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Steroid hormone synthesis by a vaccinia enzyme: a new type of virus virulence factor

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Vaccinia virus open reading frame (ORF) SalF7L has 31% amino acid identity to human 3β-hydroxysteroid dehydrogenase/$\Delta^5-\Delta^4$ isomerase (3β-HSD). Here we show that SalF7L encodes an active 3β-HSD, by the conversion of pregnenolone to the steroid hormone progesterone. The gene is transcribed early during infection into a 1.4 kb mRNA from an initiation site 12 bp upstream of the ORF. An antiserum raised against bacterially expressed SalF7L immunoprecipitated a 38 kDa polypeptide from infected cells, but not from mock infected cells or from cells infected with a mutant virus from which the SalF7L ORF had been removed. Deletion of the gene had no effect on virus replication in CV-1 cells in culture, yet the deletion mutant was attenuated when intranasally inoculated into mice. This steroid hormone synthesizing enzyme is a novel type of virus virulence factor.

Key words: attenuation/enzyme/hydroxy steroid dehydrogenase/steroid hormone/vaccinia virus

Introduction

Vaccinia virus is the most intensively studied member of the orthopoxvirus family, a group of large DNA viruses that replicate in distinct factories in the cell cytoplasm, and which provide their own enzymes for DNA replication and transcription (reviewed by Moss, 1990a). The large double-stranded DNA genome encodes ~200 genes (Goebel *et al.*, 1990) of which approximately one-third are dispensable for virus replication *in vitro* (Perkus *et al.*, 1991). Many non-essential genes are, however, important for virus replication *in vivo*. Some of these are enzymes involved in DNA metabolism e.g. thymidine kinase (Buller *et al.*, 1985), ribonucleotide reductase (Child *et al.*, 1990), thymidylate kinase (G.L.Smith, unpublished data) and DNA ligase (Kerr *et al.*, 1991). Other examples are the vaccinia growth factor (Buller *et al.*, 1988a), a 13.8 kDa secretory protein (Kotwal *et al.*, 1989) and the virus haemagglutinin (Flexner *et al.*, 1987). Vaccinia also possess several genes which aid evasion or suppression of the host immune system. These include a secretory protein that binds C4b and blocks the classical complement pathway (Kotwal and Moss, 1988; Kotwal *et al.*, 1990), a protein that is related to translation initiation factor eIF2-α and conveys resistance to interferon (Beattie *et al.*, 1991) and three serine protease inhibitors (Boursnell *et al.*, 1988; Kotwal and Moss, 1989; Smith *et al.*, 1989) that inhibit cell fusion (Law and Smith, 1992), suppress antibody responses to foreign antigens expressed by vaccinia (Zhou *et al.*, 1990) and may block antigen presentation to cytotoxic T cells (Townsend *et al.*, 1988). Additionally, vaccinia open reading frames (ORFs) have been identified with sequence homologies to soluble receptors for tumour necrosis factor (Howard *et al.*, 1991) and interleukins 1 and 6 (Smith and Chan, 1991).

Many of the genes cited above were identified by comparing ORFs deduced from nucleotide sequence data against protein databases. Subsequent functional studies of the encoded proteins are providing new insights into the mechanisms of virus pathogenesis. Moreover, the discovery of virulence genes is enabling the rational construction of stable attenuated virus strains by the deletion of specific genes. This is necessary if recombinant vaccinia viruses are to be used as new vaccines (Brown *et al.*, 1986), to prevent the rare complications that occurred during the use of vaccinia virus to eradicate smallpox (Lane *et al.*, 1969).

In sequencing vaccinia virus, three groups noted the 31% amino acid identity of a vaccinia ORF, denoted SalF7L in the Western Reserve (WR) strain and A44L in the Copenhagen strain (Goebel *et al.*, 1990; Blasco *et al.*, 1991; Smith *et al.*, 1991), to human 3β-hydroxy steroid dehydrogenase/$\Delta^5-\Delta^4$ isomerase (3β-HSD) (Thé *et al.*, 1989). This is a key enzyme in cellular steroid hormone synthesis and deficiency leads to altered hormone levels which are often fatal during the first few months of life. Here we demonstrate that the vaccinia gene encodes an active enzyme that is expressed early during infection and is non-essential for virus replication *in vitro* but increases virus virulence *in vivo*.

Results

An alignment of the predicted amino acid sequence of SalF7L with human and bovine 3β-HSD is shown in Figure 1. Overall the proteins show striking similarity. They have a very similar length with the only substantial difference being an internal deletion of six amino acids from the vaccinia virus sequence. There are blocks of completely conserved residues which might represent essential functional domains. The degree of homology suggests that SalF7L might encode an active enzyme.

Transcriptional analyses

The nucleotide sequence of the WR gene SalF7L showed the presence of a potential late RNA start site (Rosel *et al.*, 1986; Davison and Moss, 1989) ~45 nucleotides upstream of the ORF. There are no early transcriptional termination motifs [TTTTTNT (Yuen and Moss, 1987)], within the coding region, but this sequence appears twice within 65 nucleotides of the 3′ end of the ORF. In the intergenic non-coding region between SalF7L and SalF6R there are three such motifs on the opposite strand downstream of SalF6R, which is transcribed early during infection (Duncan and

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Bovine  MAGWSCLVTGGGGFLGQRIICLLVEEKDLQEIRVLDKVFRPEVREEFSKLQSKIKLT LVNVKGTQLLLEACVQASVPV
Human   MTGWSCLVTGAGGFLGQRIIRLLVKEKELKEIRVLDKAFGPELREEFSKLQNKTKLT VVNVKGTQLLLEACVQASVPV
SalF7L  M A VYAVTGGAGFLGRYIVKLLISADDVQEIRVIDIVEDEQ      PITSKVKVINYVNYYGTQTILAACVDLGIKY

Bovine  FIHTSTIEVAGPNSYREIIQDGREEEHHESAWSSPYPYSAYMHGALNNNGILTNHCKFSRVNP VYVGNVAWAHILALR
Human   FIYTSSIEVAGPNSYKEIIQNGHEEEPLENTWPAPYPHSASINEALNNNGILSSVGKFSTVNP VYVGNVAWAHILALR
SalF7L  LIYTSSMEAIGPNKHGDPFI GHEHTLYDISPGHVYAKSVFYEQCKQHGNIMYRTVDDDAVHSRVYVGNVAWMHVLAAK

Bovine  ALRDPKKVPNIQGQFYYISEIVSFLLSPIYKYNPCFNRHLVTLSNSVFTFSYKKAQRDLGYEPLYTWEEAKQKTKEWIH
Human   ALQDPKKAPSIRGQFYYISEIVSFLLRPIYTYRPPFNRHIVTLSNSVFTFSYKKAQRDLAYKPLYSWEEAKQKTVEWVQ
SalF7L  YIQYE  GSEIKGNAYFCYACKNDMKRILFRKPSLLNNYTLKISNTTEVRTNNAEILDFNYSPIFNVDVAFERTRKWL
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Fig. 1. Amino acid alignments of vaccinia SalF7L and bovine and human 3β-HSD sequences. Sequences were aligned using the programme MULTALIGN (Barton and Sternberg, 1987). Residues conserved in all three sequences are boxed.

Smith, 1992). The presence of multiple early transcription termination signals has been noted previously in intergenic regions where two early genes are transcribed towards each other (Smith *et al.*, 1991); it may prevent transcriptional interference or reduce the amount of double stranded RNA and hence interferon induction. These data suggest that the SalF7L gene might be transcribed early and possibly also late during infection. To test this, RNA from infected cells was analysed by Northern blotting using a probe specific for the coding strand of SalF7L. Figure 2B shows that this probe detects a 1.4 kb transcript in early RNA but not in late RNAs or RNA from mock infected cells. The 5′ end of this transcript was then mapped by S1 nuclease protection analysis using a 590 bp *Dde*I−*Bst*YI probe ^{32}P-labelled at the *Bst*YI site located 115 bp downstream of the first AUG in the ORF. A 127 bp fragment of this probe was protected from S1 nuclease digestion by early RNA but not late RNA or tRNA (Figure 2A). The length of this protected fragment indicated that the RNA initiated from 12 bp upstream of the ORF (Figure 2C). Allowing for the presence of a 3′ poly(A) tail, the size of the RNA is consistent with the transcription terminating ~50 nucleotides after the first TTTTTNT motif located 1060 nucleotides downstream.

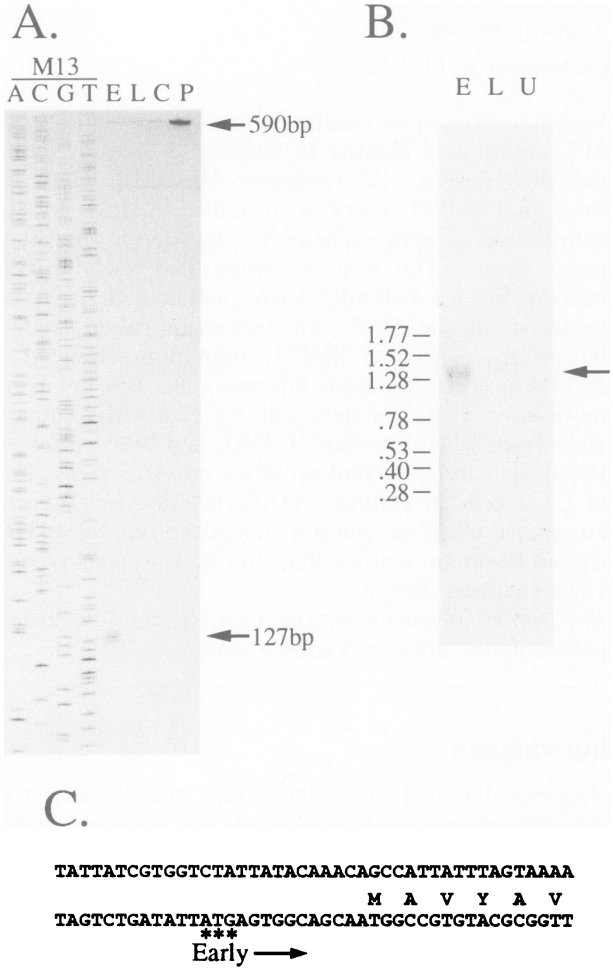

Fig. 2. Transcriptional analysis of SalF7L. **A.** S1 nuclease protection. Virus early (E) or late (L) mRNA or control tRNA (C) were hybridized with probe P, then digested with S1 nuclease and resolved on a sequencing gel alongside an M13 sequencing ladder (ACGT). The sizes of the probe and fragments protected by early RNA are indicated. **B.** Northern blot analysis. Early (E) or late (L) virus mRNA or RNA from mock-infected cells (U) were electrophoresed on a 1.3% formaldehyde agarose gel, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labelled single-stranded probe from within the SalF7L ORF. The position of the 1.4 kb early transcript is indicated. **C.** Position of the 5′ end of the early mRNA.

SalF7L is not essential for virus growth in culture

To determine the essentiality of the SalF7L ORF, we attempted to delete it using the selectable marker *Escherichia coli* guanine phosphoribosyl transferase (*Ecogpt*) (Boyle and Coupar, 1988). Plasmid pJM9, which has 86% of the SalF7L ORF replaced with the *Ecogpt* gene linked to the vaccinia 7.5K promoter, was transfected into wild type (WT)-infected cells and an MPA-resistant virus, vJM2, plaque purified in CV-1 cells. The genome of vJM2 was shown by Southern blotting to lack the SalF7L ORF but contain *Ecogpt* at this site (Figure 3A and B). When virus vJM2 was grown on CV-1 cells the plaque size was indistinguishable from WT virus (data not shown). Similarly, the replication kinetics of vJM2 and WT during single step (10 p.f.u./cell) growth curves on CV-1 and BS-C-1 cells only showed a slight (2- to 3-fold) reduction in the yield of vJM2 compared with WT (data not shown). These slight differences were not found during multiple step growth curves in CV-1 cells infected at 0.01 p.f.u./cell (Figure 4), conditions that are more likely to amplify small growth differences.

Although CV-1 cells have very little endogenous 3β-HSD activity (J.B.Moore, unpublished data), it was possible that the normal growth of vJM2 could be attributable to complementation from steroids present in serum or from phenol red, which is a weak oestrogen. Infections were therefore carried out in serum-free and phenol red-free medium. Cells were either seeded in DMEM containing 10% FCS and after growth to form a monolayer, were washed and maintained in serum-free, phenol red-free DMEM for 24 h before infection, or seeded in serum-free, phenol red-free DMEM onto plastic coated with fibronectin. Although cells seeded without serum yielded less total virus, in both conditions there was no significant difference comparing the yields of the two viruses (data not shown). SalF7L is therefore not essential for growth *in vitro*.

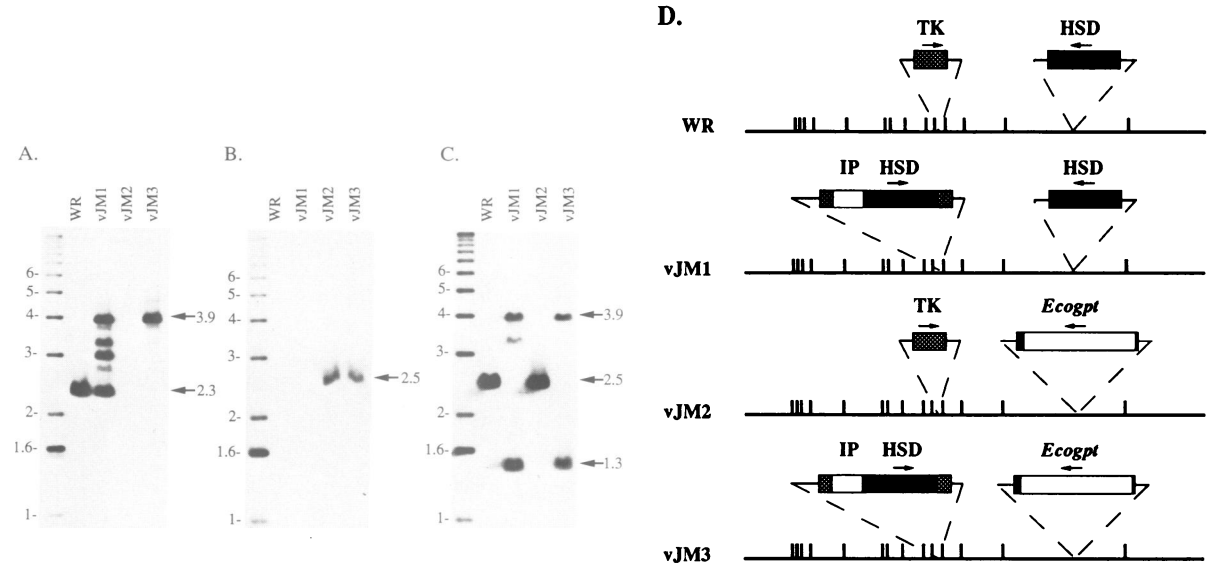

Fig. 3. Structure of recombinant virus genomes. **A–C.** Southern blot analyses. Genomic DNA was isolated from wild type (WR) or recombinant virus cores as described (Esposito *et al.*, 1981), digested with *Hin*cII, resolved by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose. Replicate filters were then probed with ^{32}P-labelled DNA from the SalF7L ORF (A), the *Ecogpt* gene (B) or the vaccinia TK gene (C). Sizes of bands in kb are indicated with arrows. The kb ladder molecular weight markers are shown. **D.** Schematic representation of WR and recombinant virus genomes, with *Hin*dIII sites indicated by vertical lines. Genes indicated are vaccinia 3β-hydroxy steroid dehydrogenase (HSD), thymidine kinase (TK), the IPTG-inducible promoter (IP) and *E.coli* guanine phosphoribosyl transferase (*Ecogpt*).

Identification of the SalF7L gene product

To characterize the SalF7L protein, a polyclonal antiserum was raised by immunization of rabbits with the SalF7L gene product expressed in *E.coli*. Additionally, a recombinant vaccinia virus was constructed which overexpressed the protein in an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible manner. This virus was made in two steps. First, a copy of the SalF7L ORF was cloned into the thymidine kinase (TK) locus of vaccinia virus under the control of an IPTG-inducible 4b promoter (Rodriguez and Smith, 1990) to give virus vJM1. Then, 86% of the endogenous copy of SalF7L was replaced with *Ecogpt* to give vJM3. The genome structures of these viruses (Figure 3D) were confirmed by Southern blot analysis. DNA isolated from virus cores was digested with *Hin*cII, and probed with DNA from within the region of the SalF7L ORF that had been deleted in plasmid pJM9 (Figure 3A), or with *Ecogpt* (Figure 3B) or TK (Figure 3C). The SalF7L probe detected the WT 2.3 kb band in WR and vJM1, and an additional 3.9 kb band in vJM1 and vJM3, representing the IPTG-inducible copy of SalF7L inserted into the TK locus. In vJM2, no band was detected, confirming the deletion of SalF7L. The *Ecogpt* probe detected a 2.5 kb band in both vJM2 and vJM3, but not in WR or vJM1, as expected. The TK probe detected the WT 2.4 kb fragment in WR and vJM2, which was replaced by two fragments of 3.9 and 1.3 kb in vJM1 and vJM3 due to a *Hin*cII site within the IPTG-inducible cassette. The additional bands in the vJM1 digests are probably the products of intramolecular recombination between the two copies of the SalF7L gene and were eliminated during the subsequent plaque purification of the recombinant virus vJM3.

The antiserum to SalF7L was used to identify the virus gene product in infected cells (Figure 5). A 38 kDa protein was precipitated from cells infected with WT (lanes 2 and 4), but not from cells infected with the deletion mutant vJM2 (lane 3) or from mock-infected cells (lane 1). In vJM3 infected cells the amount of the 38 kDa protein was substantially increased in the presence of IPTG (lanes 5 and 6), confirming the inducible nature of the SalF7L ORF in this virus.

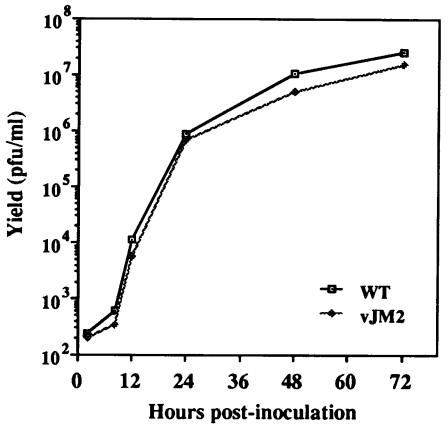

Fig. 4. Growth curves of WT and vJM2. Duplicate monolayers of CV-1 cells were infected at 0.01 p.f.u./cell with either WT or vJM2. After 1 h unbound virus was removed by washing with fresh medium and then the cells were incubated in MEM containing 2.5% FCS. At indicated times post-infection the cells were scraped into the growth medium, freeze–thawed three times, sonicated and then titrated on duplicate monolayers of BS-C-1 cells. These data shown are the mean values from duplicate samples.

SalF7L encodes an active steroid dehydrogenase

Cellular 3β-HSD catalyses many reactions in steroid hormone metabolism, all with the key events shown in Figure 6A, namely the oxidation of the 3β hydroxyl group and $\Delta^5-\Delta^4$ isomerization. A convenient assay for 3β-HSD activity relies on the different solubilities of pregnenolone and its Δ^4-3-ketosteroid metabolites, such as progesterone, in the presence of digitonin (Bauer and Bauer, 1989). When tritiated pregnenolone is added to a monolayer of cells, it

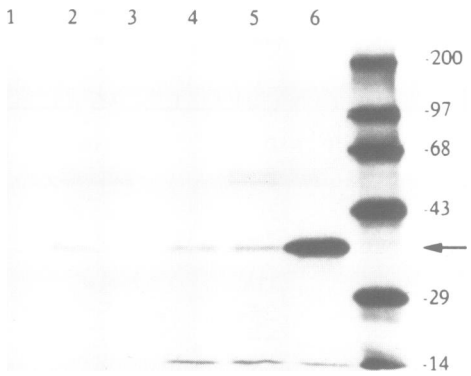

Fig. 5. Immunoprecipitation of vaccinia 3β-HSD protein. CV-1 cell monolayers were infected with WT virus (lanes 2 and 4), vJM2 (lane 3), vJM3 in the absence (lane 5) or presence (lane 6) of 5 mM IPTG, or mock-infected (lane 1). At 1 h post-infection the cells were washed and incubated in MEM lacking methionine for 30 min. The medium was then removed and replaced with the same supplemented with 40 μCi [^{35}S]methionine. At either 5 (lanes 1–3) or 20 h (lanes 4–6) post-infection, the cells were harvested and extracts prepared and immunoprecipitated with rabbit anti-SalF7L serum as described (Edbauer *et al.*, 1990).

is converted to tritiated progesterone by cellular 3β-HSD. Unconverted pregnenolone is then precipitated by addition of digitonin, while steroid hormones, such as progesterone, remain soluble. The amount of radioactivity remaining in solution is therefore a simple measure of enzyme activity.

CV-1 cells were infected with either WT virus, or the recombinants vJM1, vJM2 or vJM3, and 3β-HSD activity was measured by the above assay. Figure 6B shows that there is an increase in 3β-HSD activity in cells infected with WT virus compared to mock infected cells ($P < 0.001$). In contrast, the activity in cells infected with the deletion mutant vJM2 is the same as that in uninfected cells. Cells infected with viruses vJM1 or vJM3, both of which contain IPTG-inducible copies of the SalF7L ORF, showed substantially higher activity in the presence of IPTG. As expected, in the absence of IPTG, the removal of the endogenous copy of SalF7L in vJM3 resulted in lower 3β-HSD activity than vJM1. Under these conditions, slightly higher 3β-HSD activity was seen in vJM3 infected cells than in uninfected cells due to the leakiness of the IPTG-inducible promoter. Taken together, these data demonstrate that the vaccinia SalF7L encodes an active 3β-HSD enzyme.

Deletion of SalF7L attenuates vaccinia virus in mice

Although vaccinia virus can replicate efficiently in the absence of 3β-HSD in cultured cells, the synthesis of steroid hormones might affect virus virulence *in vivo*. This was tested by comparing the pathogenicity of WT and the deletion mutant vJM2 in mice using the intranasal route of infection (Turner, 1967; Williamson *et al.*, 1990). In one expeirment, groups of 10 mice (five female and five male) were inoculated with 10^5, 10^6, 10^7 or 2×10^7 p.f.u. of WT or vJM2 intranasally, and then observed for 21 days. The mortality of groups of mice is shown in Table IA. It is clear that deletion of the SalF7L gene substantially decreases the virulence of the virus (χ^2, $P < 0.001$). In this experiment, infection with vJM2 resulted in a higher mortality in female mice than in males (14/20 versus 9/20); however, with this sample size this was not statistically significant (χ^2, $P > 0.05$). Additionally, because of the high mortality observed

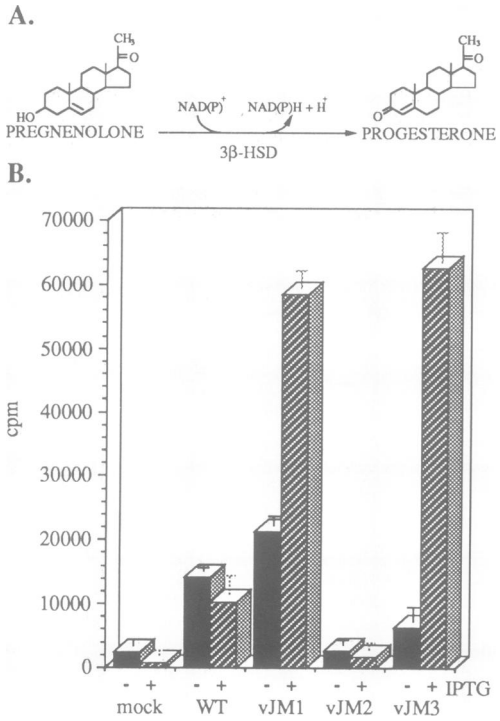

Fig. 6. Vaccinia 3β-HSD activity in infected cells. **A.** The conversion of pregnenolone to progesterone by 3β-HSD can be conveniently measured by the differential solubilities of these steroids in the presence of digitonin (Bauer and Bauer, 1989). **B.** CV-1 cells were either mock-infected or infected at 10 p.f.u./cell with WT virus, vJM1, vJM2 or vJM3, in the presence or absence of 5 mM IPTG. All infections were done in triplicate and the 3β-HSD activity of infected cells was measured as described in Materials and methods. Standard deviations are given as error bars. The background values from non-enzymatic conversion to progesterone (measured in ethanol-fixed monolayers) were subtracted from each value.

Table I. Virulence of the deletion mutant vJM2

	Dose (p.f.u.)	Virus	
		WT	vJM2
A	10^5	8/10	2/10
	10^6	10/10	5/10
	10^7	10/10	8/10
	2×10^7	10/10	8/10
B	10^3	0/10	0/10
	10^4	6/10	0/10
	10^5	8/10	0/10
	10^6	10/10	1/10
	10^7	10/10	6/10

In two experiments, 4–6 week-old BALB/c mice were anaesthetized and infected intranasally with various doses of wild type (WT) or SalF7L-deleted (vJM2) virus. Mortality of animals after 21 days is shown.

with these doses of WT, it was impossible to determine if there was a similar sex difference with WT virus.

This was examined in a second experiment with a wider range of dilutions, and mice were individually weighed daily as a general measure of health. The results are shown in Table IB and Figure 7. At low doses of vJM2 (10^3 or 10^4 p.f.u.) there was a slight weight loss in female mice compared with males (Figure 7A and B), while at higher virus doses (10^5, 10^6 and 10^7 p.f.u.) there was a substantial

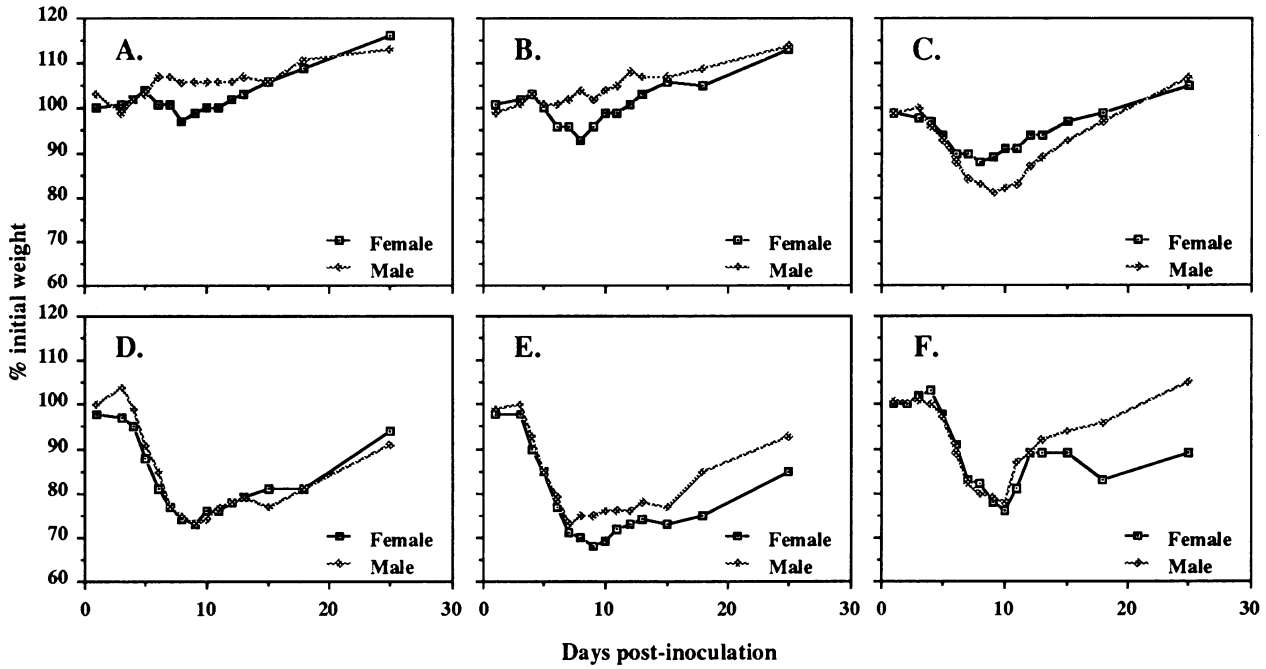

Fig. 7. Weight loss of vJM2 and WT infected mice. BALB/c mice were intranasally infected with WT or vJM2 as described in Table I and weighed individually before infection and for 21 days thereafter. On each day the mean weights of groups of five male or female mice are expressed as the proportion of the mean weight of that group of animals immediately prior to infection. Mice were infected with 10^3 (**A**), 10^4 (**B**), 10^5 (**C**), 10^6 (**D**) or 10^7 (**E**) p.f.u. of vJM2, or 10^4 p.f.u. of WT (**F**). Note that the apparent sudden increase in weight of animals in panel F on day 10 is due to the death of the sicker and lighter members of the group, so that the mean weight of survivors increased.

weight loss in both males and females, but no consistent difference between the sexes (panels C, D and E). The majority of both males and females recovered despite having lost as much as 32% of their body weight in some cases. Infection by WT virus caused more severe illness than did vJM2 as reflected in weight loss following infection (compare panels F and B) as well as mortality data (Table IB). As in the first experiment, there were fewer deaths following infection with vJM2 than there were with WR (χ^2, $P < 0.001$), confirming that attenuation resulted from the loss of the SalF7L gene, but there was no observable difference in mortality between the sexes. The LD_{50} of vJM2 was 1845 times higher than that for WT virus (probit method, Wardlaw, 1985). The deletion of other ORFs from vaccinia has been reported to result in increases of LD_{50} from 17-fold for ribonucleotide reductase to 40 000-fold for TK in intracranially inoculated mice (Buller and Palumbo, 1991), while those infected intranasally only showed an increase of between 15- and 40-fold for TK (Williamson *et al.*, 1990). These data demonstrate that SalF7L contributes substantially to virus virulence.

Discussion

Vaccinia virus gene SalF7L is shown here to encode a biosynthetic steroid enzyme. It is expressed early in the virus life cycle and is not essential for replication in culture, but contributes to virus virulence. We are not aware of any precedent for a virus encoding such an enzyme.

The normal rate of replication of the virus lacking this gene showed that the enzyme is not required for virus replication *in vitro*. However, as found with several other vaccinia genes that are non-essential *in vitro* (see Introduction), there is a clear reduction in virus virulence *in vivo* associated with the loss of this gene. SalF7L, therefore, encodes another vaccinia virulence gene, and stable virus attenuation may be obtained by its deletion. It is also another site for insertion of foreign DNA into vaccinia virus recombinants. This gene lies ~40 kb from the right genomic terminus in a region containing a cluster of other non-essential genes. These include SalF2R (S.A.Duncan, unpublished data), SalF4R (Blasco *et al.*, 1991) and SalF5R (Duncan and Smith, 1992). SalF6R is presumed not to be essential since virus Copenhagen lacks this ORF due to a small deletion and ensuing frameshift (Goebel *et al.*, 1990; Smith *et al.*, 1991; Duncan and Smith, 1992). To the right of SalF7L, genes SalF11R (thymidylate kinase; Hughes *et al.*, 1991) and SalF13R (DNA ligase; Colinas *et al.*, 1990; Kerr and Smith, 1991) are non-essential.

At the present time the function of 3β-HSD during virus replication *in vivo* is unknown. Biochemically, 3β-HSD functions at multiple steps in steroid biosynthesis, and although the assay used to demonstrate 3β-HSD activity shows that the enzyme can convert pregnenolone to progesterone, there is no indication that this is the principal reaction catalysed *in vivo*. Steroid hormones have many physiological roles in the host, either in reproduction (oestrogens, androgens and progesterone), water and salt metabolism (mineralocorticoids) or energy metabolism (glucocorticoids). At the molecular level, these hormones all work in a basically similar manner. Because of their hydrophobic nature, they are carried in the blood by serum proteins and when released from their carrier pass through the cell membrane into the cell. Here they bind to specific receptors, which were formerly thought to be cytoplasmic but are now established as being nuclear in most cases. These steroid–receptor complexes bind with high affinity to steroid responsive elements on chromatin, leading to alterations in gene regulation (reviewed by Berg, 1989). It is unlikely that vaccinia gene expression is directly enhanced by

steroid–receptor complexes, as is the case with mouse mammary tumour virus (MMTV) and glucocorticoids (Ringold *et al.*, 1975), since the virus DNA-dependent RNA polymerase recognizes only poxvirus promoters, which are fundamentally different from promoters transcribed by cellular RNA polymerase II (Moss, 1990b). There were also no alterations of vaccinia virus gene expression following infection of the steroid-responsive T47D cells and addition of progesterone or oestradiol (data not shown).

One can envisage several ways in which vaccinia might benefit from synthesis of steroid hormones. One possibility is that the steroid(s) synthesized by infected cells interact with receptors in surrounding uninfected cells and increase metabolic rates, leading to enhanced virus replication as these cells become infected. The secretory vaccinia virus growth factor also stimulates cell metabolism. It binds to cells bearing the epidermal growth factor receptor and causes these to proliferate so that the spreading virus infection encounters cells of high metabolic activity which are better able to support virus replication (Buller *et al.*, 1988a,b).

Another possible function of vaccinia-expressed steroid hormones might be to cause immunosuppression. Glucocorticoids are potent inhibitors of the immune system (Cupps and Fauci, 1982). The anti-inflammatory effect appears to be mediated through the steroid-induced increase in expression of lipocortin 1 which then inhibits phospholipase A2 (Blackwell *et al.*, 1980). This enzyme degrades cellular phospholipids into arachinodonic acid from which various mediators of inflammation such as the prostaglandins are synthesized. Prostaglandins have been shown to have varied effects on the outcome of vaccinia infection. For instance, administration of prostaglandin E_1 to BALB/c mice decreased antibody responses following vaccinia virus infection and caused higher virus titres *in vivo* and increased mortality. In contrast, administration of PGI_2 enhanced survival (Zavagno *et al.*, 1987). Glucocorticoids have also been shown to modulate the type of immune response directly by decreasing IL-2 and increasing IL-4 synthesis from T cells (Arya *et al.*, 1984; Daynes and Araneo, 1989) and also by decreasing Ia expression on macrophages (Snyder and Unanue, 1982). A possible role of glucocorticoids in vaccinia virus infections is suggested by the recent finding that vaccinia may also encode a protein related to thioredoxins (Johnson *et al.*, 1991), which can convert an inactive glucocorticoid receptor unable to bind ligand into a ligand-binding form (Grippo *et al.*, 1985).

Materials and methods

Reagents

DNA modifying enzymes were purchased from Boehringer Mannheim, AmpliTaq Polymerase from United States Biochemical and tissue culture medium from Gibco.

Cells and viruses

BS-C-1 (African green monkey kidney, epithelial) and CV-1 (African green monkey kidney, fibroblasts), TK^- 143 (human fibroblast) cells were obtained from ATCC and maintained in DMEM supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Phenol red-free DMEM (Gibco) was supplemented with 1 × non-essential amino acids, 2 mM glutamine, 0.024 mg/l biotin, 1.3 mg/l vitamin B12, 10 mg/l of thymidine, 2′-deoxycytidine, uridine and cytidine, 3.5 g/l glucose, 10 mg/l transferrin, 50 μM 2-mercaptoethanol and 30 nM sodium selenite. Vaccinia virus (WR strain) was grown in BS-C-1 monolayer cultures in MEM containing 2% FCS and partially purified through sucrose cushions as described (Mackett *et al.*, 1985). Virus titres were determined by plaque assay on BS-C-1 cells and overlaid with MEM containing 2.5% FCS and 1.5% carboxymethylcellulose.

RNA isolation

Total RNA was isolated from cells using the guanidinium method (Ausubel *et al.*, 1990). TK^- 143 cells were infected with WT vaccinia virus at 10 p.f.u./cell, or mock infected. Late virus RNA was isolated from cells 8 h post-infection, while early RNA was isolated from cells that had been incubated in 100 μg/ml cycloheximide for 1 h before and for 8 h after infection.

S1 nuclease protection

S1 nuclease protection analysis was carried out as described previously (Rodriguez and Smith, 1990). A 5′ labelled probe was prepared as follows: A 642 bp *Bst*YI fragment was excised from pJM1 and 5′ labelled with [γ^{32}-P]ATP by polynucleotide kinase. The fragment was then digested with *Dde*I and a 590 bp fragment isolated which was labelled at the *Bst*YI site 115p downstream of the beginning of the ORF and which extended 475 bp upstream of the ORF. This probe was hybridized with early or late virus mRNA or tRNA, then digested with S1 nuclease and the labelled DNA fragment separated on a polyacrylamide gel alongside an M13 sequencing ladder.

Northern analysis

Northern analysis was carried out as described elsewhere (Sambrook *et al.*, 1989). 5 μg of early, late and mock-infected RNA were run on a 1.3% agarose–formaldehyde gel with Gibco RNA ladder as standards. The RNA was transferred to a nitrocellulose filter as described. A ^{32}P-labelled, strand-specific probe was prepared by unidirectional polymerase chain reaction (PCR). A 891 bp *Bam*I–*Eco*RV fragment that mapped entirely within the SalF7L ORF was isolated from pJM2. 600 ng of this template was hybridized with an oligonucleotide primer (5′-GATTCCTAGGTCCACACA-3′) which is complementary to the coding strand of SalF7L 320 bp from the 5′ end. The primer was extended with *Taq* DNA polymerase in the presence of 200 μCi and [α-^{32}P]dATP, 10 μM cold dATP, 100 μM dCTG, dGTP and dTTP. The mixture was then heat denatured and cycles of elongation and heat denaturation repeated 25 times. The labelled DNA fragment was used to probe filters containing virus mRNAs.

Plasmid constructions

pJM1. A 1735 bp *Hin*cII–*Cla*I fragment containing the SalF7L ORF was isolated from a pUC13 plasmid contianing the 13.4 kb *Sal*I F fragment of vaccinia virus (strain WR). This fragment was then end-filled with Klenow and ligated into *Sma*I-cut pUC119 so that the *Kpn*I site of the vector was downstream of SalF7L ORF. This plasmid was called pJM1.

pJM2. A version of the SalF7L ORF with a *Bam*HI site just upstream of the ATG codon was constructed by PCR using oligonucleotides 5′-CCCGGATCCGAA**TG**GCCGTGTAC-3′ and 5′-GATTCCTAGGTCCACACA-3′ on pJM1 template. The underlined sequences indicate the *Bam*HI and *Sty*I sites and the bold characters represent the first codon of the SalF7L ORF. The PCR fragment was digested with *Bam*HI and *Sty*I and ligated into pJM1 that had been digested with these enzymes, to form pJM2. The sequence of the cloned PCR product was confirmed by DNA sequencing.

pJM3. The SalF7L ORF was excised from pJM2 as a 1187 bp *Bam*HI–*Kpn*I fragment and ligated into *Bam*HI and *Kpn*I digested pPR35 (Rodriguez and Smith, 1990), to give pJM3. This plasmid has the SalF7L ORF under the control of an IPTG-inducible vaccinia virus 4b promoter.

pJM9. An *Eco*RI–*Bam*HI fragment of pGpt07/14 containing the *E.coli* guanine phosphoribosyl transferase (*Ecogpt*) gene linked to the vaccinia virus 7.5 promoter (Boyle and Coupar, 1988) was cloned into *Eco*RI- and *Bam*HI-digested pUC119 to given pJM7. Next a 513 bp *Acc*I–*Eco*RV fragment containing 120 bp of the 3′ end of the SalF7L ORF and 393 bp downstream flanking sequence was isolated from the vaccinia virus *Sal*IF fragment, end-filled with Klenow and ligated into pJM7 that had been digested with *Bam*HI and end-filled with Klenow, giving pJM8. Lastly a 526 bp *Sna*BI–*Ban*I fragment containing 29 bp of the 5′ end of the SalF7L ORF and 497 bp upstream flanking sequences was isolated, end-filled with Klenow and ligated into pJM8 that had been digested with *Eco*RI and end-filled with Klenow, to give pJM9. This plasmid contained the 5′ and 3′ flanking sequences of the SalF7L ORF in the same orientation separated by the *Ecogpt* gene linked to the vaccinia virus 7.5K promoter.

pJM10. For expression of the SalF7L ORF in an IPTG-inducible bacterial vector, the ORF was excised from pJM2 with *Bam*HI and *Kpn*I and ligated

into pGMT7, a derivative of pET-3c (Rosenberg *et al.*, 1987) that had been digested with these enzymes, to give pJM10. This resulted in a fusion protein with M, G, S and A residues joined to the N-terminus of the complete SalF7L ORF.

Construction of recombinant viruses

A TK^- recombinant virus that overexpressed the SalF7L ORF in an IPTG-inducible manner was constructed by transfection of pJM3 into CV-1 cells infected with WT virus (strain WR) as described previously (Mackett *et al.*, 1985). TK^- plaques were screened for the presence of the *E.coli lacI* gene by DNA hybridization. The virus was called vJM1. To form vJM3, 86% of the endogenous SalF7L gene was removed from vJM1 by transfection of plasmid pJM9 into vJM1 infected cells and selection of recombinant viruses in the presence of 25 μg/ml mycophenolic acid (MPA), 250 μg/ml xanthine and 15 μg/ml hypoxanthine as previously described (Boyle and Coupar, 1988). This virus contained a single IPTG-inducible version of the SalF7L ORF within the TK gene locus. vJM2 was derived from WT virus by transfection with pJM9 and isolation of an MPA-resistant virus as above. This virus does not contain an active SalF7L ORF. Recombinant viruses were screened for the absence of plasmid sequences by DNA hybridization.

Expression of SalF7L in E.coli and preparation of antiserum

pJM10 was transformed into *E.coli* BL21(DE3)/pLysE (Studier *et al.*, 1990) and expresison of the SalF7L ORF was induced by addition of 5 mM IPTG. A prominent 38 kDa protein was induced in cells transformed with pJM10 but not the parental vector pGMT7 (data not shown). The 38 kDa protein was easily purified as insoluble inclusion bodies (Nagai and Thogerson, 1987), which were >90% composed of this protein when analysed by SDS–PAGE (data not shown). Rabbits were immunized with 500 μg of inclusion body preparations in Freund's complete adjuvant subcutaneously, and then boosted four times at roughly 2 weekly intervals and then bled.

Immunoprecipitation

Immunoprecipitations of extracts from [^{35}S]methionine-labelled cells were carried out as previously described (Edbauer *et al.*, 1990).

3β-HSD assay

3β-HSD activity in cell monolayers was measured by the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone as described (Bauer and Bauer, 1989). CV-1 cell monolayers of CV-1 cells in 24-well plates were infected in triplicate at 10 p.f.u./cell with WT virus or recombinants vJM1, vJM2 and vJM3 in the presence or absence of 5 mM IPTG. Ten hours post-infection the medium was removed and one set of samples was fixed with ethanol for 15 min at room temperature. All of the wells were then washed with 1 ml warm MEM, and the following reaction mix was added: 280 μl buffer A (10 mM HEPES, 17 mM glucose, 2 mM sucrose and 400 μg/ml BSA in PBS), 120 pmol [^{3}H]pregnenolone (Amersham, TRK 157) and 10 μl 20 mM β-nicotinamide adenine dinucleotide (NAD). The plates were rocked at 37°C for 60 min, at which time 300 μl 2 mg/ml cold carrier pregnenolone (in 96% ethanol) was added and the plates were shaken gently. Then 600 μl 1% digitonin (in 50% ethanol) was added, followed by a 60 min incubation at 37°C. The medium was removed, microfuged for 15 min, and the radioactivity in 1 ml of supernatant counted in Unisolve-E liquid scintillant (Koch-Light Ltd, Suffolk). To account for non-enzymatic conversion of [^{3}H]pregnenolone to [^{3}H]progesterone, the values obtained from the ethanol-fixed well were taken as background and subtracted from the corresponding non-fixed samples.

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