

# Vaccinia Virus Encodes an Active Thymidylate Kinase That Complements a *cdc8* Mutant of *Saccharomyces cerevisiae*\*

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A vaccinia virus open reading frame (ORF) previously predicted to encode thymidylate kinase (TmpK) is shown to encode an active enzyme. A copy of the ORF, generated by polymerase chain reaction, was cloned into an *Escherichia coli* inducible expression vector. Cell extracts of *E. coli* expressing the vaccinia gene contained high levels of TmpK activity, whereas extracts of cells without the TmpK gene did not. The vaccinia ORF expressed from a yeast vector complemented a *Saccharomyces cerevisiae cdc8* mutant, demonstrating functional compatibility of the vaccinia virus and yeast TmpK enzymes. The gene is shown to be nonessential for the replication of vaccinia virus in cultured cells by the construction of a viable virus mutant that has the coding region of the TmpK gene interrupted by the *Ecogpt* gene. Synthesis of the vaccinia TmpK protein in infected cells was demonstrated by the use of a polyclonal rabbit antiserum raised against the purified TmpK enzyme expressed in *E. coli* to immunoprecipitate a 23-kDa early polypeptide from cells infected with wild type vaccinia but not from cells infected with the TmpK mutant. Plasmid vectors that allow the construction of recombinant viruses expressing foreign gene(s) from the nonessential TmpK locus are described.

Vaccinia virus is a large DNA virus and is the most intensively studied member of the poxvirus family (see Moss (1990a) for review). These viruses replicate in the cytoplasm of infected cells and cannot, therefore, directly utilize the nuclear enzymes of the host cell for transcription or DNA synthesis. Vaccinia has circumvented this problem by encoding or packaging a complete transcriptional enzyme system (Moss, 1990b) and several enzymes required for DNA synthesis, including a DNA-dependent DNA polymerase (Moss and Cooper, 1982; Jones and Moss, 1984; Traktman *et al.*, 1984; Earl *et al.*, 1986), DNA topoisomerase (Shuman and Moss, 1987), DNA ligase (Smith *et al.*, 1989a; Kerr and Smith, 1989; Colinas *et al.*, 1990), and a nicking-joining enzyme (Lakritz *et al.*, 1985; Reddy and Bauer, 1989). Vaccinia also encodes enzymes involved in the synthesis of deoxynucleoside triphosphate precursors, such as a ribonucleotide reductase (Slabaugh and Mathews, 1984; Schmitt and Stunnenberg, 1988; Slabaugh *et al.*, 1988; Tengelsen *et al.*, 1988) and thymidine kinase (Hruby and Ball, 1982; Weir *et al.*, 1982; Hruby *et al.*,

1983; Weir and Moss, 1983). Furthermore, there are genes predicted to encode dUTPase (McGeoch, 1990) and guanylate kinase (Smith *et al.*, 1991), although in the latter case the coding region is disrupted by a frameshift mutation.

In addition to these genes the vaccinia genome (strain WR) contains an open reading frame (ORF)<sup>1</sup> which is transcribed early in infection to produce an mRNA with the potential to encode a polypeptide of 204 amino acids (23.2 kDa) exhibiting 42% amino acid identity with the thymidylate kinase (TmpK) of *Saccharomyces cerevisiae* (Smith *et al.*, 1989b). A similar ORF is present in the Copenhagen strain of vaccinia virus (Goebel *et al.*, 1990). Thymidylate kinase catalyzes a critical step in the biosynthesis of (deoxy)thymidine triphosphate and is indispensable for cell metabolism. Recently, a cDNA encoding the human TmpK enzyme has been cloned (Su and Sclafani, 1991) and the amino acid sequence of this enzyme found to have a high degree of amino acid identity with both the TmpK enzyme of *S. cerevisiae* and the predicted TmpK protein of vaccinia. In this paper the vaccinia virus TmpK gene is shown to encode an active thymidylate kinase and to complement a temperature-sensitive *cdc8* mutant of *S. cerevisiae*, which is deficient in TmpK activity (Jong *et al.*, 1984; Sclafani and Fangman, 1984). The synthesis of a 23-kDa vaccinia TmpK polypeptide is demonstrated in virus-infected cells, and the TmpK gene is shown to be nonessential for virus replication in cultured cells. Lastly, plasmid vectors are described that allow construction of recombinant vaccinia viruses that express foreign genes from the nonessential TmpK locus by the transient dominant selection method (Falkner and Moss, 1990).

## MATERIALS AND METHODS

### Recombinant DNA Methodology

Restriction endonuclease digestions, DNA ligations, and plasmid DNA preparation were performed using standard procedures (Sambrook *et al.*, 1989). DNA modifying enzymes were obtained from GIBCO/BRL and Boehringer Mannheim. DNA was amplified by the polymerase chain reaction using AmpliTaq DNA polymerase (Perkin-Elmer Cetus) according to the protocol described by the manufacturer. Oligonucleotide primers were synthesized on an Applied Biosystems 380-B DNA synthesizer.

### Bacterial Strains

The *Escherichia coli* strain TG1 (*supE hsdΔ5 thiΔ[lac-proAB] F' [traD36 proAB<sup>r</sup> lacZΔM15]*) was routinely used for DNA manipulation procedures and strain BL21(DE3)/pLysS (Studier *et al.*, 1990) was the host used for expressing the vaccinia TmpK gene cloned in the T7 expression vector pGMT7. Bacteria were grown at 37 °C in 2 × TY medium (Sambrook *et al.*, 1989) supplemented with

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<sup>1</sup> The abbreviations used are: ORF, open reading frame; bp, base pair(s); kb, kilobase pair(s); SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; MPA, mycophenolic acid; TmpK, thymidylate kinase.

50 µg/ml ampicillin for the selection of pUC-derived plasmids or 200 µg/ml ampicillin for the selection of plasmids derived from pGMT7.

#### Plasmid Constructions

*pAC1*—A 2.4-kb fragment of vaccinia DNA (strain Western Reserve, WR) containing the TmpK gene was obtained by digesting the *Sal*I fragment of genomic DNA with *Dra*I, and then cloned into the *Sma*I site of pUC13 (Messing, 1983) to form plasmid pAC1.

*pAC2*—pAC1 was digested with *Mlu*I, treated with Klenow enzyme to create blunt termini, and then ligated with a 2.0-kb *Eco*RI fragment derived from plasmid pGpt07/14 (Boyle and Coupar, 1988) that had been treated with Klenow enzyme. The latter fragment contained the *E. coli* guanine phosphoribosyltransferase (*Ecogpt*) gene linked to the vaccinia virus 7.5K gene promoter. The resultant plasmid containing the *Ecogpt* gene transcribed in the same direction as TmpK was termed pAC2.

*Transient Dominant Insertion Vectors pSJH11 and pSJH14*—The *Ecogpt* gene under the control of the vaccinia 7.5K promoter was isolated on a 2.0-kb *Eco*RI-*Bam*HI fragment from the plasmid pGpt07/14 (Boyle and Coupar, 1988), the DNA rendered blunt-ended by incubation with Klenow enzyme, and then ligated into the *Nde*I site of pUC13. The resulting plasmid, pSJH7, was digested with *Hind*III and *Eco*RI to remove the multiple cloning site polylinker, and the DNA termini end-filled using Klenow enzyme. The vaccinia TmpK gene with flanking virus DNA sequences was isolated as a 1.5-kb *Bam*HI fragment from plasmid pAC1, treated with Klenow enzyme, and blunt-end ligated to the pSJH7 vector DNA to form plasmid pSJH9. A 239-bp internal *Mlu*I-*Eco*RI fragment of the TmpK gene was removed from pSJH9 and replaced with either the vaccinia late 4b gene promoter obtained as a 120-bp *Mlu*I-*Eco*RI fragment from plasmid pRK19 (Kent, 1988), or the vaccinia constitutive 7.5K gene promoter isolated as a 290-bp *Hinc*II-*Eco*RI fragment from plasmid pGS19 (Mackett *et al.*, 1984) and ligated into the TmpK gene after the *Mlu*I terminus had been end-filled. The resultant plasmids containing the 4b or 7.5K promoter were called pSJH11 and pSJH14, respectively. Each vector contains unique *Bam*HI, *Sma*I, and *Eco*RI restriction sites downstream of the promoter for the insertion of foreign genes.

#### Yeast Strains and Media

The *S. cerevisiae* strain L194-8A (*MATa cdc8 ura3 leu2 trp1 ade*) was used throughout. The medium was 0.67% Difco yeast nitrogen base with either 2% glucose or 2% galactose and 2% raffinose as carbon sources. The appropriate nutritional requirements were added at 40 µg/ml. Cell numbers were determined using a Coulter counter (Coulter Electronics, England).

#### Viruses and Cell Culture

Vaccinia virus (strain WR) was grown in the African green monkey kidney cell line CV-1 and human TK<sup>-</sup>143 cells. Recombinant viruses expressing the *Ecogpt* gene were selected by plaque formation in the presence of mycophenolic acid, xanthine, and hypoxanthine (Boyle and Coupar, 1988; Falkner and Moss, 1988) and were purified and titered using standard protocols (Mackett *et al.*, 1985). Aliquotted virus stocks were stored at -70 °C. CV-1 and TK<sup>-</sup>143 cells were passed in minimum essential medium (GIBCO) containing 10% (v/v) fetal bovine serum.

#### Preparation of Crude *E. coli* Cell Extracts

Bacteria were harvested from 20-ml mid-exponential cultures, washed in phosphate-buffered saline, and resuspended in 0.15 ml of ice-cold extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol, 1 mM EDTA, 50 µM dTMP). The cells were disrupted in an ultrasonic disintegrator (MSE 100W) and the cell extracts clarified by centrifugation at 13,000 rpm for 5 min at 4 °C. The protein concentration of the extracts was determined by the Bradford method (Bradford, 1976).

#### Assay of Thymidylate Kinase Activity in *E. coli* Cell Extracts

0.1–1.0 µg of protein was incubated in 40 µl of reaction buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 5 mM ATP, 50 µM dTMP, and 15 µCi/ml [<sup>3</sup>H]dTMP (Amersham International PLC) at 37 °C for 10 min. The kinase reaction was terminated by heating at 75 °C for 10 min. 5-µl samples of the assay reaction mixture were spotted onto a polyethyleneimine-immregnated cellulose thin layer chromatography plate (Schleicher and

Schuell) and the reaction products, dTDP and dTTP, resolved from dTMP by ascending thin layer chromatography using 0.5 M LiCl, 2 M acetic acid as the solvent (Chen *et al.*, 1976). The regions of the plate containing dTDP and dTTP were isolated and the radioactivity determined by liquid scintillation counting in a Beckman LS 5000CE scintillation counter.

#### Southern Blot Analyses

The genomic structure of recombinant viruses was analyzed by Southern hybridization (Sambrook *et al.*, 1989). DNA was isolated from purified virus particles as described (Mackett *et al.*, 1985) and digested with the appropriate restriction endonucleases. Following electrophoresis on a 0.8% agarose gel the DNA was transferred to a nitrocellulose membrane and analyzed by hybridizing with radioactive DNA probes labeled to high specific activity using [ $\alpha$ -<sup>32</sup>P]dATP (Amersham International PLC) and a commercial random primed DNA labeling kit (Boehringer Mannheim).

#### Antisera and Immunoprecipitation

New Zealand White × Sandy Lop rabbits were immunized with 100 µg of TmpK inclusion body protein in complete Freund's adjuvant, and then boosted with the same amount of protein in incomplete Freund's adjuvant after 14 and 28 days. Immune sera were collected at 40 days and tested for reactivity with antigen by immunoblotting (Harlow and Lane, 1988).

In immunoprecipitation experiments, CV-1 cells infected with vaccinia at 50 plaque-forming units/cell were incubated in methionine-free minimum essential medium (GIBCO) 1.5–2 h or 2.5–3 post-infection and then radioactively labeled by incubating for 1 h in the same medium supplemented with 100 mCi/ml L-[<sup>35</sup>S]methionine (Amersham International PLC). The radiolabeled cells were harvested, washed in phosphate-buffered saline, and lysed by incubating for 5 min in ice-cold Nonidet P-40 lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 13,000 rpm for 5 min at 4 °C. The supernatants were pre-cleared by incubating with preimmune rabbit serum for 30 min on ice and the immune complexes precipitated by incubation with protein A-Sepharose beads for 2 h at 4 °C. The beads were removed by centrifugation at 13,000 rpm for 30 s and the supernatants then incubated with rabbit antiserum for 30 min on ice. The immune complexes were precipitated by incubation with protein A-Sepharose for 2 h at 4 °C. The beads were collected by centrifugation, washed twice in Nonidet P-40 lysis buffer and once in 2 M urea, 0.4 M LiCl, 10 mM Tris-HCl, pH 8.0, and then incubated in protein sample buffer (10% glycerol, 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue) for 15 min at room temperature. The beads were pelleted by centrifugation and the proteins in the supernatants resolved by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and analyzed by autoradiography.

#### RESULTS

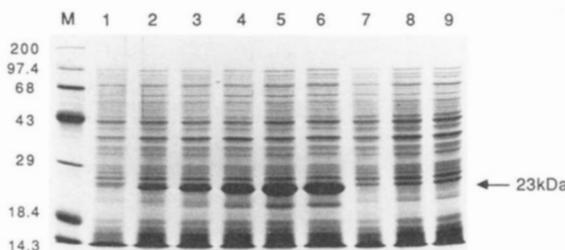
*Expression of the Vaccinia Virus TmpK Gene in E. coli*—The plasmid pGMT7, a derivative of the bacteriophage T7 expression vector pET-3c (Rosenberg *et al.*, 1987) containing a multiple cloning site polylinker immediately downstream of the T7 gene 10 translational start site, was used to express the vaccinia TmpK gene in *E. coli*. *Bam*HI restriction sites were created at the 5' and 3' termini of the predicted coding sequence of the TmpK gene (excluding the region upstream of the early RNA start site (Smith *et al.*, 1989b)) by PCR using the oligonucleotide primers CCGGATCCATGTC-TCGTGGGGC (5' primer) and CCGGATCCTTACATCCA-CAGTTGC (3' primer) and plasmid pAC1 ("Materials and Methods") as the template. The TmpK gene was then ligated into a unique *Bam*HI site within the polylinker of pGMT7 to generate plasmid pSJH3. Messenger RNA transcripts initiated at the gene 10 promoter of pSJH3 would encode a fusion protein consisting of the entire TmpK gene product with the tripeptide Met-Gly-Ser fused to the N terminus. The TmpK gene was expressed by transformation of *E. coli* BL21(DE3)/pLysS (Studier *et al.*, 1990) (a λ lysogen containing gene 1 of bacteriophage T7, encoding RNA polymerase, under the con-

trol of the *lacUV5* promoter) with plasmid pSJH3 and induced by addition of IPTG.

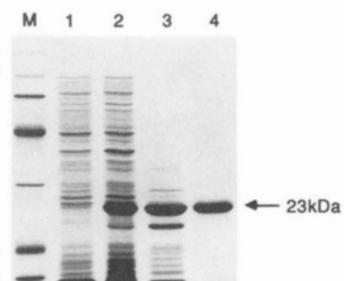
The time course of TmpK protein synthesis following IPTG induction was analyzed by removing aliquots of a culture at intervals after IPTG addition, lysing the cells in protein sample buffer, and analyzing the lysates by SDS-polyacrylamide gel electrophoresis (Fig. 1). The synthesis of a protein of the expected size (23.5 kDa) was induced in *E. coli* containing plasmid pSJH3 (*lanes 2–6*), but not in cells containing the parental vector pGMT7 (*lanes 8 and 9*). The levels of TmpK protein synthesized increased with prolonged incubation, such that at 4 h after induction (*lane 6*) it constituted 30–40% of the total cellular protein as estimated from the Coomassie-stained polyacrylamide gel.

Polypeptides overexpressed at high levels in *E. coli* often form insoluble aggregates or inclusion bodies within the cytoplasm of the bacterial cell (Marston, 1986). Such inclusion bodies may be easily isolated and can provide a good source of antigen for immunizing animals to raise antisera against the protein. Inclusion bodies of vaccinia TmpK protein were found to be produced in IPTG-induced *E. coli* BL21(DE3)/pLysS containing plasmid pSJH3, and were isolated and purified (Fig. 2) as described (Nagai and Thogersen, 1987). These TmpK inclusion bodies were used to immunize rabbits in order to raise a polyclonal antiserum against the vaccinia TmpK protein.

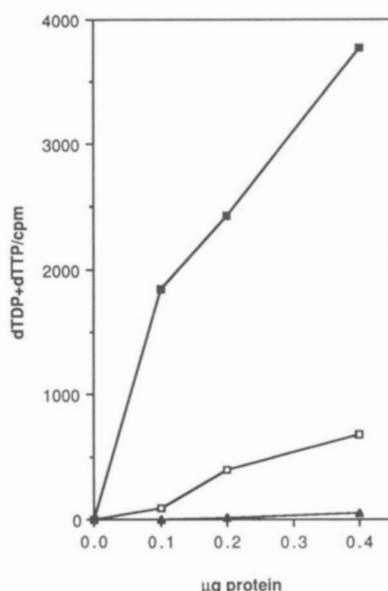
**The Vaccinia TmpK Protein Synthesized in *E. coli* Is Enzymatically Active—**To determine if the vaccinia TmpK protein can phosphorylate dTMP, an *in vitro* enzyme assay was used to measure the levels of thymidylate kinase activity in cell extracts of *E. coli* expressing the vaccinia TmpK gene (Fig. 3). Extracts of IPTG-induced *E. coli* containing the expression vector pGMT7 exhibited a low basal level of thymidylate kinase activity resulting from the action of the endogenous cellular TmpK enzyme (Nelson and Carter, 1969). In contrast, high levels of kinase were detected in IPTG-induced cells containing plasmid pSJH3, indicating that the vaccinia TmpK protein is an active thymidylate kinase. Non-induced *E. coli* containing pSJH3 also showed elevated levels of kinase activity, presumably a result of a low level of



**FIG. 1. Expression of the TmpK gene in *E. coli*.** Cultures of BL21(DE3)/pLysS containing pSJH3 (TmpK) or the parental vector pGMT7 were grown to an  $OD_{600}$  of 0.4, and then the *lac* inducer IPTG was added to a final concentration of 1 mM to induce synthesis of the T7 RNA polymerase and thereby initiate the transcription of the TmpK gene. Aliquots of cultures were removed at time intervals after addition of IPTG and the cells lysed by heating to 100 °C in protein sample buffer. Proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis, and visualized by Coomassie staining. *Lanes 1–6* show the extract of the cells containing pSJH3 made at zero (*lane 1*), 30 (*lane 2*), 60 (*lane 3*), 120 (*lane 4*), 180 (*lane 5*), and 240 (*lane 6*) min after induction, and *lanes 7–9* show the extracts of cells carrying pGMT7 made at zero (*lane 7*), 60 (*lane 8*), and 120 (*lane 9*) min after addition of IPTG. The position of a protein of approximately 23 kDa induced by IPTG in cells containing pSJH3 is indicated by an arrow. Protein molecular mass markers (*lane M*, GIBCO/BRL) were lysozyme (14.3 kDa),  $\beta$ -lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorlyase b (97.4 kDa), and myosin (200 kDa).



**FIG. 2. Purification of the vaccinia TmpK protein synthesized in *E. coli*.** Inclusion bodies of vaccinia TmpK protein were isolated from an IPTG-induced culture of BL21(DE3)/pLysS containing pSJH3 by the method of Nagai and Thogersen (1987). To further purify the TmpK protein from the inclusion body preparation, a sample was resolved on a preparative 12% SDS polyacrylamide gel, the TmpK protein sliced from the gel, and purified by electroelution. A Coomassie-stained SDS polyacrylamide gel depicting the various stages in the purification of the TmpK protein is shown. *Lanes 1 and 2* contain extracts of non-induced and IPTG-induced *E. coli*, respectively. *Lane 3* contains an aliquot of the TmpK inclusion body preparation, and *lane 4* contains the TmpK protein sample following preparative gel electrophoresis and electroelution. Molecular mass markers (*lanes M*) are as described in Fig. 1.



**FIG. 3. Thymidylate kinase activity in *E. coli* expressing the vaccinia TmpK gene.** The levels of thymidylate kinase activity in cell extracts of *E. coli* BL21(DE3)/pLysS containing pSJH3 or the vector pGMT7 were assayed as described under “Materials and Methods.” The amounts of [<sup>3</sup>H]dTDP and [<sup>3</sup>H]dTTP formed (in counts/min) versus the amount of cell extract protein added to the assay reaction mixture are shown for assays on IPTG-induced *E. coli* carrying the vector pGMT7 (closed triangles), non-induced *E. coli* containing pSH3 (open squares), and IPTG-induced *E. coli* containing pSJH3 (closed squares). The plots are derived from a single experiment in which the cell extracts were assayed in parallel and are representative of the observations made in three separate experiments in which fresh cell extracts were used in each case.

expression of the vaccinia TmpK gene due to the basal level of synthesis of T7 RNA polymerase which occurs in the host cells in the absence of a *lac* inducer (Studier *et al.*, 1990).

**The Vaccinia TmpK Gene Complements the cdc8 Mutation in *S. cerevisiae*—**The thymidylate kinase of the budding yeast *S. cerevisiae* is encoded by the *CDC8* gene (Sclafani and Fangman, 1984; Jong *et al.*, 1984). Temperature-sensitive *cdc8* mutants are defective in nuclear (Hartwell, 1971), mitochondrial (Newlon and Fangman, 1975), and 2  $\mu$ M plasmid (Liv-

ington and Kupfer, 1977) DNA replication at the nonpermissive temperature. The herpes simplex virus TK gene, which encodes a multifunctional enzyme with thymidine kinase, thymidylate kinase, and deoxycytidine kinase activities (Cooper, 1973; Chen and Prusoff, 1978), has been shown to complement the *cdc8* defect when introduced into *cdc8* mutants in an expression vector (Sclafani and Fangman, 1984). Recently, a cDNA encoding the human thymidylate kinase has been cloned by functional complementation of a *cdc8* mutant (Su and Sclafani, 1991). To provide further evidence that the vaccinia TmpK gene encodes an active enzyme, we examined whether its expression in a *cdc8* mutant of *S. cerevisiae* could complement the *cdc8* defect at the nonpermissive temperature.

The yeast expression vector pEMBLyex4 (Murray *et al.*, 1987) has a multiple cloning site polylinker located immediately downstream of a hybrid *GAL-CYC1* promoter. Within yeast cells expression of a gene cloned in the multiple cloning site can be induced by growth in the presence of galactose, or repressed by growth in medium containing glucose. The PCR-derived version of the vaccinia TmpK gene with terminal *Bam*H I restriction sites was ligated into a unique *Bam*H I site within the polylinker of pEMBLyex4 to generate plasmid pSJH1. This plasmid, together with a control consisting of pEMBLyex4 alone, was transformed into the temperature-sensitive *cdc8* strain L194-8A and transformants were selected at the permissive temperature of 25 °C and on minimal medium containing glucose. The colonies resulting from each transformation were transferred to the restrictive temperature of 37 °C and onto medium containing either 2% glucose as carbon (C) source or a combination of 2% raffinose and 2% galactose (the latter is a poor C source, and the raffinose allows better growth of the cells without affecting induction by the galactose). The control colonies containing the vector alone showed no growth at all at 37 °C, while those carrying the vaccinia TmpK gene showed clear growth on both the galactose and glucose-containing medium.

The growth at 37 °C in the presence of glucose was unexpected. However, pEMBLyex4 carries the 2 μM plasmid origin of replication (Murray *et al.*, 1987) and therefore has a copy number of approximately 30, and this, together with some residual expression, probably accounts for the observed growth. Microscopic examination of the cells growing at 37 °C in glucose-containing medium suggested that the levels of TmpK being produced were limiting in amount as the cells were enlarged (which is often associated with a nonlethal defect in the cell cycle) and some had even formed the "dumbbell" morphology associated with the *cdc8* defect. In fact, determination of the growth rate of the transformants at 37 °C showed that glucose supported only limited growth (Fig. 4). In contrast, the growth of cells with galactose and raffinose as C sources was much more rapid (Fig. 4), and moreover, the cells were of normal size and morphology, consistent with an abundant supply of TmpK. Thus, the vaccinia gene clearly does complement the *cdc8* mutant, and the vaccinia TmpK protein is able to substitute for the defective yeast enzyme.

**Construction of VAC1 and VACHB Mutant Viruses**—To determine if the TmpK gene was essential for vaccinia virus replication, we attempted to construct recombinant viruses that had the TmpK ORF interrupted by the insertion of exogenous DNA. The DNA chosen comprised the *Ecogpt* gene linked to the vaccinia virus 7.5K promoter (Boyle and Coupar, 1988) and enabled the selection of recombinant viruses in the presence of mycophenolic acid, xanthine, and hypoxanthine (Boyle and Coupar, 1988; Falkner and Moss, 1988). Plasmid

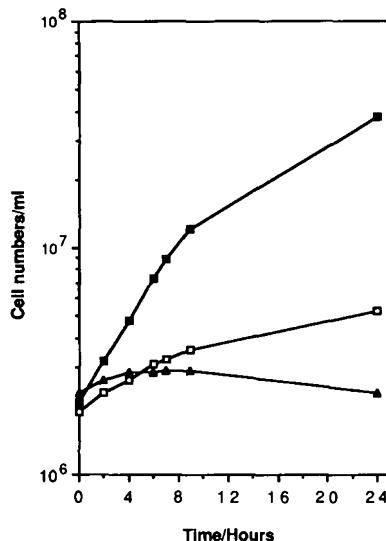
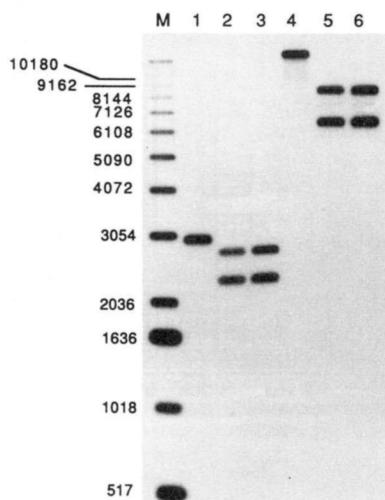


FIG. 4. Growth rates at 37 °C of *S. cerevisiae* L194-8A transformants containing plasmids pSJH1 and pEMBLyex4. Cultures were grown in yeast nitrogen base containing the appropriate C sources at 25 °C, and at time zero they were switched to 37 °C and the cell numbers monitored. Closed triangles show L194-8A containing pEMBLyex4 grown in the presence of 2% galactose and 2% raffinose, open squares show L194-8A containing pSJH1 grown in the presence of 2% glucose, and closed squares show L194-8A containing pSJH1 grown in medium containing 2% galactose and 2% raffinose.

pAC2 ("Materials and Methods") was transfected into CV-1 cells infected with either wild-type virus (strain WR) or recombinant virus vHBs4 (Smith *et al.*, 1983). The latter contains the hepatitis B virus surface antigen inserted into the vaccinia virus thymidine kinase gene and is therefore thymidine kinase-negative. In each case, recombinant viruses were plaque-purified three times in the presence of MPA, xanthine, and hypoxanthine, and the tertiary plaques were amplified in 24-well trays of CV-1 cells in the presence of these drugs. After extensive cytopathic effect was observed, DNA was extracted from these infected cells and subjected to dot blot analysis using radioactive probes representing the *Ecogpt* gene or unrelated plasmid DNA. This screening was designed to identify virus clones that were *Ecogpt*-positive but plasmid-negative. If the TmpK gene was essential for virus replication all isolates grown under MPA selection would contain the *Ecogpt* gene, a functional copy of the TmpK gene, and the entire plasmid integrated into the virus genome via a single recombination event. If, on the other hand, the gene was nonessential it should be possible to identify recombinant viruses produced by double cross-overs which retain the *Ecogpt* gene within an interrupted TmpK locus and which have lost all plasmid sequences. This strategy recently enabled the isolation of recombinant vaccinia viruses lacking the DNA ligase gene (Kerr and Smith, 1991), but that study also demonstrated that virus genomes formed by single cross-overs can resolve and reform plasmids. Since these plasmids are nonspecifically replicated in vaccinia virus infected cells they can persist, recombine with viral DNA, and give rise to virus heterogeneity. It was therefore important to screen for the absence of all plasmid sequences to be sure a stable TmpK deletion mutant had been identified. Using either parent virus recombinant plaques were obtained with the desired phenotype. These viruses, designated VAC1 (wild-type parent) and VACHB (vHBs4 parent), were then amplified and purified and their genomes subjected to Southern blot analysis.

Fig. 5 shows the result of these analyses. Digestion of wild-type DNA with *Bam*H I or *Sal*I produced bands of 3.0 and

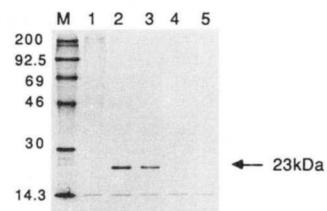


**FIG. 5. Southern blot analysis of virus genomes.** Genomic DNA was isolated from purified WR, VACHB, and VAC1 viruses and digested with *Bam*HI or *Sall*. The products were analyzed by Southern hybridization, using the 620-bp PCR product of the TmpK structural gene as the probe. An autoradiograph is shown. Lanes 1–3 contain the *Bam*HI digests of WR, VACHB, and VAC1, respectively, and lanes 4–6 contain the *Sall* digests of WR, VACHB, and VAC1, respectively.  $^{32}\text{P}$ -labeled DNA markers (lane M) are the Gibco/BRL 1-kb ladder.

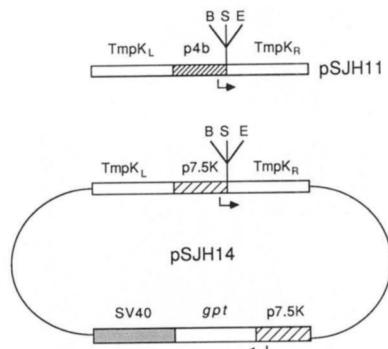
13.4 kb, respectively, which hybridized with a TmpK probe. In contrast, digestion of VAC1 or VACHB DNA with these enzymes produced bands of 2.3 and 2.7 kb (*Bam*HI) or 6.7 and 8.7 kb (*Sall*), consistent with the recombinant viruses having the *Eco*gpt gene inserted into the TmpK gene. The absence of plasmid DNA sequences in the recombinant virus genomes was confirmed by Southern blot analysis using a plasmid DNA probe and by PCR analysis of the TmpK locus using specific oligonucleotide primers (data not shown). The above data confirmed that stable virus mutants in which the *Eco*gpt gene is integrated into the TmpK gene had been generated, and thus demonstrated that the TmpK gene is non-essential for virus replication in cultured cells. Virus VACHB is thymidine kinase- and TmpK-negative, while VAC1 is TmpK-negative and thymidine kinase-positive.

**Identification of the TmpK Polypeptide**—The vaccinia TmpK gene has previously been shown to be transcribed early during infection (Smith *et al.*, 1989b). To confirm that the TmpK mRNA is translated in infected cells, the rabbit polyclonal antiserum raised against the TmpK inclusion body protein was used to immunoprecipitate the TmpK protein from extracts of virus-infected cells radiolabelled with L-[ $^{35}\text{S}$ ] methionine (Fig. 6). The antiserum did not precipitate any protein from an extract of mock-infected cells (lane 1). However, a protein of approximately 23 kDa was precipitated from extracts of cells infected with WR virus (lanes 2 and 3), but not from cells infected with the mutant virus VAC1 (lanes 4 and 5) in which the TmpK gene has been inactivated by insertional mutagenesis. These data confirmed that a virus-encoded thymidylate kinase is synthesized in infected cells.

**Plasmid Vectors for Expression of Foreign DNA from the TmpK Locus by Transient Dominant Selection**—Transient dominant selection allows the construction of recombinant vaccinia viruses expressing foreign genes without the stable integration of a selectable marker into the virus genome (Falkner and Moss, 1990). This technique allows the sequential insertion of several foreign genes at different nonessential loci or the sequential alteration of several endogenous genes. The procedure requires a plasmid containing the foreign gene



**FIG. 6. Immunoprecipitation of vaccinia TmpK protein.** L-[ $^{35}\text{S}$ ]Methionine-labeled proteins precipitated by the rabbit anti-TmpK antiserum from extracts of CV-1 cells infected with either WR or VAC1 virus were resolved by 12% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The samples were: lane 1, mock-infected cells labeled at 3–4 h; lanes 2 and 3, WR-infected cells labeled 2–3 and 3–4 h post-infection, respectively; lanes 4 and 5, VAC1-infected cells labeled at 2–3 h and 3–4 h post-infection, respectively. The arrow indicates the position of a protein of approximately 23 kDa that is immunoprecipitated from cells infected with WR. *Methyl-<sup>14</sup>C*-labeled protein molecular mass markers (lane M, Amersham International PLC) were lysozyme (14.3 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), phosphorylase b (92.5 kDa), and myosin (200 kDa).



**FIG. 7. Schematic maps of plasmids pSJH11 and pSJH14.** Plasmids pSJH11 and pSJH14 are vectors for constructing TmpK-recombinant viruses by transient dominant selection. TmpK<sub>L</sub> and TmpK<sub>R</sub> are 5' and 3' segments of the vaccinia TmpK gene flanking the vaccinia 4b and 7.5K promoters, shown as p4b and p7.5, respectively. The direction of transcription driven from these promoters is indicated by an arrow. The cloning region containing *Bam*HI (B), *Sma*I (S), and *Eco*RI (E) restriction sites for the insertion of foreign genes under the control of the vaccinia promoters is shown. The *Eco*gpt gene and downstream SV40 sequences isolated from the plasmid pGpt07/14 (Boyle and Coupar, 1988) are shown as gpt and SV40.

linked to a vaccinia promoter and flanked by vaccinia DNA sequences from a nonessential locus, and a dominant selectable marker, such as the *Eco*gpt gene, external to the flanking vaccinia virus sequences. In this study the TmpK gene is shown to be nonessential for virus growth in tissue culture and is therefore a locus that may be used for the insertion of foreign genes. Two vectors have been constructed for introducing genes at the TmpK locus by transient dominant selection ("Materials and Methods") (Fig. 7); one vector (pSJH14) in which the foreign gene is placed under the control of the vaccinia constitutive 7.5 K promoter (Mackett *et al.*, 1984), and the second (pSJH11) in which the gene is under the control of the late 4b promoter (Rosel *et al.*, 1986; Kent, 1988). Each vector contains unique *Bam*HI, *Sma*I and *Eco*RI restriction sites for the insertion of foreign genes.

## DISCUSSION

A vaccinia virus ORF transcribed early in infection was formerly predicted to encode a 23.2-kDa protein showing 42% amino acid identity with the thymidylate kinase of *S. cerevisiae* (Smith *et al.*, 1989b). This ORF, originally designated

SalF13R, has been renamed SalF11R (Smith *et al.*, 1991). The protein encoded by this gene is now shown by two independent methods to be an active thymidylate kinase. First, the gene was expressed at high levels in *E. coli* and extracts from these bacteria contained (in different experiments) between 200- and 1000-fold higher levels of thymidylate kinase activity in an *in vitro* assay. The level of activity was inducible with IPTG as expected. Furthermore, the vaccinia TmpK gene can fully complement a *cde8* mutant of *S. cerevisiae*, which is defective in thymidylate kinase activity. This confirmed the activity of the enzyme and demonstrated functional complementarity between the yeast and vaccinia virus enzymes. Attempts to detect the virus encoded TmpK activity in crude extracts of vaccinia virus infected cells using an *in vitro* enzyme assay have been unsuccessful (data not shown). However, the synthesis of the 23-kDa vaccinia TmpK protein in infected cells has been demonstrated by immunoprecipitation using a polyvalent antisera raised against the protein expressed in bacteria.

A cDNA encoding the human thymidylate kinase has recently been cloned (Su and Sclafani, 1991) and the predicted amino acid sequence of the human enzyme found to share 42% amino acid identity with the vaccinia TmpK protein. Likewise, the vaccinia thymidine kinase enzyme shows 68% amino acid identity with the human thymidine kinase enzyme (Weir *et al.*, 1983; Hruby *et al.*, 1983). From these levels of homology it seems that vaccinia may have acquired a cellular TmpK and thymidine kinase gene sometime during evolution. Since the thymidine kinase gene from several species contains multiple introns (Gudas *et al.*, 1990), while vaccinia virus genes are unspliced, it is likely that the latter are derived from cDNA copies of cellular genes. Vaccinia virus also encodes a two subunit ribonucleotide reductase, and genes which potentially encode dUTPase and guanylate kinase have been identified. Given the presence of dUTPase, thymidine kinase, and TmpK, the presence of a thymidylate synthase may have been predicted. However, assays for a novel thymidylate synthase polypeptide in vaccinia virus-infected cells, by incubation of cell extracts with [<sup>32</sup>P]FdUMP which binds covalently to thymidylate synthase enzymes (Honess *et al.*, 1986), only detected the endogenous cellular thymidylate synthase, the levels of which decreased as the infection progressed.<sup>2</sup> In contrast, in herpes virus simairi-infected cells the novel virus thymidylate synthase polypeptide was clearly seen by this assay. Additionally, the determination of the complete nucleotide sequence of the Copenhagen strain of vaccinia revealed that the virus genome does not contain an ORF with an amino acid homology to the highly conserved thymidylate synthase proteins (Goebel *et al.*, 1990).

Concerning the role of TmpK in the virus life cycle, two mutant viruses, VAC1 and VACHB, were constructed in which the TmpK gene was disrupted by insertional mutagenesis. The ability of these viruses to grow in cultured cells indicates that the TmpK gene is nonessential for virus replication. Two other vaccinia enzymes involved in synthesis of deoxynucleoside triphosphates (thymidine kinase and ribonucleotide reductase) are also nonessential for virus replication in cultured cells, and loss of these genes causes virus attenuation (Dubbs and Kit, 1964; Weir *et al.*, 1982; Buller *et al.*, 1985; Child *et al.*, 1990). This is relevant to the use of the thymidine kinase gene as a popular site for the insertion of foreign genes into the virus genome (Mackett *et al.*, 1982; Smith *et al.*, 1983). Preliminary data with viruses WR, VAC1, VACHB, and vHBs4 indicate that the TmpK<sup>-</sup> viruses are

attenuated in a similar manner to the thymidine kinase-negative viruses, but that loss of both genes causes increased attenuation.<sup>3</sup> It is likely that these genes are important to vaccinia *in vivo* because they enable the virus to replicate efficiently in nonproliferating host tissues in which the cellular pools of deoxynucleoside triphosphates and their precursors may be low.

The nonessentiality of the TmpK gene for virus growth enables this locus to be used as a potential site for the insertion of foreign DNA into the virus genome. Therefore, two plasmid vectors, pSJH11 and pSJH14, which can be used to generate TmpK<sup>-</sup> virus recombinants by transient dominant selection (Falkner and Moss, 1990) were constructed. pSJH11 allows expression of a foreign gene late in infection from the vaccinia 4b promoter, whereas pSJH14 contains the early and late vaccinia 7.5K promoter for foreign gene expression. These vectors may be useful for the insertion of additional foreign genes in those viruses in which other sites (such as thymidine kinase) are already utilized.

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<sup>2</sup> Unpublished data obtained by a collaboration with M. Stoffel and the late R. W. Honess.

<sup>3</sup> S. J. Hughes, A. de Carlos, and G. L. Smith, unpublished data.

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