Deletion of fowlpox virus homologues of vaccinia virus genes between the 3β-hydroxysteroid dehydrogenase (A44L) and DNA ligase (A50R) genes

Michael A. Skinner,[1]* Jeffrey B. Moore,[2] Matthew M. Binns,[3] Geoffrey L. Smith[2] and Michael E. G. Boursnell[4]

[1] *Institute for Animal Health, Compton Laboratory, Nr. Newbury, Berkshire RG16 0NN,*
[2] *Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE,*
[3] *Animal Health Trust, Lanwades Park, Kennett, Newmarket, Suffolk CB8 7PN and*
[4] *Cantab Pharmaceuticals Limited, Cambridge Science Park, Milton Road, Cambridge CB4 4GN, U.K.*

A fragment of 4156 bp of fowlpox virus (FPV) genomic DNA contains homologues of vaccinia virus 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD; A44L) and DNA ligase (A50R) genes. The FPV locus has clearly been rearranged relative to that of vaccinia virus as homologues of genes A45R to A49R, including the thymidylate kinase and a gene with homology to superoxide dismutase, are deleted. The deleted genes are replaced by two open reading frames: for a serine proteinase inhibitor with homology to vaccinia virus gene K2L and for a protein with no significant homology to proteins in the databases. In addition, the FPV homologues of A44L and A50R are in the same polarity in FPV whereas they are in opposite polarities in vaccinia virus. Increased 3β-HSD activity has been demonstrated in cells infected with either of two different strains of FPV or with canarypox virus.

The nucleotide sequences of a number of regions of fowlpox virus (FPV) genomic DNA have been obtained (reviewed in Mockett *et al.*, 1992). Except for the terminal and near-terminal sequences, these blocks show considerable local collinearity of homologous genes when compared with the genome of vaccinia virus, although the translocation of the thymidine kinase gene is a notable exception (Binns *et al.*, 1992). We have already shown that there is considerable reorganization of the genome when the locations of the blocks of homologous genes are compared in FPV and vaccinia virus (Mockett *et al.*, 1992).

The vaccinia virus DNA ligase locus (Smith *et al.*, 1989*a*) lies at the boundary of the conserved core of essential genes and the terminal region defined as non-essential by the derivation of large deletion mutants (Goebel *et al.*, 1990; Smith *et al.*, 1991; Johnson *et al.*, 1993). The ligase gene itself has been shown to be non-essential (Kerr & Smith, 1991; Colinas *et al.*, 1990). Other nearby genes have also been shown to be non-essential, such as the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD; Moore & Smith, 1992). The status of this locus in FPV is therefore of interest, in particular given our demonstration that the locus appears to have translocated (relative to flanking loci) to the opposite end of the genome in FPV compared to vaccinia virus (Mockett *et al.*, 1992).

The translated sequence of an M13 clone (MFP298), from a random library of sonicated fragments of FPV genomic DNA, showed homology to the vaccinia virus DNA ligase (A50R; Kerr & Smith, 1989). A 'prime-cut' probe generated from MFP298 hybridized to a 5·6 kb *Pvu*II fragment of FPV genomic DNA (fragment I; Mockett *et al.*, 1992). A library of *Pvu*II-digested FPV genomic DNA was constructed in pUC13 (at the *Sma*I site) and was probed by colony hybridization (Grunstein & Hogness, 1975) with the probe generated from MFP298. A positive plasmid clone, pMB438, was identified as carrying the 5·6 kb fragment. Random fragments of pMB438, generated by sonication, were cloned into M13 and sequenced using methods described previously (Binns *et al.*, 1992).

The sequence of 4·1 kb of *Pvu*II fragment I contains four open reading frames (ORFs) greater than 300 bp in length (Fig. 1). All four ORFs are present on the same strand of DNA (reading left to right). Comparison of the predicted protein products of the ORFs with sequence databases revealed that three ORFs were homologous to ORFs of vaccinia virus. ORF 1 encoded a 42K protein homologous to the 3β-HSD homologue (ORF SalF7L or A44L) of vaccinia virus (Goebel *et al.*, 1990; Blasco *et*

The nucleotide sequence data reported in this paper appears in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number Z29716.

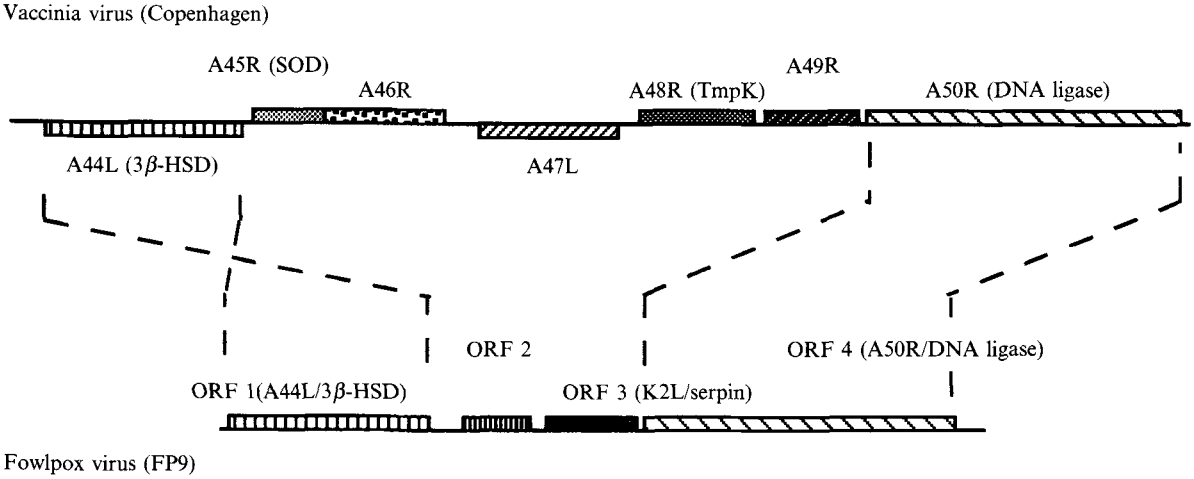

Fig. 1. Comparison of genome organization around the 3β-HSD/DNA ligase locus in FPV (sequence reported in this paper) and vaccinia virus (strain Copenhagen; using the gene nomenclature and sequence data of Goebel *et al.*, 1990). Drawn to scale. Shaded blocks represent ORFs; those above the line are transcribed left-to-right, those below it are transcribed right-to-left. Identical shading represents homologous ORFs. SOD, superoxide dismutase-like protein; TmpK, thymidylate kinase. Prepared using CAD Gene (Genetic Technology Corporation).

al., 1991; Smith *et al.*, 1991). The ORF 3 product (19K) showed homology with the serine protease inhibitor (serpin) encoded by vaccinia virus gene K2L (Boursnell *et al.*, 1988) and the 64K predicted product of ORF 4 was homologous to the vaccinia virus DNA ligase (SalF13R or A50R; Kerr & Smith, 1989). The predicted product of the remaining ORF 2 (15K) showed no significant homology with any proteins in the database (Swissprot v. 26), the highest initial and optimized score being 44 for neurofibromin (Swiss:Nf1_Human), with which it shares 23·5% identity over 34 amino acids.

The predicted amino acid sequence encoded by the FPV 3β-HSD gene (ORF 1) showed 33 to 35% amino acid identity (over a length of 352 to 358 residues) with mammalian 3β-HSDs (murine, rat, bovine, macaque and human). It showed 36% to 37% amino acid identity with 3β-HSDs from the Copenhagen and WR strains of vaccinia virus (Goebel *et al.*, 1990; Blasco *et al.*, 1991; Smith *et al.*, 1991) and 28% identity with that from fish lymphocystis disease virus, an iridovirus (Baker & Blasco, 1992). Comparison of the three viral 3β-HSDs shows that there are two absolutely conserved blocks of four or more amino acids: Gly-13 to Arg-17 and Gly-226 to His-232 (using coordinates for the FPV protein). More than four amino acids of both of these blocks are also conserved, as shown by Moore & Smith (1992), between the vaccinia virus, bovine and human type I proteins (isoforms of four types have been identified in rodents, encoded by a multigene family). The first of these blocks corresponds to the nucleotide binding domain of the steroid dehydrogenases, as discussed by Rutherfurd *et al.* (1991), present as both the GXGXXG and GXXGXXG motifs in viral and bovine proteins but only as GXGXXG in the human protein. The second of the two conserved blocks coincides with the steroid isomerase binding site that was shown to be conserved between the mammalian proteins and steroid isomerases from *Pseudomonas putida* and *P. testosteroni* (Rutherfurd *et al.*, 1991). Two residues are completely conserved between viral, bacterial and mammalian proteins: those equivalent to Gly-226 and Ala-235 of the FPV protein.

To see whether FPV (and canarypox virus), like vaccinia virus, expressed functional 3β-HSD, cells infected with FPV or with canarypox virus were assayed for 3β-HSD activity, as described by Moore & Smith (1992). The results (Fig. 2) demonstrated that infection of chick embryo fibroblasts (CEFs) with FPV strain FP9 caused an increase in 3β-HSD activity, at 6 h post-infection (p.i.), of almost threefold. This compares to a level of induction of fourfold for wild-type vaccinia virus (WR) in mammalian cell lines (Moore & Smith, 1992 and unpublished). The activity had decreased by 50% at 24 h p.i. and was insensitive to the presence of cytosine arabinoside in the culture medium (data not shown), indicating that expression of 3β-HSD occurs early, as reported previously for vaccinia virus (Moore & Smith, 1992). Similar levels of activity were seen for CEFs infected by the Duphar 'poxine' vaccine strain of FPV, which is less attenuated than FP9, or by canarypox virus, another avipoxvirus. Although it cannot be ruled out that infection of CEFs by avipoxviruses induces a cellular enzyme, such an event was not observed when CV-1 cells were infected with a 3β-HSD-deficient vaccinia virus mutant (Moore & Smith, 1992). Given the presence of the conserved gene in FPV, we consider it more likely that the induced activity was due to the virus-encoded

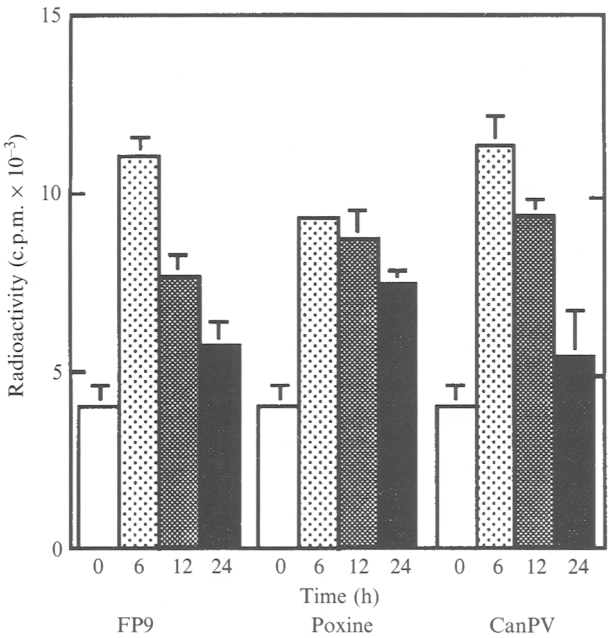

Fig. 2. The activity of 3β-HSD in monolayers of CEFs was measured by the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone as described by Moore & Smith (1992). Secondary cultures of CEFs in 24-well plates were mock-infected or infected, in triplicate, with 5×10^6 p.f.u. of FPV (FP9 or Duphar 'poxine' strains) or canarypox virus (CanPV), in the presence (data not shown) or absence of cytosine arabinoside (40 µg/ml). At 6, 12 and 24 h p.i., cells were washed and the assay was performed as described by Moore & Smith (1992). A set of samples was also fixed with ethanol at room temperature for 10 min, prior to washing and assay, to provide background values for the non-enzymatic conversion to progesterone. Standard deviations are shown.

enzyme. It remains to be determined whether 3β-HSD is involved in avipoxvirus virulence in avian hosts as was shown to be the case for vaccinia virus in mice (Moore & Smith, 1992).

The FPV DNA ligase encoded by ORF 4 has predicted amino acid identities of 48% (over 555 residues) with the vaccinia virus DNA ligase (encoded by A50R; Kerr & Smith, 1989), 32% with those of human and yeast (over 389 and 409 residues, respectively) and less than 20% with DNA ligases from African swine fever virus (Hammond *et al.*, 1992) and bacteriophages. The sequence around the active site at Lys-236 (VEFKYDGERIQIH) is well conserved compared to ligases of human, yeast and viral origin (Hammond *et al.*, 1992).

A poxvirus serpin homologue was initially identified in cowpox virus (CPV) as a gene responsible for the red-plaque phenotype of wild-type CPV (Pickup *et al.*, 1986). This serpin (CrmA, CPV 38K) was subsequently shown to function by inhibiting activation of interleukin-1β by interleukin-1β-converting enzyme (ICE), a type of cysteine protease (Ray *et al.*, 1992). Recently it has been shown that CrmA can also inhibit the induction of apoptosis by ICE (Miura *et al.*, 1993). In addition, the expression of CrmA was correlated with the inhibition of formation of a chemoattractant biochemically similar to (14R,15S)-dihydroxyeicosatetraenoic acid (diHETE), an arachidonic acid metabolite (Palumbo *et al.*, 1993). Serpin genes have also been identified in myxoma virus (Upton & McFadden, 1986), vaccinia virus (*spi*-1, *spi*-2 and *spi*-3; Boursnell *et al.*, 1988; Kotwal & Moss, 1989; Smith *et al.*, 1989*c*) and in the 11 kb near-terminal *Bam*HI fragment of FPV (Tomley *et al.*, 1988). Like the FPV serpin identified by Tomley *et al.* (1988) which is 148 amino acids long, the ORF 3 product reported here is considerably shorter (168 amino acids) than other serpins, including those from other poxviruses, which are in excess of 350 amino acids long. The ORF 3 product shares 19 to 27% amino acid identity with a wide range of serpins and overall is most closely related to Spi-3 (encoded by K2L) of vaccinia virus (the ORF 3 product shares 27, 24 and 20% identity, respectively, with Spi-3, Spi-1 and Spi-2 from vaccinia virus and 18% with the other FPV serpin). The ORF 3 product aligns well with Spi-3 up to the conserved aspartate at residue 118 and from the conserved asparagine at residue 143 but the region around and towards the N terminus from the serpin active site is poorly conserved, in common with the serpin-like 47K heat shock proteins. The region of the ORF 3 product aligning with the active site (reviewed by Gettins *et al.*, 1993), EKISN, contains a serine residue which may be equivalent to the active-site serine of Spi-1 and 3.

The genes found between A44L and A50R in vaccinia virus but not in FPV encode a homologue of superoxide dismutase (A45R; Goebel *et al.*, 1990; Blasco *et al.*, 1991; Smith *et al.*, 1991), a protein with an integrin lipid-binding motif (A47L; Johnson *et al.*, 1993), thymidylate kinase (A48R; Smith *et al.*, 1989*b*; Hughes *et al.*, 1991) and two proteins of unknown function (A46R and A49R; Goebel *et al.*, 1990; Smith *et al.*, 1991). Of these, only the thymidylate kinase gene has thus far been identified as non-essential in tissue culture (Hughes *et al.*, 1991). It remains to be seen whether it and the other genes are indeed absent from FPV, which has a genome some 30% larger than that of vaccinia virus, or whether, like the thymidine kinase gene (Binns *et al.*, 1992), they are present at a different genomic location.

The change in relative orientation of the FPV homologues of vaccinia genes A44L and A50R is notable if not particularly surprising. In considering the relative positions of blocks of genes in vaccinia virus and FPV, we previously concluded (Mockett *et al.*, 1992) that a number of recombination events (including the transposition of homologues of A44L and A50R, relative to flanking markers, from one end of the genome to the other) separated the two viruses, which have probably

diverged from a common ancestor. It is therefore not possible to ascertain which of the two viruses, if either, has the genes in the ancestral positions.

We wish to acknowledge the skilled technical assistance of Bridgette Mockett and the help of Pal Somogyi. J.B.M. was funded by the MRC (programme grant PG8901790 to G.L.S.).

References

BAKER, M. E. & BLASCO, R. (1992). Expansion of the mammalian 3 beta-hydroxysteroid dehydrogenase/plant dihydroflavonol reductase superfamily to include a bacterial cholesterol dehydrogenase, a bacterial UDP-galactose-4-epimerase, and open reading frames in vaccinia virus and fish lymphocystis disease virus. *FEBS Letters* **301**, 89–93.

BINNS, M. M., BOURSNELL, M. E. & SKINNER, M. A. (1992). Gene translocations in poxviruses: the fowlpox virus thymidine kinase gene is flanked by 15 bp direct repeats and occupies the locus which in vaccinia virus is occupied by the ribonucleotide reductase large subunit gene. *Virus Research* **24**, 161–172.

BLASCO, R., COLE, N. B. & MOSS, B. (1991). Sequence analysis, expression, and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein. *Journal of Virology* **65**, 4598–4608.

BOURSNELL, M. E. G., FOULDS, I. J., CAMPBELL, J. I. & BINNS, M. M. (1988). Non-essential genes in the vaccinia virus *Hin*dIII K fragment: a gene related to serine protease inhibitors and a gene related to the 37K vaccinia virus major envelope antigen. *Journal of General Virology* **69**, 2995–3003.

COLINAS, R. J., GOEBEL, S. J., DAVIS, S. W., JOHNSON, G. P., NORTON, E. K. & PAOLETTI, E. (1990). A DNA ligase gene in the Copenhagen strain of vaccinia virus is nonessential for viral replication and recombination. *Virology* **179**, 267–275.

GETTINS, P., PATSTON, P. A. & SCHAPIRA, M. (1993). The role of conformational change in serpin structure and function. *BioEssays* **15**, 461–467.

GOEBEL, S. J., JOHNSON, G. P., PERKUS, M. E., DAVIS, S. W., WINSLOW, J. P. & PAOLETTI, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179**, 247–266.

GRUNSTEIN, M. & HOGNESS, D. S. (1975). Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proceedings of the National Academy of Sciences, U.S.A.* **72**, 3961–3965.

HAMMOND, J. M., KERR, S. M., SMITH, G. L. & DIXON, L. K. (1992). An African swine fever virus gene with homology to DNA ligases. *Nucleic Acids Research* **20**, 2667–2671.

HUGHES, S. J., JOHNSTON, L. H., DE CARLOS, A. & SMITH, G. L. (1991). Vaccinia virus encodes an active thymidylate kinase that complements a cdc8 mutant of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **266**, 20103–20109.

JOHNSON, G. P., GOEBEL, S. J. & PAOLETTI, E. (1993). An update on the vaccinia virus genome. *Virology* **196**, 381–401.

KERR, S. M. & SMITH, G. L. (1989). Vaccinia virus encodes a polypeptide with DNA ligase activity. *Nucleic Acids Research* **17**, 9039–9050.

KERR, S. M. & SMITH, G. L. (1991). Vaccinia virus DNA ligase is nonessential for virus replication: recovery of plasmids from virus-infected cells. *Virology* **180**, 625–632.

KOTWAL, G. J. & MOSS, B. (1989). Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily (published erratum appears in *Journal of Virology* **64**, 966). *Journal of Virology* **63**, 600–606.

MIURA, M., ZHU, H., ROTELLO, R., HARTWEIG, E. A. & YUAN, J. (1993). Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homologue of the *C. elegans* cell death gene ced-3. *Cell* **75**, 653–660.

MOCKETT, B., BINNS, M. M., BOURSNELL, M. E. G. & SKINNER, M. A. (1992). Comparison of the locations of homologous fowlpox and vaccinia virus genes reveals major genome reorganization. *Journal of General Virology* **73**, 2661–2668.

MOORE, J. B. & SMITH, G. L. (1992). Steroid hormone synthesis by a vaccinia enzyme: a new type of virus virulence factor. *EMBO Journal* **11**, 1973–1980.

PALUMBO, G. J., GLASGOW, W. C. & BULLER, R. M. (1993). Poxvirus-induced alteration of arachidonate metabolism. *Proceedings of the National Academy of Sciences, U.S.A.* **90**, 2020–2024.

PICKUP, D. J., INK, B. S., HU, W., RAY, C. A. & JOKLIK, W. K. (1986). Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 7698–7702.

RAY, C. A., BLACK, R. A., KRONHEIM, S. R., GREENSTREET, T. A., SLEATH, P. R., SALVESEN, G. S. & PICKUP, D. J. (1992). Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. *Cell* **69**, 597–604.

RUTHERFURD, K. J., CHEN, S. & SHIVELY, J. E. (1991). Isolation and amino acid sequence analysis of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. *Biochemistry* **30**, 8108–8116.

SMITH, G. L., CHAN, Y. S. & HOWARD, S. T. (1991). Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. *Journal of General Virology* **72**, 1349–1376.

SMITH, G. L., CHAN, Y. S. & KERR, S. M. (1989*a*). Transcriptional mapping and nucleotide sequence of a vaccinia virus gene encoding a polypeptide with extensive homology to DNA ligases. *Nucleic Acids Research* **17**, 9051–9062.

SMITH, G. L., DE CARLOS, A. & CHAN, Y. S. (1989*b*). Vaccinia virus encodes a thymidylate kinase gene: sequence and transcriptional mapping. *Nucleic Acids Research* **17**, 7581–7590.

SMITH, G. L., HOWARD, S. T. & CHAN, Y. S. (1989*c*). Vaccinia virus encodes a family of genes with homology to serine proteinase inhibitors. *Journal of General Virology* **70**, 2333–2343.

TOMLEY, F., BINNS, M., CAMPBELL, J. & BOURSNELL, M. (1988). Sequence analysis of an 11·2 kilobase, near-terminal, *Bam*HI fragment of fowlpox virus. *Journal of General Virology* **69**, 1025–1040.

UPTON, C. & MCFADDEN, G. (1986). Identification and nucleotide sequence of the thymidine kinase gene of Shope fibroma virus. *Journal of Virology* **60**, 920–927.

(Received 11 February 1994; Accepted 7 April 1994)