Volume 17 Number 19 1989 Nucleic Acids Research

Vaccinia virus encodes a thymidylate kinase gene: sequence and transcriptional mapping

Geoffrey L.Smith*+, Alejandro de Carlos§ and Y.Sang Chan

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

Received August 8, 1989; Revised and Accepted August 31, 1989 EMBL accession no. X16259

ABSTRACT

The nucleotide sequence and deduced amino acid sequence of a vaccinia virus gene from the SalI F fragment are shown. The predicted polypeptide shares 42% amino acid identity over a 200 amino acid region with *Saccharomyces cerevisiae* thymidylate kinase (TmpK) and has low homology with herpes simplex virus deoxypyrimidine kinase. Northern blotting and S1 nuclease protection showed that the TmpK gene is transcribed early during infection and mapped the mRNA 5′ end to immediately upstream of the second inframe ATG codon of the open reading frame (ORF). The encoded polypeptide is predicted to be 204 amino acids long (23.2 kD) and is almost colinear with yeast TmpK. Vaccinia virus possesses genes for TK and TmpK, separated by 57 kilobases of DNA, which are co-ordinately expressed and the encoded enzymes perform sequential steps in the same biochemical pathway.

INTRODUCTION

Vaccinia virus is a large DNA virus which replicates in the cytoplasm of infected cells and is the most intensively studied poxvirus (1). Poxviruses encode many enzymes involved in nucleic acid metabolism, a feature shared with some other large DNA viruses, such as herpes simplex virus (HSV) and bacteriophage T4. The requirement for these enzymes is most acute in poxviruses because they have evolved to replicate cytoplasmically and consequently cannot directly utilize the cell's nuclear enzymes for RNA and DNA synthesis. For virus mRNA synthesis, vaccinia packages a multisubunit DNA-dependent RNA polymerase (2,3), capping and methylating enzymes (4) and a poly A polymerase (5). For DNA synthesis, the virus packages or encodes DNA polymerase (6−8), DNA ligase (9), topoisomerase (10,11) and a nicking-joining enzyme (12, 13). In addition there are enzymes expressed within infected cells which synthesize precursors of DNA; these include a heterodimeric ribonucleotide reductase (14−16) and thymidine kinase (17−19).

A molecular characterization of a region of the 185 kb vaccinia virus DNA genome adjacent to the right hand inverted terminal repeat has been undertaken. Analysis of the nucleotide sequence of the 13.4 kb SalI F fragment revealed more than twenty open reading frames one of which SalF 15R encodes DNA ligase (9). In this report the structure, deduced amino acid sequence and transcriptional mapping of SalF 13R are reported. This gene encodes a predicted protein with high homology to yeast thymidylate kinase, an enzyme not previously known to be expressed by vaccinia virus or other poxviruses. Herpes viruses also encode a TmpK enzyme but, unlike vaccinia virus, the same polypeptide contains both TK and TmpK activity (hereafter referred to as HSV TK/TmpK, although the enzyme also possesses deoxycytidine kinase activity). In poxviruses and herpes viruses TK is a determinant of virus pathogenicity. Since TK and TmpK perform consecutive steps in the

same pathway of purine biosynthesis it is likely that TmpK may also affect vaccinia virus virulence.

MATERIALS AND METHODS

Nucleotide sequencing

The 13.4 kb SalI F fragment of a rifampicin resistant mutant of vaccinia virus (strain WR) was cloned from cosmid 6 (20) into SalI cut pUC13. The vaccinia DNA was excised, circularised and sonicated to create random fragments >300 bp in length. These were cloned into SmaI cut M13mp18 and sequenced using the dideoxynucleoside triphosphate chain termination method (21) with ^{35}S-dATP and buffer gradient polyacrylamide gels (22).

Computer analysis

Nucleotide sequence data were read directly from autoradiographs using a sonic digitiser and assembled into continuous sequence using programmes DBUTIL and DBAUTO (23, 24). The nucleotide sequence was screened for open reading frames using programme ORFFILE (M.E.G. Boursnell, Institute of Animal Health, Houghton) and individual files created using programme DELIB (M.E.G.Boursnell). Comparison of deduced open reading frames against protein databases was made using programme FASTP (25).

Virus and cells

Vaccinia virus strain WR was grown in human TK$^-$143 cells and virus plaque assays performed on monolayers of CV-1 cells. Aliquoted virus stocks were stored at −70°C. Cells were passed in Glasgow Modified Eagles Medium (GMEM) with 10% foetal bovine serum (FBS).

Virus mRNA preparation

Monolayers of TK$^-$143 cells were infected with vaccinia virus at 30 plaque forming units (pfu) per cell in the presence or absence of cycloheximide (100μg/ml). After 2h adsorption, the virus inoculum was removed and replaced with GMEM/2.5% FBS with, or without, cycloheximide. Early and late mRNA was isolated 6h post infection from cells incubated with cycloheximide or 10h post infection without drug, respectively. Total cell RNA was obtained by lysing cells in guanidinium isothiocyanate/sarkosyl and pelleting through CsCl by overnight centrifugation (26).

Northern Blotting

Virus mRNA samples were resolved by electrophoresis through denaturing agarose gels, transferred to nitrocellulose membranes and probed with ^{32}P-labelled single stranded DNA (27).

S1 nuclease protection

A 635 bp BamHI to BclI fragment, 5′-labelled at the BclI site, was purified and hybridized to early or late virus mRNA or tRNA as described (27). S1 nuclease digestion of hybridized nucleic acid was performed at either 37°C or 30°C for 1h. Protected DNA fragments were electrophoresed on a 6% polyacrylamide, tris-borate-EDTA (TBE) buffer gradient, sequencing gel using a sequencing ladder from an unrelated M13 clone as size markers.

RESULTS

The position of the 13.4 kb SalI F restriction fragment within the vaccinia virus genome is indicated in Figure 1. The complete nucleotide sequence of this DNA fragment was determined to an average character density of 6.1 and will be presented elsewhere. The sequence of an 800 nucleotide fragment, which lies between nucleotides 6313 and 7113

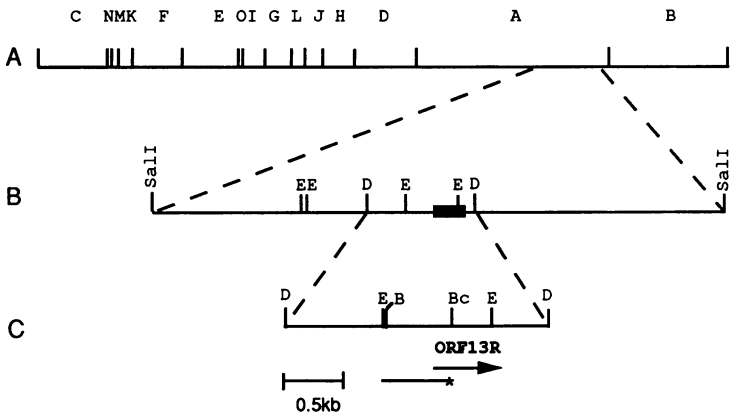


Figure 1. Position of ORF SalI 13R within the 185 kb vaccinia virus genome. A. HindIII restriction map. B. Expanded 13.4 kb SalI F fragment with position of ORF 13R shown as filled box. C. Expanded 2.4 kb DraI fragment with position and direction of transcription of ORF 13R shown. The scale refers to this fragment. Line with asterisk indicates the [^{32}P]-labelled probe used for S1 analysis. Letters in parts B and C indicate restriction enzyme sites: EcoRI, E, D, DraI, B, BamHI and Bc, BclI.

from the left end of SalI F, is shown in Figure 2 together with the deduced sequence of a 227 amino acid ORF. The nucleotide sequence from which the ORF was derived was completely determined from both DNA strands. The ORF is designated SalF 13R to indicate that it is the thirteenth ORF initiating within the SalF fragment and is transcribed rightwards. Approximately 40 nucleotides upstream of the ATG codon at the beginning of the ORF and 20 nucleotides downstream of the termination codon there are sequences TTTTTGT and TTTTTAT, respectively, which represent termination signals for early transcription (28). The next downstream T_5NT motif is located a further 540 nucleotides

```
                                                                M  T  Y  I  F  I  F  S  F  I   10
CTTAGATGGTTTAATATTTTTGTTACCCATGATCTATAAAGGTAGACCTAATCGTCTCGGATGACCTATATATTTATTTTCAGTTTTATT  90

 I  R  I  N  C  K  K  Y  V  R  F  T  K  M  S  R  G  A  L  I  V  F  E  G  L  D  K  S  G  K   40
ATACGCATAAATTGTAAAAAATATGTTAGGTTTACAAAAATGTCTCGTGGGGCATTAATCGTTTTTGAAGGATTGGACAAATCTGGAAAA 180
                                *****
 T  T  Q  C  M  N  I  M  E  S  I  P  A  N  T  I  K  Y  L  N  F  P  Q  R  S  T  V  T  G  K   70
ACAACACAATGTATGAACATCATGGAATCTATACCGGCAAACACGATAAAATATCTTAACTTTCCTCAGAGATCCACTGTCACTGGAAAG 270

 M  I  D  D  Y  L  T  R  K  K  T  Y  N  D  H  I  V  N  L  L  F  C  A  N  R  W  E  F  A  S  100
ATGATAGATGACTATCTAACTCGTAAAAAAACCTATAATGATCATATAGTTAATCTATTATTTTGTGCAAATAGATGGGAGTTTGCATCT 360

 F  I  Q  E  Q  L  E  Q  G  I  T  L  I  V  D  R  Y  A  F  S  G  V  A  Y  A  A  A  K  G  A  130
TTTATACAAGAACAACTAGAACAGGGAATTACTTTAATAGTTGATAGATACGCGTTCTCTGGAGTAGCGTATGCCGCCGCTAAAGGCGCG 450

 S  M  T  L  S  K  S  Y  E  S  G  L  P  K  P  D  L  V  I  F  L  E  S  G  S  K  E  I  N  R  160
TCAATGACTCTCAGTAAGAGTTATGAATCTGGATTGCCTAAACCCGACTTAGTTATATTCTTGGAATCTGGTAGCAAAGAAATTAATAGA 540

 N  V  G  E  E  I  Y  E  D  V  T  F  Q  Q  K  V  L  Q  E  Y  K  K  M  I  E  E  G  D  I  H  190
AACGTCGGCGAGGAAATTTATGAAGATGTTACATTCCAACAAAAGGTATTACAAGAATATAAAAAAATGATTGAAGAAGGAGATATTCAT 630

 W  Q  I  I  S  S  E  F  E  E  D  V  K  K  E  L  I  K  N  I  V  I  E  A  I  H  T  V  T  G  220
TGGCAAATTATTTCTTCTGAATTCGAGGAAGATGTAAAGAAGGAGTTGATTAAGAATATAGTTATAGAGGCTATACACACGGTTACTGGA 720

 P  V  G  Q  L  W  M                                                                      227
CCAGTGGGGCAACTGTGGATGTAATAGTGAAATTACATTTTTTATAAATAGATGTTAGTACAGTGTTATAAATGGATGAAG          800
```

Figure 2. Nucleotide sequence of a 800 bp region of the vaccinia virus SalI F fragment. Deduced amino acid sequence of a 227 amino acid open reading frame. Numbers on the upper and lower lines refer to amino acids from the beginning of the ORF or to the nucleotides from start of DNA fragment, respectively. Underlined nucleotides represent potential early transcriptional termination sequences and asterisks represent the 5′ ends of early mRNA determined by S1 nuclease protection (Figure 4).

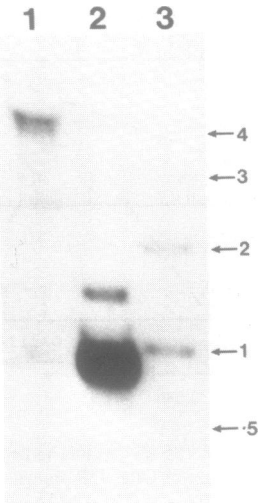


Figure 3. Northern Blot. Early (lane 2) or late (lane 1) virus mRNA, prepared as described in Materials and Methods, was electrophoresed through a denaturing agarose gel, transferred to nitrocellulose and probed with a single-stranded [^{32}P]-labelled DNA fragment. The probe was complementary to the sequence shown in Figure 2 and was derived from an M13 clone containing vaccinia virus DNA from entirely within the coding region of ORF SalF 13R. Lane 3 shows the positions of double stranded DNA molecular weight markers in kilobases. An autoradiograph is shown.

away within the promoter region of the DNA ligase gene (9) and contains two overlapping termination signals within the sequence TTTTTTTAT. The location of these early transcriptional termination signals and the absence of the sequence TAAAT(G) (a late transcription initiation site (29,30)) at the at the 5′ ends of SalF 13R suggests that the gene may be transcribed early during infection.

Transcriptional analyses

Messenger RNA transcribed from SalF 13R was analysed by Northern blotting and S1 nuclease protection. For the Northern analysis (Figure 3), early and the late virus mRNAs were electrophoresed through an agarose gel, transferred to nitrocellulose and probed with a ^{32}P-labelled, single stranded DNA fragment. The probe was prepared from an M13 clone containing vaccinia DNA entirely from within the SalF 13R coding region and is complementary to the sequence shown in Figure 2. Early during infection (lane 2) there is a major mRNA of approximately 850 nucleotides and a minor mRNA of approximately 1500 nucleotides. These mRNAs have a size consistent with transcription initiating near the 5′ end of SalF 13R and terminating 50 nucleotides downstream of the first and second T_5NT motifs, respectively (allowing for addition of 3′ poly A of up to 200 nucleotides and for the slightly different mobility of double stranded DNA molecular weight markers). For late mRNA (lane 1) there is a very small amount of a 850 nucleotide mRNA, probably representing residual early transcripts, and a small amount of RNA migrating at the 28S rRNA band front. At most there is only a very small amount of late RNA being transcribed from the SalF 13R region.

The 5′ end of mRNA from SalF 13R was accurately mapped by S1 nuclease protection

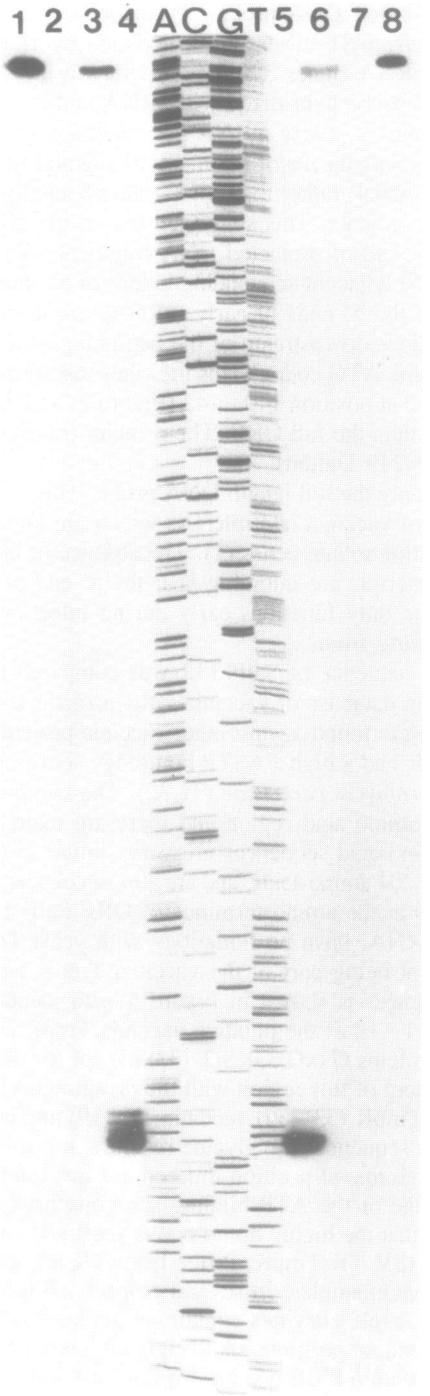
of a 616 base pair BamHI-BclI DNA probe ^{32}P-labelled at the BclI site (see Figure 1). DNA fragments protected from S1 nuclease digestion after hybridization with early mRNA (lanes 4 and 5) or late mRNA (lanes 3 and 6) are shown in Figure 4. The undigested probe (lanes 1 and 8) and probe hybridized with tRNA and then digested with S1 (lanes 2 and 7) are shown as controls. Early mRNA protected a cluster of DNA fragments of 181 to 187 nucleotides long with the major fragments of 182 and 183 nucleotides S1 nuclease digestion at 30°C (lanes 1 and 4) rather than 37°C (lanes 5 and 8) increased the proportion of the longer protected fragments. This suggested that at the higher temperature the S1 nuclease is nibbling the 3′ end of protected DNA fragments. Lengths of the DNA were determined by counting the adjacent sequencing ladder of an unrelated M13 clone (lanes ACGT). The positions of the 5′ ends of early mRNAs are marked in Figure 2 and are approximately 65 nucleotides downstream of the beginning of the ORF but immediately upstream of the next inframe ATG codon. This indicates that translation is likely to initiate from the methionine codon at position 140−142 (Figure 2) and that the expressed protein is 23 amino acids shorter than the full ORF. The primary translation product would have a molecular weight of 23,219 Daltons.

Late mRNA protected only the full length DNA probe. This is consistent with the large and heterogenous length of vaccinia late mRNAs which are known to run through early transcription units in addition to late genes (1). The absence of other protected fragments indicates that no late transcripts are initiating near the 5′ end of the gene SalF 13R and the promoter for this gene only functions early during infection.

Analysis of the open reading frame

The deduced amino acid sequence of SalF 13R was compared against protein database SWISSPROT and our own database of vaccinia virus proteins using programme FASTP (25). No strong matches were found against other vaccinia proteins but the deduced amino acid sequence of SalF 13R had a high FASTP homology score (371) against thymidylate kinase (TmpK) of *Saccharomyces cerevisiae* (31,32). The two proteins share 42% amino acid identity over a 200 amino acid region and there are many additional conservative changes. The aligned amino acid sequences are very similar in length, yeast 216 amino acids versus vaccinia virus 204 amino acids, and are almost colinear. The computer predicted extra amino acid residues at the amino terminus of ORF SalF 13R which are upstream of the 5′ end of early mRNA, have no homology with yeast TmpK. This is consistent with these amino acids not being part of the vaccinia TmpK enzyme. An alignment of the two amino acid sequences is shown in Figure 5 with identical amino acids boxed.

Amino acids residues 11−18 of the putative vaccinia TmpK enzyme fit the consensus motif for ATP binding proteins GxxGxGKS/T (33) except for the second glycine, where there is lysine. An alignment of this region with the presumed ATP binding sites of yeast TmpK (31,32) HSV TK/TmpK (33−39) vaccinia TK (19) and human TK (40) is shown in Figure 6A. In all these sequences the glycine residues at positions five and ten, lysine at position eleven and threonine at position thirteen are invariant. Only HSV TK/TmpK contains the second glycine of the ATP binding site consensus (above). The alignment of this region also shows that the highly homologous yeast and vaccinia TmpK sequences and the more divergent HSV TK/TmpK, differ from TK sequences, of which vaccinia and man are representative examples, in several respects. First, immediately preceding the first glycine all with TmpK enzymes contain an acidic residue while TKs contain a hydrophobic residue. Second, at positions six to eight all poxvirus (19,41−43) and cellular (40,44,45) TK enzymes contain PMF residues while yeast and vaccinia TmpK sequences


1 2 3 4 A C G T 5 6 7 8

contain LDK/R. Here the HSV enzyme fits neither pattern and this may reflect its broader substrate specificity. Third, at position fourteen poxvirus and cellular TKs contain glutamic acid while vaccinia and yeast TmpK contain glutamine and HSV has threonine.

Outside the ATP binding site there is no detectable homology between the vaccinia TmpK and TK sequences. However, homology exist between vaccinia TmpK and HSV TmpK/TK at a second nucleotide/nucleoside binding region. The alignment of the sequences from yeast TmpK, vaccinia TmpK and HSV TK/TmpK in this region is shown in figure 6B. Although the yeast and vaccinia enzymes are clearly more homologous, a TLI triplet is conserved between vaccinia and HSV (positions three to five). These data support the proposal that the HSV TK/TmpK originated from a cellular TmpK and may subsequently have evolved to have TK activity (46). In contrast, the vaccinia TmpK, which is more closely related to TmpK of yeast, has had less evolutionary pressure to diversify and acquire TK activity since the virus contains a separate TK enzyme.

DISCUSSION

DNA sequence analysis of the 13.4 kb SalI F fragment of vaccinia virus has identified an ORF (SalF 13R) with 42% amino acid identity to *Saccharomyces cerevisiae* TmpK (31,32). There is also limited homology to HSV TK/TmpK in regions of the herpes enzyme that are involved in nucleotide and nucleoside binding (37,46). If the product of SalF 13R possesses TmpK activity, as seems likely but is unproven, then this enzyme may be added to the growing list of enzymes encoded by vaccinia virus. Genes encoding enzymes which are involved with DNA metabolism and which have been sequenced are DNA polymerase (8), DNA topoisomerase (29) DNA ligase (9), DNA-dependent ATPase (47), two subunits of ribonucleotide reductase (14–16) and thymidine kinase (TK) (19).

The vaccinia TK enzyme is located 57 kilobases leftward in the central, conserved region of the virus genome but there was no indication that the virus also had a gene for TmpK. The presence in vaccinia virus of two distinct genes performing sequential steps in the same biochemical pathway may be contrasted with the situation of HSV (and other herpes viruses (46)) where the same polypeptide performs both enzymatic reactions. Both vaccinia enzymes are closely related to the cellular homologues (42% amino acid identity between vaccinia virus and yeast TmpK, and 68% identity between vaccinia and human TK), while the homology between HSV TK/TmpK and either cellular enzyme is low. The degree of homology of either virus protein with mammalian TmpKs will be interesting but awaits the cloning and sequencing of the latter. It seems that vaccinia virus has acquired copies of both cellular enzymes, possibly derived from cDNA since the virus-encoded genes have contiguous protein coding sequences while some cellular genes have introns (for instance, chicken TK has six introns (45)). The origin of the herpes virus TK/TmpK is probably a cellular TmpK gene as previously proposed (46). This conclusion is reinforced by the sequence of a second TmpK protein which is highly related to the yeast TmpK sequence but which also has limited homology to the HSV enzyme. The sequences within the presumed ATP binding site of the yeast and vaccinia TmpK differ somewhat from the HSV enzyme. These differences may have arisen as HSV TmpK evolved to acquire TK

Figure 4. S1 nuclease mapping of the 5′ end of mRNA to ORF SalF 13R. Early (lanes 4 and 5) or late (lanes 3 and 6) virus mRNA or tRNA (lanes 2 and 7) was hybridized with a 365 bp BamHI to BclI DNA fragment [^{32}P]-labelled at the BclI site (see Figure 1). Hybridized nucleic acid was digested with S1 nuclease at 30°C (lanes 2–4) or 37°C (lanes 5–7) and run on a 6% polyacrylamide gel with a sequencing ladder from an unrelated M13 clone (lanes ACGT) as size markers. Undigested probe is includes in lane 1 and 8. An autoradiograph is shown.

Figure 5. Aligned amino acid sequences of vaccinia virus ORF SAlF 13R (VV) and *Saccharomyces cerevisiae* (SC) TmpK (31) Identical amino acid residues are boxed. Numbers above or below the aligned sequences refer to amino acid positions of VV or SC respectively.

activity and have resulted in the HSV enzyme having broader substrate specificity which makes it an excellent target for antiviral chemotherapy. Vaccinia virus, which has separate genes for these two enzymes, have proteins much more closely related to the cellular enzymes. This may be because either mutations which broadened the biochemical specificity conferred no advantage since the other enzyme was present, or the genes were acquired more recently.

Transcriptional mapping of the TmpK gene demonstrates that it is transcribed early and not late during infection. The 5′ ends of early mRNA map immediately upstream of the second inframe ATG codon of the computer predicted ORF so that the primary translation product is a polypeptide of 204 amino acids with a molecular weight of 23,219 Daltons. Vaccinia virus TK and TmpK genes are, therefore, co-ordinately expressed. This is predictable as (a) they perform sequential steps in the biosynthesis of dTDP from thymidine and (b) the products of there enzyme activities are required prior to, or during, DNA synthesis. TK is a nonessential gene for *in vitro* replication and has been widely used as a site for insertion of foreign DNA into recombinant vaccinia viruses (48). It is also a

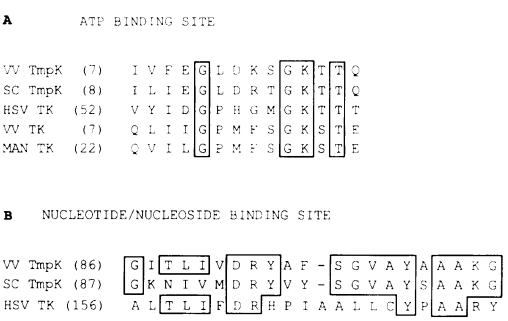


Figure 6. A. Aligned amino acid sequences for the presumed ATP binding site of vaccinia (VV) and *Saccharomyces cerevisiae* (SC) TmpK, HSV TK/TmpK and human and VV TK. Residues identical in all five sequences are boxed. Numbers indicate the amino acids between the amino terminus and the region shown. B. Amino acid sequences for region of HSV TK/TmpK involved in nucleoside/nucleotide binding, aligned with corresponding regions of vaccinia virus (VV) or *Saccharomyces cerevisiae* (SC) TmpK proteins. Amino acids conserved between two, or all, of the sequences are boxed. Numbers are as described in 6A.

determinant of virus pathogenicity for both vaccinia (49) and HSV (50,51). Likewise the vaccinia TmpK is a non-essential gene (de Carlos and Smith, unpublished data) that may be a useful site for insertion of foreign DNA and may be a gene which affects virus virulence *in vivo*. The relative degrees of attenuation of $TK^-/TmpK^+$ $TK^+/TmpK^-$ and $TK^-/TmpK^-$ viruses remains to be determined. However, it may be speculated that $TmpK^-$ viruses would have their growth more restricted in quiescent cells than TK^- viruses because TmpK utilises biosynthetic precursors derived from both the *de novo* synthetic and salvage pathways of purine metabolism, while TK utilises only the latter.

ACKNOWLEDGMENTS

We thank Helen Newman and Henrietta Mountain for typing and Shona Kerr for critical reading of the manuscript. This work was supported by MRC project grant G8425735CA, AdC was a holder of a student short term fellowship from the Ministerio de Educacion y Ciencia, Spain, and GLS is a Lister Institute–Jenner Research Fellow.

*To whom correspondence should be addressed

Present addresses: [+]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK and [§]Centro de Investigaciones Biologicas, Velazquez 144, 28006 Madrid, Spain

REFERENCES

1. Moss, B. (1985) In Fields, B.N. (Ed.), Virology. Raven Press, New York, pp 658–703.
2. Munyon, W.E., Paoletti, E. and Grace, J.T. (1967) Proc. Natl. Acad. Sci. USA., **58**, 2280–2288.
3. Kates, J.R. and McAuslan, B.R. (1967) Poxvirus DNA–dependent RNA polymerase. Proc. Natl. Acad. Sci. USA., **58**, 134–141.
4. Martin, S.A., Paoletti, E. and Moss, B. (1975) J. Biol. Chem., **250**, 9322–9329.
5. Moss, B., Rosenblum, E.N. and Gershowitz, A. (1975) J. Biol. Chem., **250**, 4722–4729.
6. Challberg, M.D. and Englund, P.T. (1979) J. Biol. Chem., **254**, 7812–7819.
7. Traktman, P., Sridhar, P., Condit, R.C. and Roberts, B.E. (1984). J. Virol., **49**, 125–131.
8. Earl, P.L., Jones, E.V. and Moss, B. (1986) Proc. Natl. Acad. Sci. USA., **83**, 3659–3663.
9. Smith, G.L., Chan, Y.S. and Kerr, S.M. submitted for publication.
10. Schuman, S. and Moss, B. (1987) Proc. Natl. Acad. Sci. USA., **84**, 7478–7482.
11. Shaffer, R. and Traktman, P. (1987) J. Biol. Chem., **262**, 9309–9315.
12. Lakritz, N., Fogelson, P.D., Reddy, M., Baum, S., Hurwitz, J. and Bauer, W.R. (1985) **53**, 935–943.
13. Reddy, M.K. and Bauer, W.R. (1989) J. Biol. Chem., **264**, 443–449.
14. Schmitt, J.F.C. and Stunnenberg, H.G. (1988) J. Virol., **62**, 1889–1897.
15. Slabaugh, M., Roseman, N., Davis, R. and Matthews, C. (1988) J. Virol., **62**, 519–527.
16. Tengelsen, L.A. Slabaugh, M.B., Bibler, J.K. and Hruby, D.E. (1988) Virology, **164**, 121–131.
17. Hruby, D.E. and Ball, L.A. (1982) J. Virol., **43**, 403–409.
18. Weir, J.P. Bajszar, G. and Moss, B. (1982) Proc. Natl. Acad. Sci. USA., **79**, 1210–1214.
19. Weir, J.P. and Moss, B. (1983) J. Virol. **46**, 530–537.
20. Baldick, C.J. and Moss, B. (1987) Virology, **156**, 138–145.
21. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA., **74**, 5463–5467.
22. Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983) Proc. Natl. Acad. Sci. USA., **80**, 3693–3695.
23. Staden, R. (1980) Nucleic Acids Res., **8**, 3673–3694.
24. Staden, R. (1982) Nucleic Acids Res., **10**, 4731–4751.
25. Lipman, D.J. and Pearson, W.R. (1985) Science, **227**, 1435–1441.
26. Ausubel, I. and Frederick, M. (1987) Current Protocols in Molecular Biology. Green Publishing Associates and Wiley-Interscience, New York.
27. Smith, G.L., Howard, S.H. and Chan, Y.S. (1989) J. Gen. Virol., **70**, In press.
28. Yuen, L. and Moss, B. (1987) Proc. Natl. Acad. Sci. USA., **84**, 6417–6421.
29. Rosel, J.L., Earl, P.L. and Moss, B. (1986) J. Virol., **60**, 436–449.
30. Hanggi, M., Bannwarth, W. and Stunnenberg, H.G. (1986) EMBO J., **5**, 1071–1076.
31. Jong, A.Y.S., Kuo, C.L. and Campbell, J.L. (1984) J. Biol. Chem., **259**, 11052–11059.

32. Rothstein, R., Helms, C. and Rosenberg, N. (1987) Mol. Cell Biol. **7**, 1198–1207.
33. Otsuka, M. and Kitt, S. (1984) Virology, **135**, 316–330.
34. McKnight, S.L. (1980) Nucleic Acids Res., **8**, 5949–5964.
35. Wagner, M.J., Sharp, J.A. and Summers, W.C. (1981) Proc. Natl. Acad. Sci. USA., **78**, 1441–1445.
36. Gompels, U. and Minson, A.C. (1986) Virology., **153**, 230–247.
37. Darby, G., Larder, B.A. and Inglis, M.M. (1986) J. gen. Virol., **67**, 753–758.
38. Kit, S., Kit, M., Qavi, H., Trkula, D. and Otsuka, H. (1983) Biochem. Biophys. Acta, **741**, 158–170.
39. Swain, M.A. and Galloway, D.A. (1983) J. Virol., **46**, 1045–1050.
40. Bradshaw, H.D. and Deininger, P.L. (1984) Mol. Cell Biol., **4**, 2316–2320.
41. Boyle, D.B., Coupar, B.E.H., Gibbs, A.J., Seigman, L.J. and Both, G.W. (1987) Virology, **156**, 355–367.
42. Esposito, J.J. and Knight, J.C. (1984) Virology, **135**, 561–567.
43. Upton, C. and McFadden, G. (1986) J. Virol., **60**, 920–927.
44. Lin, P.F. Lieberman, H.B., Yeh, D.B., Xu, T., Zhao, S.Y. and Ruddle, F.H. (1985) Mol. Cell Biol. **5**, 3149–3156.
45. Kwoh, T.J. and Engler, J.A. (1984) Nucleic Acids Res., **12**, 3959–3971.
46. Robertson, G.R. and Whalley, J.M. (1988) Nucleic Acids Res., **16**, 11303–11317.
47. Rodriguez, J.F., Kahn, J.S. and Esteban, M. (1986) Proc. Natl. Acad. Sci. USA, **83**, 9566–9570.
48. Mackett, M. and Smith, G.L. (1986) J. gen. Virol. **67**, 2067–2082.
49. Buller, R.M.L., Smith, G.L., Cremer, K., Notkins, A.L. and Moss, B. (1985) Nature, **317**, 813–815.
50. Field, H.J. and Wildy, P. (1978) J. Hyg. Camb. **81**, 267–277.
51. Kit, S., Qavi, H., Dubbs, D.R. and Otsuka, H. (1983) J. Med. Virol., **12**, 25–36.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.