 and 4).

The L1L ORF began at nt 1452 (Fig. 4) in a leftward orientation and extended beyond nt 1 in the sequence. The 1452 nt of the 5' terminus of L1L encoded a polypeptide of 484 amino acids with an isoelectric point to 7 in Fig. 3) hybridized with total RNA isolated from FPV-infected QT-35 cells at early and late times of infection (Fig. 3). All probes hybridized to 1·1 kb late transcripts and probe 7 hybridized most strongly to a 1.1 kb late transcript. Probes 3, 4 and 5, which were beyond the right end of the 2.00 kb EcoRI XbaI fragment (probe 1) and to the left of the 0.63 kb XbaI fragment (probe 7), hybridized strongly to the early/late 0·6 kb transcript. Probe 6, which overlapped probes 3, 4 and 5, also hybridized strongly to the early/late 0·6 kb transcript. Probes 3 and 4, mapping within the 1·84 kb XbaI fragment, hybridized weakly to a 2·9 kb late transcript. Probes 1 and 2 hybridized very strongly to a 2·9 kb transcript.
of 6.28 and predicted molecular mass of 57.8 kDa. The
amino terminus of the predicted L1L protein shared
homology with the RNA-polynucleotide-associated trans-
scription specificity factor (RAP9) genes of VV H4L
(Rosel et al., 1986; 54.3% identity and 76.0% similarity
over the 5' most 463 amino acids) and of orf virus F2L
(Fleming et al., 1993; 49.4% identity and 74.4% similarity
over the 5' most 413 amino acids).

The L2R ORF (nt 1596–2117; Fig. 4) at the far right
of the 1.84 kb Xbal fragment (probe 3, which hybridized
strongly to the 0.6 kb early/late transcript; Fig. 3) was
522 nt in length and in a rightward orientation. The L2R
ORF encoded a polypeptide of 174 amino acids with a
predicted molecular mass of 19.3 kDa and an isoelectric
point of 9.44 which was predominantly hydrophilic for
about two-thirds of its length from its amino terminus
and more hydrophobic along the one-third at the
carboxy-terminal end, but the extreme terminus was still
hydrophilic (Fig. 5b). The L2R protein product shared
low homology with the VV H5R protein, Ag35 (Rosel et al.,
1986; 17% identity and 52.7% similarity) and the orf virus F3R
(Fleming et al., 1993; 17.2% identity and 52.0% similarity).
L2R was 53 amino acids shorter than orf virus F3R and 29 amino acids shorter than the
VV H5R with differences occurring primarily between
residues 25 to 60 of F3R (Fig. 5a).

The L3R ORF, immediately downstream of L2R, was
948 nt (nt 2121–3068) long and in a rightward orien-
tation, encoding a protein of 316 amino acids with a
predicted molecular mass of 37.1 kDa and an isoelectric
point of 10.22. The L3R protein was predominantly
hydrophilic and shared homology with the topo-
isomerases of VV (Rosel et al., 1986; 52.7% identity and
79.5% similarity), Shope fibroma virus (Upton et al.,
1990; 54.3% identity and 79.5% similarity) and orf
virus, F4R (Fleming et al., 1993; 47.0% identity and
77.3% similarity).

Identification of regulatory sequences for L1L, L2R and
L3R

A TAAATG poxvirus promoter motif which overlaps
the translational ATG start codon in most late VV genes
(Davison & Moss, 1989b), was found upstream of the
FPV L1L and L3R genes starting at nt 1454 and 2118,
respectively. A TAAAT motif in the L2R gene started
96 nt (nt 1486) and 24 nt (nt 1572) upstream of the
translational start codon. A VV-like early promoter
critical region (AAAATTGAAAAAAAAA) was also
found upstream of the L2R gene, starting at nt 1518.

Since the L2R transcript was the most abundant we
mapped the 5' end of this transcript by primer extension.
The L2R early transcript 5' end mapped to nt 1547 (Fig.
4) representing a C residue 49 nt upstream of the L2R
ORF (Fig. 6). The 5' end of the L2R late transcripts
mapped to nt 1538–1541 as well as to nt 1547 identified

Fig. 6. Primer extension analysis of the FPV L2R gene. The primer was 5'
CATGATGACTGCTATCCG 3' corresponding to nt 1663–1680 (Fig. 4). (a) A 6%
polyacrylamide sequencing gel. Lanes E and L represent products of the primer extension
of early (E) and late (L) RNA isolated from FPV-infected QT-35 cells and the adjacent
lanes are for the sequencing reactions for G, A, T and C with the same primer and pE68
as the template. The symbol ▲ represents the position of the primer extension product
of both early and late mRNAs corresponding to nt 1547 (-49 relative to the L2R ORF
ATG) and ▲ the position for late mRNA corresponding to nt 1537–1541. (b) Diagram
depicting the primer location and the strategy used in the primer extension analysis of
L2R. Rectangle marked with an asterisk represents the labelled primer. Filled areas
upstream of the L2R ORF DNA are the untranslated region. Underlined nucleotides at
L and E/L below the sequence indicate the position of the 5' end primer extension
products from late and early/late transcripts respectively relative to the L2R gene
sequence and the arrowhead indicates orientation of the ORF. The numbers above the
sequence are relative to the A of ATG (underlined) as +1.
for the L2R early transcripts. Since typical poxvirus late mRNAs contain short untranslated poly(A) leader sequences at their 5' ends (Bertholet et al., 1987; Schwer et al., 1987) this could also occur for L2R late transcripts. The actual position of the transcriptional start site of late L2R transcripts would therefore be downstream of that determined by primer extension. The observation of multiple bands at nt 1538–1541 (residues ATTA) is typical for primer extension analysis of late poxvirus mRNAs because the 5' poly(A) leader sequences often vary in length by a few nucleotides. The ATTA residues were 31 to 34 nt upstream of the VV-like TAAAT late transcriptional initiation element. Nevertheless, the major primer extension product of late L2R transcripts was a single discrete size band which mapped at the 5' end to nt 1547, the same as for early mRNA. This suggested that most of the late mRNAs were initiated from the same site as the early L2R mRNA.

**Transcriptional analysis of the putative early/late L2R FPV promoter**

The relative strength of the FPV L2R promoter was measured by a transient transcription assay. The L2R promoter (L2R) and the VV promoters for P11 and P7.5 were fused upstream of the NDV HN reporter gene in plasmids designated pL2RHN, pP11HN and pP7.5HN (Fig. 7a). The strategy used to construct the pL2RHN expression vector resulted in the loss of the VV-like TAAAT critical late promoter sequence at nt 1572–1576 (Fig. 4) generating a TGATC sequence instead. HN mRNA was detected from CEF cells infected with NDV
as expected but not from mock-infected or FPV-infected CEF cells transfected with p18RHN which contained the HN gene without an upstream promoter. HN RNA was not detected either in extracts from uninfected CEF cells transfected with either pP11HN, pP7.5HN or pL2RHN (data not shown), but was present in FPV-infected cells transfected with these plasmids (Fig. 7b, c).

The highest amount of HN mRNA seen in FPV-infected and transfected cells was from AraC-treated cells (i.e. early) transfected with pL2RHN. In these cells there was 6.5- and 8.2-fold more HN mRNA detected relative to cells transfected with pP7.5HN and pP11HN, respectively. In infected cells not treated with AraC (i.e. late) the amount of HN transcripts observed for pL2RHN transfected cells was lower but was still 2.4- and 1.2-fold the amount detected for p7.5HN and p11HN, respectively.

Discussion
In this study at least eight specific small restriction enzyme fragments of FPV DNA were shown to contain strongly transcribed genes and several fragments (e.g. B-3.6, B-3.1, S-3.3 and S-2.7) hybridized strongly with cDNA derived from RNA transcribed throughout the viral infection cycle. The degree of hybridization with labelled cDNA presumably reflected the relative abundance of specific FPV mRNA species during the infection cycle as suggested for herpesvirus transcription (van Santen, 1991; Raab-Traub et al., 1983). Nevertheless, some highly expressed genes may have been missed. While most early VV mRNA has an average size of 1 kb (Oda & Joklik, 1967), late poxvirus mRNAs have enormous length heterogeneity and can occur as double-stranded RNA (Colby & Duesberg, 1969), a poor template for reverse transcriptase. The corresponding cDNAs would therefore be present in relatively low amounts and the corresponding genes would not have been identified as being highly expressed in our assay.

Although the relative level of cDNA, as determined by hybridization, may also reflect turnover rates and half-lives of some of the mRNAs, we felt that this preliminary study was adequate for the identification of some specific FPV DNA fragments with early, early/late and late strongly expressed genes. The high level of transcription from these fragments was further confirmed by Northern blot analysis.

The fine mapping and Northern blot analysis of the 6.5 kb EcoRI L fragment (and subsequent sequence data) indicated that there were up to five genes within or overlapping this DNA fragment. The order and orientation of the three FPV genes from the EcoRI L fragment, designated L1L, L2R and L3R, were the same as their respective homologues in vaccinia (H5L, H6R and H7, respectively) and orf (F2R, F3R and F4R, respectively) viruses (Rosel et al., 1986; Goebel et al., 1990; Fleming et al., 1993).

The homology between LIL and the VV RAP94 proteins (encoded by genes H5L and H4L in the WR and Copenhagen strains, respectively) and the orf virus F2L, suggested that LIL was the FPV equivalent of the RAP94 gene. RAP94 is thought to confer on the RNA polymerase the ability to transcribe dsDNA from early VV promoters in the presence of the early transcription factor (Zhang et al., 1994).

A 52.0% similarity was noted between the deduced amino acid sequence of FPV L2R and VV Ag35 (H6R and H5R in the WR and Copenhagen strains, respectively). Ag35 is a major surface antigen with an apparent molecular mass of 35 kDa (Gordon et al., 1991), although its predicted molecular mass is only 22.3 kDa (Rosel et al., 1986). Ag35 is found early in VV-infected cells, is associated with localized areas on the spherical surface of developing particles and then becomes evenly distributed over the virion surface. This protein possesses a hydrophilic N-terminal half and an amphipathic C-terminal helix having both hydrophilic and hydrophobic domains on opposite sides of the same helix (Gordon et al., 1991). Like the Ag35 protein of VV, the predicted 19.3 kDa L2R gene product in FPV was very basic and was predominantly hydrophilic.

The homology between the FPV L3R and other poxvirus topoisomerase genes suggested that L3R was the FPV topoisomerase gene homologue. Poxvirus topoisomerases are suspected to be essential for transcription, replication, recombination and the resolution of viral telomeres (McFadden, 1988).

The critical region (AAAAATTGAAAAA) for the FPV L2R gene promoter began 30 nt upstream of the early/late RNA transcriptional start site identified by primer extension. This region closely resembled the critical region within the P7.5 promoter (AAAAAGTAAAATATA; Davison & Moss, 1989a) and the optimized sequence of VV early promoters (AAAAATGAAAAA(C/T)TA; Davison & Moss, 1989a) and the critical region identified within the strong early/late FPV promoter (AAAAATTGAATGTAA; Kumar & Boyle, 1990a, b).

FPV appears to have early gene transcriptional regulation sequences similar to those of VV. The sequence TTTTTTTT, 14 nt downstream of the L2R gene, is a subset of TTTTTNT which controls termination of transcription in VV early genes. The presence of VV-like early transcription termination sequences just downstream of early FPV genes has been described by others as well (Kumar & Boyle, 1990a, b; Binns et al., 1988).

Most strong late VV promoters are characterized by the presence of the TAAATG element overlapping the
The TAAAC or TAAAA (mapping at -47 and -54 nt, TAAAC or TAAAA sequences upstream of the FPV initiation codon) may have been sufficient for late early and late mRNA) may have been reactivated. Also the L2R critical region and 110 nt upstream of the ATG initiation codon and promoter, may have been reactivated. Also the L2R putative late promoter was also active late in infection. Evidence supporting early promoter reactivation during late VV infection was found by Garces et al. (1993) for the VV early promoter of the 7.5K and rpo30 genes. Also, transcriptional initiation occurring from identical positions for both early and late transcription has already been observed for the FPV early/late promoter (Kumar & Boyle, 1990b) and the same may occur for the L2R promoter.

The ability of the FPV L2R promoter in pL2RHN to transiently express HN transcripts both early and late during FPV infection of CEF cells, despite the elimination of the putative late promoter TAAAT element, suggests that the TAAAT element was not essential for late expression. Perhaps the second TAAAT element, starting 34 nt upstream of the putative early promoter critical region and 110 nt upstream of the ATG initiation codon (and present in pL2RHN), was sufficient. Alternatively, the early L2R promoter, like the VV early P7.5 promoter, may have been reactivated. Also the L2R TAAAC or TAAAA (mapping at -47 and -54 nt, upstream of the L2R ATG initiation codon and corresponding to the transcriptional start site for both early and late mRNA) may have been sufficient for late FPV expression, as is the situation for VV intermediate gene promoters (Balduck et al., 1992). Fleming et al. (1993) identified an orf virus early/late F3R gene which initiates late RNA transcripts from a TAAAG sequence instead of the VV-like TAAAT sequence. Therefore, the TAAAC or TAAAA sequences upstream of the FPV L2R gene may also behave like the TAAAT sequence in VV.

The two strongly expressed late ORFs, L1L and L3R, had the highly conserved TAAAT element with the transcriptional start sites overlapping the ATG start codon (TAAATG). This further demonstrates the conservation of promoter sequences among poxviruses. However the upstream regions of the FPV L1L and L3R putative late promoters were A+T rich, unlike VV strong late promoter upstream regions which are only T rich.

Early in FPV infection, transient expression of HN RNA controlled by the FPV L2R promoter was at least 6-fold higher than that of the VV P7.5 promoter. This promoter is also efficiently expressed, although only about half as strongly, late in infection. This strong transcription at both early and late times by the FPV L2R promoter is well suited for the expression of foreign genes in rFPVs constructed for use as replicating and non-replicating vaccines. The potential use of FPV as a vector for non-replicating vaccines was demonstrated by the ability of a rFPV that expressed a rabies virus glycoprotein under the control of the VV H6 promoter to protect mammals against rabies (Taylor & Paoletti, 1988). Strong early/late promoters have been preferentially used in non-replicating rFPV vaccines because in non-avian cell lines late FPV gene expression is delayed and at a lower level when compared to late gene expression in avian cells (Somogyi et al., 1993).

We thank Paul Huber for his excellent photographic assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (Grant-STR0016018) and by the Ontario Ministry of Agriculture, Food and Rural Affairs.

References


(Received 18 August 1995; Accepted 27 November 1995)