Comparison of a Conserved Region in Fowlpox Virus and Vaccinia Virus Genomes and the Translocation of the Fowlpox Virus Thymidine Kinase Gene

By MATTHEW M. BINNS,* FIONA M. TOMLEY, JOAN CAMPBELL AND MICHAEL E. G. BOURSNELL

Institute for Animal Health, Houghton Laboratory, Houghton, Huntingdon, Cambridgeshire PE17 2DA, U.K.

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SUMMARY

The DNA sequence of a clustered set of genes which are conserved in orthopoxviruses has been determined for the avipoxvirus, fowlpox virus. The arrangement of the genes in fowlpox virus is nearly identical to that in vaccinia virus, and genes which are overlapping in vaccinia virus overlap in fowlpox virus. One major difference exists however, as the thymidine kinase (TK) gene is absent in fowlpox virus from the position it occupies within this cluster of genes in vaccinia virus. Instead, in fowlpox virus there is a 32 bp non-coding region present between the genes that flank the TK gene in vaccinia virus. The fowlpox virus TK gene has been cloned and sequenced. The sequences immediately flanking the TK gene show no homology to any previously reported poxvirus gene. These results are discussed in terms of genome stability in poxviruses and the use of the TK gene as a non-essential region for the introduction of foreign genes into poxviruses.

INTRODUCTION

Poxviruses have a complex dsDNA genome of approximately 130 to 280 kb, and replicate in the cytoplasm of infected cells. Members of the poxvirus family infect a wide range of animal species and are classified into six genera, orthopoxviruses, parapoxviruses, leporipoxviruses, capripoxviruses, avipoxviruses and suipoxviruses (Baxby, 1984). Interest in poxviruses has largely concerned the eradication of smallpox by vaccination with vaccinia virus. Accordingly, the orthopoxviruses, to which smallpox virus and vaccinia virus belong, are the best characterized. The demonstration that foreign immunogen genes could be introduced into and expressed in vaccinia virus (Mackett et al., 1982, Panicali & Paoletti, 1982) has restimulated interest in poxviruses. In particular both avipoxviruses and capripoxviruses are being studied as potential vectors for new veterinary vaccines, a role to which vaccinia virus is also being applied (Brown et al., 1986).

The poxviruses are quite poorly characterized at the molecular level. Studies involving restriction cleavage patterns have indicated that the different members of the orthopoxvirus group have a highly conserved central genome region (Mackett & Archard, 1979). The same studies indicated that these conserved restriction fragments were not present in parapoxviruses or avipoxviruses. Approximately 50 kb of DNA sequence from the vaccinia virus genome (186 kb) has been published, and a number of functions have been assigned to the sequenced genes. Comparisons of gene sequences for poxviruses not belonging to the orthopoxvirus group have been carried out for the thymidine kinase (TK) (Boyle et al., 1987; Upton & McFadden, 1986), growth factor (Chang et al., 1987; Upton et al., 1987) and DNA polymerase (Binns et al., 1987) genes. In general the genes show little conservation of sequence at the nucleotide level while maintaining strong homology at the amino acid level. In addition several genes have been mapped in vaccinia virus but again there is a lack of information for other poxviruses.
Two recent publications suggest that the overall genomic organization of poxviruses outside the orthopoxvirus group may vary significantly. First, Upton & McFadden (1986) have mapped the TK gene of Shope fibroma virus (SFV), a leporipoxvirus, to a position approximately 50 kb from the right terminal hairpin. In vaccinia virus the TK gene maps approximately 75 kb from the left terminal hairpin. Also, in SFV the open reading frame (ORF) upstream of the TK gene shares homology with that of vaccinia virus but the ORF downstream shows no homology to that of vaccinia virus. Second, when we examined the data of Boyle et al. (1987) we noted that the TK gene of fowlpox virus (FPV), an avipoxvirus, is flanked by sequences that show no homology to either the upstream or downstream sequences of vaccinia virus or SFV. Taken together, these findings suggest that the TK gene may have been mobile in the evolution of the poxviruses. Given that the TK gene is frequently used as a non-essential region for gene insertion in poxviruses we have sought to clarify whether the FPV TK gene has been translocated. In the process we have demonstrated the presence of the highly conserved orthopoxvirus region in FPV but found that this region no longer contains the FPV TK gene.

METHODS

Molecular cloning of FPV DNA. DNA from FPV strain HP444 was isolated as previously described (Binns et al., 1987). Samples of DNA were partially digested with MboI to determine conditions yielding fragments of 30 to 40 kb. Fragments of this size were ligated with BamHI-cleaved pJB8 cosmid (Ish-Horowicz & Burke, 1981). The ligated cosmids were then packaged and transfected into Escherichia coli DH5 by established protocols (Maniatis et al., 1982). The cosmid-cloned FPV DNA was analysed by performing alkaline ‘mini-preps’ (Maniatis et al., 1982) on the transfectants. DNA from a series of suitable cosmids was then purified on caesium chloride/ethidium bromide (CsCl/EtBr) gradients. PstI fragments from cosmid clone pMB304 were cloned into PstI-cleaved pBR322 and the cloned inserts analysed using STET preparations (Holmes & Quigley, 1981). Plasmid DNA from one such clone, pMB359 containing an 8 kb PstI fragment, was isolated on CsCl/EtBr gradients.

Sequencing strategy. Random fragments of HP444, pMB359 and pMB361 DNA were generated by sonication (Deininger, 1983), end-repaired, fractionated to collect fragments of 300 to 600 bp (Dretzen et al., 1981) and cloned into Smal-cleaved phosphatase-treated M13mp10 (Amersham). A series of 250 recombinants from HP444 were sequenced by the M13/dideoxynucleotide chain termination method (Sanger et al., 1977) using buffer-gradient gels and 35S label (Biggin et al., 1983). These sequences were translated in all six reading frames and compared to a library of published vaccinia virus sequences as previously described (Binns et al., 1987). Colonies of random M13 clones from pMB359 were probed with a 32P-labelled 8 kb PstI insert of pMB359, prepared from low-gelling temperature agarose, to identify M13 clones with viral inserts. Positive M13 clones were sequenced as before and the data analysed on a VAX11/750 using the programs of Staden (1982, 1984). Protein homologies were examined using FASTP (Lipman & Pearson, 1985).

Hybridizations. Dot blots were carried out by denaturing 0-5 μg of cosmid and plasmid DNAs in 0-25 M-NaOH at 60 °C for 1 h followed by neutralization with 1 M-Tris–HCl buffer pH 7-5. Five microlitre samples were dotted onto nitrocellulose filters which were baked under vacuum at 80 °C for 4 h. Probes from M13 clones were labelled by carrying out a normal sequencing reaction in the absence of dideoxynucleotides and with [32P]dCTP instead of [35S]dATP. Prehybridization, hybridization and filter washes were carried out as described by Binns et al. (1987). Southern blots, colony hybridizations and preparation of nick-translated probes were all carried out by established methods (Maniatis et al., 1982).

RESULTS

Identification of the conserved region

From the screening of the random clones of HP444, two were identified that showed homology at the amino acid level to two genes within the region conserved among different orthopoxviruses. Clone MFP393 showed homology with the F11 gene of vaccinia virus described by Plucienniczak et al. (1985) and clone MFP432 with the F9 gene. This was of interest because F9 is the gene next to the TK gene in vaccinia virus. Clone MFP432 was therefore 32P-labelled and used to probe a set of cosmid clones in a dot blot hybridization. The probe hybridized strongly to pMB304 which was subsequently studied in more detail.

Initially RsaI fragments of pMB304 were cloned into Smal-cleaved M13mp10. The resulting clones were found to contain other sequences from the conserved region, including the RNA polymerase 147K large subunit (F12), F2, F4, F5, F6 and F7. Subsequently we demonstrated

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Clustered genes of fowlpox virus

Table 1. Percentage identity between FPV (FP) and vaccinia virus (F) genes

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Identical amino acids (%)</th>
<th>Conservative changes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP1/F1</td>
<td>37.6</td>
<td>25.7</td>
</tr>
<tr>
<td>FP2/F2</td>
<td>55.2</td>
<td>35.9</td>
</tr>
<tr>
<td>FP3/F3</td>
<td>23.5</td>
<td>38.2</td>
</tr>
<tr>
<td>FP4/F4</td>
<td>45.3</td>
<td>27.1</td>
</tr>
<tr>
<td>FP5/F5</td>
<td>33.3</td>
<td>33.3</td>
</tr>
<tr>
<td>FP6/F6</td>
<td>41.7</td>
<td>19.7</td>
</tr>
<tr>
<td>FP7/F7</td>
<td>39.2</td>
<td>28.4</td>
</tr>
<tr>
<td>FP9/F9</td>
<td>53.3</td>
<td>24.9</td>
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</table>

that clone MFP432 (F9) hybridized to an 8 kb PstI fragment of pMB304. This fragment was cloned into PstI-cleaved pBR322 and the resulting clone, pMB359, was sequenced.

Sequence analysis

The sequence of the 6.1 kb fragment of pMB359 is presented in Fig. 1 together with the translation of the major ORFs, FP0 to FP9. The sequence starts 146 bases upstream of the initiation codon for the FP0 ORF and ends at the PstI site used for cloning the 8 kb fragment, which lies within the F9 ORF. When the gene organization of this region in FPV is compared with that in vaccinia virus it can be seen that the two are very similar, even to the extent that genes which overlap in the latter (F3/F4 and F6/F7) also overlap in FPV (see Fig. 2). The one major difference in the gene organization in this region is that whereas in vaccinia virus the TK gene (F8) lies between F7 and F9, in FPV the gene for F7 is next to that for F9. There are only 32 bp between the termination signal for F7 and the initiation codon for F9.

In vaccinia virus this conserved region encodes a set of mainly late genes (Plucienniczak et al., 1985) and many of the FPV sequences 5' to the ORFs closely resemble the consensus sequence $\AA\AA\AA TAAAT_{\AA\AA}$ which has been proposed for vaccinia virus late promoters (Hanggi et al., 1986). A comparison of the probable promoter sequences present 5' to the genes in FPV and vaccinia virus is presented in Fig. 3 (FP and F respectively). Also included in the figure is a comparison of the sequence 5' to the vaccinia virus TK gene with that of the FPV sequence we have recently determined. The comparisons are influenced by the fact that the tight packing of genes in this region places some of the promoter sequences within coding regions for the preceding gene. The comparisons show that the FP2/F2, FP4/F4, FP5/F5 and FP7/F7 promoter sequences are particularly closely related. Of these the FP2/F2 and FP7/F7 promoters lie within coding regions. FP4/F4 and FP5/F5 are encoded on opposite strands such that the non-coding region between the ATG initiation codons may serve as a promoter on both strands (see Fig. 1).

The sequence presented in Fig. 1 shows that another gene, FP0, can be added to this tightly packed gene cluster; this gene again has a typical late promoter sequence 5' to its coding region. In addition the sequence of the amino end of the FP1 gene, which was not included in the vaccinia virus sequence (Plucienniczak et al., 1985), is presented and this is also preceded by a late promoter (see Fig. 3).

The percentage identity between the FPV and vaccinia virus proteins is presented in Table 1 and ranges from 55% for FP2/F2 to 23% for FP3/F3.

Sequence of the FPV TK gene

A BamHI clone, pMB310, containing the FPV TK gene was identified from a BamHI library using a 35 residue oligonucleotide derived from the sequence of the FPV TK gene (Boyle et al., 1987). A HindIII fragment of pMB310 containing the TK gene was subcloned into pUC13 to generate pMB361, and this clone was sequenced. The sequence of the HP444 TK gene, along with flanking sequences including the complete upstream gene, is presented in Fig. 4. The sequence within the TK coding region is identical to that of the Webster mild vaccine strain (Boyle et al., 1987). Furthermore the flanking sequences are similar indicating that the TK gene
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Fig. 1. Nucleotide sequence of 6.1 kbof FPVDNA encoding a set of conserved genes FP0 to FP9. The sequence starts 146 bp upstream of the FO gene and ends within F9 at the PstI site which was used in the cloning. The mNor ORFs have been translated using the single-letter amino acid code. The 32 bp non-coding region between FP7 and FP9 has been underlined.
**Fig. 2.** Comparison of the gene organization in vaccinia virus (F1 to F9) with that in FPV (FP0 to FP9). The genes are shown in the reading frames in which they occur, with F4/FP4 encoded on the opposite DNA strand.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>FP0</td>
<td>ATACGTGAATAATATACCATATCAAGTTTTAATAACAAATGAAAAATAAATAGT</td>
</tr>
<tr>
<td>FP1</td>
<td>CTATAAAGTCAGCTTTTTTGGGTTTCAGGATATATACTACGTAATAATAGT</td>
</tr>
<tr>
<td>FP2</td>
<td>TTTTTGGATTTTTATTATGATAGCGTTAATATTAATTGTTTTACTTTAAATG</td>
</tr>
<tr>
<td>FP3</td>
<td>ACCGGTGTTGGTTTAGAGAATTAAATTCAACTAATAATACATTAATAGT</td>
</tr>
<tr>
<td>FP4</td>
<td>GATTTTCTAATAGAAGATCGTTCATTTAGTAGATTCTTTATTGTCATCATG</td>
</tr>
<tr>
<td>FP5</td>
<td>CTGTAAGTTGCAATTATTTACTCAATTTAACCTTTAAAATACTTACGTAATAGT</td>
</tr>
<tr>
<td>FP6</td>
<td>TATTTAATACGATGAAAAACATTTGTCTTTGTCGAATTTATAGTATCTAAATAGT</td>
</tr>
<tr>
<td>FP7</td>
<td>TGTTCAACATCGGTACCGGTATTCAATTATCAGAAACTTTTTACTTTAAATAGT</td>
</tr>
<tr>
<td>FP8</td>
<td>TATTTATACGATGAAAAACATTTGTCTTTGTCGAATTTATAGTATCTAAATAGT</td>
</tr>
<tr>
<td>FP9</td>
<td>CTAAAATAATACGATGAAAAACATTTGTCTTTGTCGAATTTATAGTATCTAAATAGT</td>
</tr>
</tbody>
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**Fig. 3.** Comparison of the 5' upstream sequences of FPV (FP) and vaccinia virus (F) genes. Identical bases are indicated by colons. The 5' upstream sequence of the FPV TK gene (FPTK) which we have recently determined is compared to the vaccinia virus sequence (F8) and the sequences for FP0 and FP1, are included for completeness.
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Fig. 4. Nucleotide sequence and predicted amino acid sequence of the FPV HP444 TK gene. The six bases in the 3' downstream region which are absent from the Webster mild vaccine strain are underlined.

DISCUSSION

In this paper we have demonstrated that a tight cluster of genes present in a conserved region of orthopoxviruses is present in an almost identical arrangement in the avipoxvirus, FPV. We have extended the cluster to include the initiation codon for FP1 and another closely packed gene FP0. Both FP0 and FP1 appear to be late genes, judged by analysis of their 5' non-coding sequences, which conform to the consensus sequence AA AAT G recently determined for vaccinia virus late genes (Hanggi et al., 1986). The 5' non-coding sequences of FP2, FP3, FP4, FP5, FP6 and FP7 also conform to the consensus although a C residue is present at position 2 in FP5 and FP6, and at position 9 in FP3 (see Fig. 3).

In vaccinia virus several of the proteins encoded in the region equivalent to that sequenced here have been identified by in vitro translation of hybrid-selected mRNA (Weir & Moss, 1984; Belle Isle et al., 1981). These include polypeptides of 30K and 27K which probably correspond to the FP1 and FP20RFs, and a major late protein of 28K which corresponds to FP5. The conservation of ORFs FP1 to FP9 in FPV suggests that these proteins are translated in both FPV and vaccinia virus. Plucienniczak et al. (1985) reported homologies between F2 and variable surface glycoprotein 3 of Trypanosoma brucei, F4 and VP2 of foot-and-mouth disease virus and VP1 of poliovirus, and F9 with anthranilate synthase from E. coli and Neurospora crassa.

All of the FPV ORFs have been compared with the National Biomedical Research Foundation databases using FASTP. A match was observed between FP9 and anthranilate synthase of E. coli (optimized score 90). This match is stronger than that previously noted with the vaccinia virus F9 protein (optimized score 52). The shared homologies, involving different amino acids in many cases, suggest that the poxvirus F9 proteins are genuinely related to the anthranilate synthases. Poor matches between FPV FP4 and poliovirus VP1 or foot-and-mouth
disease virus VP2 at the same level as those previously reported for the vaccinia virus proteins were also noted.

By sequencing the region FP0 to FP9 we have demonstrated that one gene, the TK locus, is absent from this gene cluster in FPV. The TK gene from FPV HP444 has been cloned and sequenced and is flanked by sequences the same as those reported for the Webster mild vaccine strain of FPV by Boyle et al. (1987). These sequences show no homology to the genes that flank the TK gene in the orthopoxviruses vaccinia, variola or monkeypox (Esposito & Knight, 1984), or to any other published poxvirus sequence. In the leporipoxvirus SFV, the TK gene maps in a different position to that in vaccinia virus but the SFV TK gene is flanked on the 5' side by the same gene (F7) as in vaccinia virus (Upton & McFadden, 1986). On the 3' side the SFV TK is flanked by a gene that is not homologous to the vaccinia virus gene or to those which flank the TK gene in FPV. These results indicate that the TK gene in poxviruses exists in three different genetic loci in the three poxvirus genera studied. It would be of interest to know more about the gene organization in SFV, to determine whether more genes have been translocated along with F7 and TK, and also the structure of the region around the SFV F9 gene. In a recent set of experiments (Ball, 1987), designed to study homologous recombination in vaccinia virus, its TK gene (on an 888 bp Sau3A fragment) was flanked by direct repeats of a 1503 bp λ phage fragment and this construct was insertered into the original TK locus. This arrangement was inherently unstable under non-selective conditions. Interestingly, examination of two mutants isolated after eight non-selective passages that had achieved a stable TK + phenotype showed that the TK gene had relocated without any flanking λ sequences to a site in the HindIII F fragment about 45 kb from its original position. Although derived under artificial conditions, these mutants demonstrate the facility poxviruses possess for rearranging their genomes.

TK genes are frequently used as non-essential regions into which foreign gene sequences are introduced to form recombinant poxviruses. The location of the TK gene in three different positions in three different genera of poxviruses suggests that this gene may be mobile and care should be taken to ensure that recombinants constructed by insertion into TK genes are stable.

Whilst this manuscript was in preparation Drillien et al. (1987) published the sequence of the FP6 to FP12 genes from the Salsbury vaccine strain of FPV. They also found that between FP7 and FP9 there is 32 bp of non-coding DNA which is identical to that presented here for the HP444 FPV strain. Taken together, our data and those of Drillien et al. (1987) demonstrate that the genes FP1 to FP12 (comprising approximately 7 kb) are highly conserved between FPV and vaccinia virus with the exception of the TK gene which is relocated elsewhere. Studies are in progress to map the position of the FPV TK gene to see whether it maps in a region similar to that of the vaccinia virus HindIII G fragment.

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REFERENCES


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