Comparison of the locations of homologous fowlpox and vaccinia virus genes reveals major genome reorganization

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We have derived a restriction enzyme map for the fowlpox virus FP9 strain. Sites for BamHI, PvuII, PstI and NcoI have been mapped mainly by Southern blotting. The size of the genome derived from the restriction maps (254 kb) corresponds to the figure of 260 ± 8 kb determined from analysis of genomic DNA by pulsed-field electrophoresis. The map can be compared with a previously published map for a different strain of fowlpox virus using the PstI digest which is common to both studies. Some 65 kb of fowlpox virus sequence, in 11 blocks, as well as individual M13 clones have been aligned with the map. Where those blocks correspond with blocks of homologous genes in vaccinia virus, it is possible to compare the genomic locations for those genes in the two viruses. This comparison reveals that, whereas there are blocks of sequence within which genes exist in the same relative position in the two viruses, the genomic location of those sequence blocks differs widely between the two viruses.

Introduction

The complete genome sequence has been determined for only one member of the poxvirus family, vaccinia virus (VV) strain Copenhagen, type species of the Orthopoxvirus genus (Goebel et al., 1990). Restriction enzyme analysis of the orthopoxviruses has shown that the central 60% of the genome is conserved with variation between species occurring mainly near the termini (Witteke et al., 1977; Mackett & Archard, 1979; Esposito et al., 1979). Subsequent sequence analysis has shown that this region contains the genes essential for virus replication (Plucienniczak et al., 1985; Broyles & Moss, 1986; Niles et al., 1986; Rosel et al., 1986; Schmitt & Stunnenberg, 1988). That the central region of the genome is conserved in a different genus was demonstrated by Gershon et al. (1989), who exploited the cross-hybridization at low stringency between DNA from VV and capripox virus (CPV) to show that the two viruses share a relatively conserved, collinear central region of 105 to 120 kb.

DNA sequence analysis of parts of the genomes of various poxviruses has demonstrated the similar organization of small numbers of genes within the cloned sequences. Genes are normally collinear within these cloned sequences (Drillien et al., 1987; Binns et al., 1988, 1990a, 1992; Gershon et al., 1989; Tartaglia et al., 1990; Upton et al., 1991), except when the cloned sequences have been derived from the inverted terminal repeat (ITR) regions (Upton & McFadden, 1986; Upton et al., 1987; Tomley et al., 1988; Campbell et al., 1989; Goebel et al., 1990; Howard et al., 1991; Smith et al., 1991). Despite the collinearity of most genes within these cloned sequences, there are examples of individual genes which are found at different positions in different viruses, such as thymidine kinase (TK; Upton & McFadden, 1986; Boyle et al., 1987; Drillien et al., 1987; Binns et al., 1988, 1992; Gershon & Black, 1989; Schnitzlein & Tripathy, 1991; Jackson & Buls, 1992), the gene downstream of TK in CPV and leporipoxviruses, which is homologous to the VV host range gene C7L (Kotwal & Moss, 1988; Gershon & Black, 1989; Goebel et al., 1990; Schnitzlein & Tripathy, 1991; Jackson & Buls, 1992) and genes which are found in some but not all viruses such as the gene encoding A-type inclusion body protein ATI (Funahashi et al., 1988; Gershon et al., 1989; Goebel et al., 1990), and genes X and Y in fowlpox virus (FPV, Binns et al., 1990a, 1992; Tartaglia et al., 1990). There are also examples of gene families occurring, with members found at various sites within the genome of a virus, such as serine protease inhibitors (serpins) (Pickup et al., 1986; Boursnell et al., 1988; Tomley et al., 1988; Kotwal & Moss, 1989; Smith et al., 1989, 1991; Upton et al., 1990), growth factors (Venkatesan et al., 1982; Blomquist et al., 1984; Chang et al., 1987; Porter & Archard, 1987), host range proteins (Gillard et al., 1986; Upton et al., 1987; Kotwal & Moss, 1988; Spehner et al., 1988; Tamin et al., 1988; Tomley et
al., 1988; Perkus et al., 1990; Howard et al., 1991), proteins containing ankyrin repeats (Lux et al., 1990; Howard et al., 1991; Smith et al., 1991) and lectins (Tomley et al., 1988; Smith et al., 1991). Members of these gene families are often found at different sites in different viruses (Tomley et al., 1988; Goebel et al., 1990; Smith et al., 1991). None of these results, however, contradict the presence of a collinear core of essential genes being present in the vertebrate poxviruses. Recent data by Hall & Moyer (1991), however, indicate that the same might not be true of the entomopoxviruses. A 7 kb DNA fragment from the central 50% of the genome of *Amsacta moorei* entomopoxvirus (AmEPV) contained sequences from six genes: the spheroidin gene, two genes with no known homologues and homologues of three genes which in VV are widely separated.

Here we present results which indicate that the collinear core of essential genes found in the mammalian poxviruses is considerably rearranged in FPV, type species of the Avipoxvirus genus.

**Methods**

*Virus and cells.* The attenuated HP438 strain of FPV, derived from the pathogenic HP1 strain by 438 passages (Mayr & Malicki, 1966) in chick embryo fibroblasts (CEF), was a gift from Professor A. Mayr. A twice plaque-purified isolate (FP9) was derived after two further low multiplicity passages in CEFs. Virus was grown and purified as described in Binns et al. (1987).

*Purification, digestion and Southern blotting of FPV DNA.* Isolation of FPV DNA from sucrose gradient-purified virus was as described by Tomley et al. (1988). Digested FPV DNA (1 μg) was electrophoresed on 0.75% agarose gel (Type V; Sigma) in TAE buffer at 20 V for 16 h, then stained with ethidium bromide and photographed. Gels were denatured for 30 min in 0.5 M-NaOH, 1.5 M-NaCl then neutralized for 30 min in 2 M-NaCl, 1 M-Tris-HCl pH 7.5. Each gel was blotted onto two Hybond-N filters (Amersham) in 20 x SSC overnight, rinsed in 2 x SSC and exposed to u.v. light (365 nm) for 5 min on a transilluminator. Where it was necessary to re-use blots, they were washed three times for 20 min in 2 x SSC at 42 °C then exposed to Kodak XAR film (flashed and with screens as necessary).

*Results and Discussion*

**Restriction enzyme map**

The products of restriction digests of FPV FP9 genomic DNA were analysed by conventional and pulsed-field agarose gel electrophoresis (Fig. 1). The estimated sizes of the DNA fragments, obtained following digestion with *PstI*, *BamHI*, *PvuII* and *NcoI* and electrophoresis on several conventional and pulsed-field agarose gels, are shown in Table 1. The genome size estimated from the maps is 251 to 267 kb, 244 to 262 kb, 244 to 262 kb and 248 to 258 kb for *PstI*, *BamHI*, *PvuII* and *NcoI*, respectively, with a mean size of 254 kb. We have derived restriction enzyme maps (Fig. 2) for these enzymes mainly by Southern blotting of digested FPV DNA using either purified or cloned restriction enzyme fragments as probes. Many fragments have been cloned and further characterized (M. Binns, unpublished data) and some have been sequenced (Binns et al., 1987, 1988, 1989, 1990a, b, 1992; Tomley et al., 1988; Campbell et al., 1989; M. A. Skinner and others, unpublished data).

*Pulsed-field gel electrophoresis.* Pulsed-field electrophoresis was performed using a CHEF DR II apparatus (Bio-Rad). Electrophoresis of restriction fragments was performed in 1% Seakem ME agarose (FMC) in 0.5 x TBE buffer, at 10 °C, 180 V, for 22 h using either 10 s or 1 to 5 s pulses.

For pulsed-field electrophoresis of intact FPV genomic DNA, sucrose gradient-purified FPV (2 x 108 p.f.u.) was pelleted and resuspended in 20 μl 10 mM-Tris-HCl pH 7.5, 125 mM-EDTA, 1% sarkosyl, 10% 2-mercaptoethanol. Molten low melting point agarose (60 μl, BRL) in 10 mM-Tris-HCl pH 7.5, 125 mM-EDTA, 1% sarkosyl at 50 °C was added to the virus suspension prewarmed to 37 °C, then the virus/agarose pellet was suspended in 0.5 ml of 10 mM-Tris-HCl pH 7.5, 125 mM-EDTA, 1% sarkosyl, 5% 2-mercaptoethanol, proteinase K (1 mg/ml, Boehringer), at 37 °C overnight. The virus/agarose pellet was pelleted by centrifugation at 6500 r.p.m. for 5 min in a microfuge and then washed repeatedly in 0.5 ml 50 mM-EDTA pH 8.0 for 15 min at room temperature. The pellet was drained, cut up and placed in two wells of a 1% agarose gel. The pellet was sealed into the wells using molten agarose, then the samples were electrophoresed in 0.5 x TBE buffer, at 10 °C, 160 V, for 36 h using 20 to 50 s pulses. Gels were stained in ethidium bromide (5 μg/ml) for 30 min prior to viewing on a transilluminator.

**DNA size markers.** For electrophoresis of restriction fragments of FPV, a combination of λ DNA cut with *HindIII* (BRL) plus φX174 RF DNA cut with *HaeIII* (BRL) plus uncut λ DNA (Boehringer) was used as a marker next to each digast track of FPV DNA. For pulsed-field electrophoresis of FPV genomic DNA, λ concatemers (low molecular weight markers, New England Biolabs) were used.

**Probes.** Following restriction enzyme digestion of 2 to 3 μg FPV DNA, specific bands were purified from 0.5% agarose gels (electrophoresed in TAE) using Gene clean II (Bio 101) following the manufacturer's protocol. Purified plasmid or FPV DNA was labelled with [α-32P]dCTP using Prime-it (Stratagene) and separated from unincorporated nucleotides on a Sephadex G50 column (Pharmacia).

**Hybridization.** Membranes were pre-hybridized and hybridized at 65 °C in 3 x SSC, 1 x Denhardt's solution, 0.2% SDS, 100 μg herring sperm DNA/ml for 1 h and overnight, respectively. Membranes were washed three times for 20 min in 2 x SSC at 42 °C then exposed to Kodak XAR film (flashed and with screens as necessary).
Fig. 1. Analysis of the products of digestion of FPV genomic DNA with restriction endonucleases. Markers (M) used in all cases are λ DNA uncut and digested with HindIII, giving the sizes shown. (a) Products were electrophoresed by conventional agarose gel electrophoresis (see Methods) and viewed by u.v. illumination of ethidium bromide-stained gels. (b) Pulsed-field electrophoresis of ethidium bromide-stained digestion products (see Methods) using 1 to 5 s pulses. In both panels, FPV DNA was digested with PstI (lane 1), BamHI (lane 2), PvuII (lane 3) or NcoI (lane 4). The faint band at about 39 kb in the BamHI digest (b, lane 2) is present at less than equimolar concentrations and is considered to be derived from a small proportion of a variant virus in this thrice plaque-purified population. (c) Pulsed-field electrophoresis of FPV genomic DNA (lane 1) following lysis of purified virions embedded in agarose (see Methods). Marker (M) sizes are shown.

Genomic DNA, using a modification of the method of Schwartz & Cantor (1984). Intact genomic DNA could then be analysed by pulsed-field electrophoresis (Fig. 1c), following which an apparent size of 260 ± 8 kb was estimated, which is in good agreement with the mean size of 254 kb estimated from the restriction enzyme maps.

Comparison of the PstI maps of FP9 and of FPV-M and FPV-M3

Previously published restriction enzyme maps (Coupar et al., 1990) for the FPV mild vaccine strain (FPV-M) and for a derivative (FPV-M3), using enzymes PstI, SalI, SmaI and NcoI, can be compared with that for FP9 through the common PstI maps. The FPV-M virus had inverted terminal repeats (ITRs) of ≥16.5 kb (as represented by the presence of PstI fragment G at both ends of the genome). FPV-M3 had ITRs of <16.5 kb, as the PstI G fragment was now found at only one end of the genome, but ≥8.2 kb, as represented by the presence of the 8-2 kb BamHI fragment L/M at both ends of the genome. As has been demonstrated previously by sequence analysis and hybridization (Tomley et al., 1988), the ITRs of FP9 are about 10 kb in length, containing the BamHI 6-2 kb fragment K/L. FP9, like FPV-M3 but unlike FPV-M, has only one terminal PstI fragment G (16-3 kb). In FP9, the largest PstI fragment (42 kb, A) is found at one terminus, whereas in FPV-M3 the largest fragment (45 kb, A') is found internal to fragment D' (24-5 kb), A'/D' being derived from A/G in FPV-M. Thus, fragment A of FP9 is equivalent to A'/D' of FPV-M3. The internal PstI fragments show the same relative position and similar estimated sizes for FP9, FPV-M3 and FPV-M. However, it is clear that PstI L in FP9 is smaller than in FPV-M (8-3 kb compared to 11-2 kb), comigrating with PstI K in FPV-M. It also appears that the relative sizes of fragments F, G and H
Table 1. Size of restriction endonuclease fragments of FPV FP9 genomic DNA estimated after conventional and pulsed-field agarose gel electrophoresis

<table>
<thead>
<tr>
<th>PstI</th>
<th>BamHI</th>
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<th>NcoI</th>
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<tr>
<td>Fragment</td>
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<td>Size (kb)</td>
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<td>A</td>
<td>48-52</td>
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<tr>
<td>B</td>
<td>37-40</td>
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<tr>
<td>C</td>
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<tr>
<td>D</td>
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<td>D</td>
<td>19-21</td>
</tr>
<tr>
<td>E</td>
<td>20-22</td>
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<td>1-8</td>
<td>Z</td>
<td>1-8</td>
</tr>
<tr>
<td>α†</td>
<td>0-9†</td>
<td>α†</td>
<td>0-9†</td>
</tr>
</tbody>
</table>

Total size (kb): 251 to 267
Average size (kb): 259

* A band migrating at the position of NcoI fragment D was previously thought to be a doublet. This is now known not to be the case but the previous nomenclature has been maintained for consistency, thus there is no band E.
† Fragments found within the inverted terminal repeats.
‡ Size derived from sequence analysis only.

Differ between the viruses: G in FP9 migrates closer to H than to F, whereas G in FPV-M almost comigrates with F.

Comparison of genome sizes of FP9, FPV-M and FPV-M3

We estimate the genome size of FP9 to be 254 kb, compared to 309 kb for FPV-M and 299 kb for FPV-M3 (Coupar et al., 1990). The 10 kb size difference between FPV-M and FPV-M3 was attributed to a deletion relative to FPV-M in PstI A/G (63 + 16-5 = 79-5 kb) to give PstI A'/D' in FPV-M3 (45 + 24-5 = 69-5 kb). Comparing FP9 with FPV-M3, it is clear that PstI A of FP9 (42 kb) is further deleted (by some 28 kb) relative to the equivalent PstI A'/D' of FPV-M3 (69-5 kb). In addition, fragment PstI B of FP9 is some 4 to 5 kb shorter than the equivalent fragment PstI B of FPV-M3 and fragment PstI L of FP9 is some 3 kb shorter than fragment PstI L of FPV-M3. These major differences therefore account for FP9 DNA being 35 kb shorter than that of FPV-M3. The other minor size differences between equivalent bands probably account for the rest of the observed difference in genome size.

Localization of FPV homologues of VV genes on the FPV restriction enzyme map

Seven blocks of FPV genomic DNA sequence that contain homologues of VV genes have been localized on the FPV restriction map. These sequence blocks are represented by genomic clones described in Table 2.
**Fowlpox virus genome organization**

![Restriction enzyme map of FPV FP9 genomic DNA](image)

**Fig. 2. (a)** Restriction enzyme map of FPV FP9 genomic DNA. The figure is drawn to scale using the estimated average size for each fragment. Where fragments of nearly identical size have not been distinguished (such as PvuII A and A' or B and B'), they are both drawn as the average size of the two fragments. (b) Comparison of the location of blocks of sequence and of some single ORFs in FPV and VV. Sequence blocks from FPV which have homologous blocks in VV (filled rectangles below the FPV BamHI map) are aligned with the VV HindIII map. The relative position of these blocks is indicated by the joining dashed lines. Where known, the relative orientation of the blocks is indicated by arrows. The position of FPV restriction enzyme fragments used to map random FPV M13 clones homologous to VV ORFs (in brackets) is shown by (, 0. The relative position of these is also indicated by the joining dashed lines, the position of the VV genes being indicated by filled bars. The blocks of FPV sequence shown above the FPV map are those which have no overall homology to VV sequences. The position of the inverted terminal repeats is shown by (, - ). The position of the TK genes in both viruses is also indicated.

**Table 2. FPV genomic clones**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Reference</th>
<th>VV homologues</th>
<th>Hybridizing fragments</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>BamHI</td>
<td>PstI</td>
</tr>
<tr>
<td>371</td>
<td>Binns et al. (1989, 1990b)</td>
<td>A3L(4b) to A5R</td>
<td>A</td>
</tr>
<tr>
<td>294</td>
<td>Binns et al. (1987)</td>
<td>E9L (DNA polymerase)</td>
<td>P</td>
</tr>
<tr>
<td>429</td>
<td>M. A. Skinner (unpublished data)</td>
<td>H3L to H7R</td>
<td>A</td>
</tr>
<tr>
<td>438</td>
<td>M. A. Skinner (unpublished data)</td>
<td>A44L, A50R</td>
<td>C</td>
</tr>
</tbody>
</table>

**Comparison of the genome locations of homologous genes of FPV and VV**

Map positions, obtained during this study for the clones described above, are shown in Fig. 2(b). Clones 294, 445, 359, 429, 379/389 and 371 contain homologues of genes from the central portion of the VV genome (HindIII fragments E to the internal end of A; Fig. 2b). In FPV, however, these clones form two blocks, either side of the centre, separated by some 50 kb (Fig. 2b). Furthermore, the relative position of these clones is different from the
relative position of the homologous VV sequences. Thus, clones 359, 429 and 371 are found in FPV in the same order as are their VV homologues (although in VV, 379/389 comes between 429 and 371). Clones 438, 379, 389, 445 and 294 are present in FPV in the opposite order to their VV homologues. Indeed, clones 438 and 379/389 are present in the opposite half of the FPV genome relative to their VV homologues. The different location of these blocks is clearly illustrated by considering 429 (homologues of VV H3L to H7R and D1R), 379/389 (homologues of VV genes D6R, D7R, D9R to D13L and A1L) and 371 (homologues of VV A3L to A12L, A20R, A24R, A29L, A32L and B1R, some ORFs being reversed relative to others (e.g. 294 and 445 relative to 359 and 429). Other blocks have been translocated relative to their neighbours; block 379/389 has been translocated from its position between the equivalents of 429 and 371 in VV to the other end of the genome in FPV, and block 438 has been translocated from near one end of the genome in VV to the other end in FPV. In moving to opposite ends of the genome, blocks 379/389 and 438 have reversed their positions relative to each other, block 438 being maintained as the distal block in both viruses. Unfortunately, it has not been possible to orientate the 379/389 and 438 blocks on the FPV genome. It would be intriguing if these two blocks, like block 445, have reversed their orientation in FPV relative to VV so that blocks found to the left of centre in the FPV genome would be in a reverse orientation to their VV homologues. It is notable that random FPV clones in M13 homologous to VV genes E1L, F9L and F10L map to the right of 294 in FPV but to the left in VV (Fig. 2b). Although it has also not been possible to orientate blocks 429 and 371, their position relative to block 359 (which has the same orientation in FPV as in VV) suggests that these blocks, which are found to the right of centre in FPV, are in the same orientation as in VV.

These results pose something of a quandary as to which is the ‘correct’ orientation of the FPV genome relative to that of VV, whether as drawn in Fig. 2(b), with blocks 359, 429 and 371 in the same order in both viruses or in the reverse orientation with blocks 294, 445, 379/389 and 438 in the same order. That the ‘correct’ orientation is as represented in Fig. 2(b) is supported by experiments to map random FPV clones in M13. Of 61 M13 clones mapped to restriction fragments PstI F, PvuII C, PvuII D (equivalent to the right half of BamHI A and BamHI E; Fig. 2a, b) and BamHI G, 21 showed significant homology at the amino acid level with VV ORFs using FASTP. Of those 21, 18 showed homology with ORFs from VV HindIII A and B [namely A3L (which is present in clone 371), A7L, A10L, A12L, A20R, A24R, A29L, A32L and B1R, some ORFs being identified by more than one M13 clone]. This indicates that the region to the right of block 371, as shown in Fig. 2(b), shows extensive homology to the VV HindIII A homologues in the other genome, such as the replacement of the D8L homologue in FPV by ORF Y in clones 379/389 (Binns et al., 1990a). The mechanism for such replacements is not known but we have reported recently (Binns et al., 1992) that the TK gene in FPV is flanked by 15 bp direct repeats, suggesting that the translocation event might have involved transposition.

On a larger scale, it is apparent from our results that blocks of genes have been rearranged relative to each other. The orientations of some blocks of genes have been reversed relative to others (e.g. 294 and 445 relative to 359 and 429). Other blocks have been translocated relative to their neighbours; block 379/389 has been translocated from its position between the equivalents of 429 and 371 in VV to the other end of the genome in FPV, and block 438 has been translocated from near one end of the genome in VV to the other end in FPV. In moving to opposite ends of the genome, blocks 379/389 and 438 have reversed their positions relative to each other, block 438 being maintained as the distal block in both viruses. Unfortunately, it has not been possible to orientate the 379/389 and 438 blocks on the FPV genome. It would be intriguing if these two blocks, like block 445, have reversed their orientation in FPV relative to VV so that blocks found to the left of centre in the FPV genome would be in a reverse orientation to their VV homologues. It is notable that random FPV clones in M13 homologous to VV genes E1L, F9L and F10L map to the right of 294 in FPV but to the left in VV (Fig. 2b). Although it has also not been possible to orientate blocks 429 and 371, their position relative to block 359 (which has the same orientation in FPV as in VV) suggests that these blocks, which are found to the right of centre in FPV, are in the same orientation as in VV.

Comparison of genome organization of FPV and VV

It would therefore appear that differences in the genome organization of FPV and VV occur at a number of levels. One or a few genes may be deleted from within a sequence of otherwise contiguous homologues, for instance TK from clone 359 (Drillien et al., 1987; Binns et al., 1988) and homologues of A45R to A49R from clone 438 (M. A. Skinner and others, unpublished data). These deleted genes may occur in a different position in the genome (e.g. TK in clone 445). Deleted genes might be replaced by genes from elsewhere in the genome, such as the replacement of the I4L homologue in FPV by TK (Binns et al., 1992) or by genes which have no
that right-hand ends of the genomes are equivalent as shown in Fig. 2(b) and that the FPV genome is shown in the ‘correct’ orientation.

The situation found in comparing FPV (as an avipoxvirus) to VV (as an orthopoxvirus) is similar to that found in comparing varicella-zoster virus (VZV, an alphaherpesvirus) to Epstein-Barr virus (EBV, a gammaherpesvirus). Davison & Taylor (1987) showed that three regions exist within the U₅ component of these viruses which show a collinear arrangement of homologous genes. Denoted as A, B and C, the arrangement of these regions in VZV was ABC, whilst in EBV it was CA'B, where A' denotes A in reverse orientation. There was no detectable gene conservation within the U₅ component, the repeats or sequences near the ends of U₅, a situation comparable with that found in the near-terminal regions of FPV and VV. For both of these viral families, it is clear that the divergent members are derived from common ancestors, and that large-scale rearrangements have occurred in one or both lineages. The complexity of the rearrangements observed between FPV and VV supports the contention of Davison & Taylor (1987) that none of the present-day members may represent the prototype genome organization. It is interesting that these two families of large DNA viruses both show such a similar evolutionary fate, given that one replicates in the nucleus, the other in the cytoplasm. That a similar situation has arisen may be due to the fact that viral DNA is replicated by virus-encoded DNA polymerases in both cases, although the detailed molecular mechanisms for the rearrangements may well be different.

**Location of FPV-specific sequences**

The near-terminal regions are likely to provide most of the FPV-specific sequences and thus account for the extra 65 kb of genomic DNA, as shown by the BamHI H and L sequences (Tomley et al., 1988; Campbell et al., 1989) and by a 6.5 kb fragment (clone 564; M. A. Skinner and others, unpublished data) to the left of block 438. It is clear that although the central 50 kb of FPV contains some sequences homologous to those in VV HindIII F and E, there is probably capacity for FPV-specific sequences. This is illustrated by the fact that the 2.2 kb BamHI S fragment (clone 284; F. Tomley, personal communication), which lies within this region (Fig. 2b), has been found to have no homology to VV genes (M. A. Skinner, unpublished data). FPV-specific sequences will probably also lie between the blocks of VV homologues, such as between 379/389 and 445 or between 429 and 371. Indeed a 0.7 kb fragment (US1), which contains ORFs with no VV homologues (J. Campbell, personal communication) maps between 438 and 379/389 (Fig. 2b).

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**References**


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