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Members of a Novel Family of Mammalian Protein Kinases Complement the DNA-Negative Phenotype of a Vaccinia Virus *ts* Mutant Defective in the B1 Kinase

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Temperature-sensitive (*ts*) mutants of vaccinia virus defective in the B1 kinase demonstrate a conditionally lethal defect in DNA synthesis. B1 is the prototypic member of a new family of protein kinases (vaccinia virus-related kinases, or VRK) that possess distinctive B1-like sequence features within their catalytic motifs (R. J. Nichols and P. Traktman, J. Biol. Chem., in press). Given the striking sequence similarity between B1 and the VRK enzymes, we proposed that they might share overlapping substrate specificity. We therefore sought to determine whether the human and mouse VRK1 enzymes (hVRK1 and mVRK1, respectively) could complement a B1 deficiency in vivo. Recombinant *ts2* viruses expressing hVRK1, mVRK1, or wild-type B1 were able to synthesize viral DNA at high temperature, but those expressing the more distantly related human casein kinase 1α2 could not. Complementation required the enzymatic activity of hVRK1, since a catalytically inactive allele of hVRK1 was unable to confer a temperature-insensitive phenotype. Interestingly, rescue of viral DNA synthesis was not coupled to the ability to phosphorylate H5, the only virus-encoded protein shown to be a B1 substrate in vivo. Expression of hVRK1 during nonpermissive *ts2* infections restored virus production and plaque formation, whereas expression of mVRK1 resulted in an intermediate level of rescue. Taken together, these observations indicate that enzymatically active cellular VRK1 kinases can perform the function(s) of B1 required for genome replication, most likely due to overlapping specificity for cellular and/or viral substrates.

Vaccinia virus, the prototypic member of the *Poxvirus* family, encodes a 192-kb double-stranded DNA genome that encodes ~200 proteins. This expansive coding capacity enables vaccinia virus to replicate in the cytoplasm of host cells with significant autonomy from cellular factors within the nucleus. A requirement for this compartmentalization is that vaccinia virus must encode and synthesize the majority of components required for productive viral infection, including those involved in transcription and genome replication. Through genetic and biochemical analysis of complementation groups of temperature-sensitive (*ts*) mutants, five viral open reading frames (ORFs) essential for genome replication have been described. These include the viral DNA polymerase (E9) (6, 7, 26–28, 38, 40, 41, 44), a component of the processive DNA polymerase complex (A20) (17, 32), a DNA-independent nucleotide triphosphatase (D5) (10, 11), uracil DNA glycosylase (D4) (39), and a serine/threonine protein kinase (B1) (33, 34, 43). B1 is an essential 34-kDa serine/threonine protein kinase which is made at early times postinfection, prior to the onset of viral DNA synthesis. Phenotypic analysis of two *ts* mutants with defined lesions in the B1 ORF (*ts2* and *ts25*) has demonstrated that, in the absence of B1, the virus is deficient in its ability to synthesize viral DNA at high temperature (33). The severity of this defect varies with cell type and multiplicity of infection (MOI). Accumulation of viral DNA and production of infectious progeny are severely restricted when murine L929 fibroblasts are infected under restrictive conditions, whereas these processes are only moderately affected during propagation in primate BSC40 epithelial cells (33). Pulse-chase experiments demonstrated that the *ts2*- and *ts25*-encoded B1 proteins are highly unstable in both cell types under all growth conditions (34), whereas in vitro kinase assays utilizing purified recombinant ts2- and ts25-B1 proteins established that the mutations eliminate or severely impair B1's enzymatic activity (34). It is not known why the B1 mutants exhibit a temperature-sensitive phenotype despite the fact that the ts2- and ts25-B1 proteins appear to be labile and/or inactive at permissive and nonpermissive temperatures. We hypothesized that host cell factors might complement the defect at the permissive but not the nonpermissive temperature, perhaps due to a temperature dependency in their availability or activity. A precedent for such a model can be found in the demonstrated ability of a cellular protein to complement a herpes simplex virus ribonucleotide reductase mutant in a temperature-sensitive manner (30).

Recent genomic and bioinformatic approaches have led to the classification of a novel family of protein kinases with remarkable sequence similarity to the vaccinia virus B1 protein kinase (29a). The B1 protein sequence contains several distinctive sequence variations within its catalytic domain which set B1 apart from the majority of cellular Ser/Thr protein kinases (43). These variations are most obvious in the catalytic loop (subdomain VI) ^{145}HGDIK^{149}, the active site (subdomain VII) ^{167}DYG^{169}, and the substrate recognition domain (sub-

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domain VIII) ^{199}PID^{201} (amino acid residues refer to the position in B1; underlined residues are the unique B1 residues). Traditional Ser/Thr kinases contain the motifs HLDIK, DFG, and SIN (the SIN motif is casein kinase 1 [CK1] specific; an APE motif is found in the majority of other kinases) in these catalytic subdomains (13, 14). The first members of this new kinase family to be described were the products of two previously unidentified human genes termed human vaccinia virus B1-related kinase 1 (hVRK1) and hVRK2 (29). hVRK1 was isolated by screening a fetal liver-specific cDNA library with subtractive hybridization to identify novel genes involved in cell cycle regulation. hVRK2 was identified by database searches designed to identify hVRK1-like genes. At the amino acid level, hVRK1 and hVRK2 were found to be ~40% identical to B1. This is a striking observation, because the most closely related mammalian kinase previously found was human CK1 (hCK1) with 20% homology to B1. Independently, a murine 51-kDa nuclear Ser/Thr kinase (51PK) with ~40% identity to B1 was identified (47). Protein alignments indicate that the 51PK kinase is a murine homologue of hVRK1, with these two proteins possessing 86% identity to each other. Therefore, we will henceforth refer to 51PK as mVRK1. Little is known about the cellular functions of these mammalian kinases. Both hVRK1 and mVRK1 have been shown to be active Ser/Thr protein kinases that localize to the nucleus (23, 29a, 47). In vitro, hVRK1 has been shown to phosphorylate fragments of the murine p53 protein prepared as glutathione *S*-transferase fusions (1, 23), but the functional relevance of this activity remains to be determined. Our laboratory has shown that hVRK1 and mVRK1 show robust autophosphorylation activity and will also phosphorylate casein as an exogenous substrate (29a). Furthermore, our laboratory has shown that three differentially spliced isoforms of mVRK1 are expressed within murine L929 fibroblasts: full length (mVRK1), lacking exon 12 (mVRK1Δ), or lacking exons 12 and 13 (mVRK1ΔΔ) (29a). The mVRK1ΔΔ isoform is identical to hVRK1 in length; detailed genomic analysis of these genes can be found in a report by Nichols and Traktman (29a).

The high degree of structural conservation seen in this new family of viral and cellular protein kinases stimulated us to determine whether they also exhibited functional overlap. Given our long-standing interest in vaccinia virus DNA replication, we sought to determine whether the VRK1 proteins were able to compensate for B1 during vaccinia virus DNA replication. In this report, we describe our construction and analysis of recombinant viruses engineered to express the wild type (wt) or a catalytically inactive form of hVRK1, mVRK1, mVRK1Δ, mVRK1ΔΔ, hCK1α, or wt B1 within the context of the *ts2* genome. We show that expression of hVRK1, all isoforms of mVRK1, and wt B1, but not hCK1α or catalytically inactive hVRK1, restores viral DNA synthesis at the nonpermissive temperature. We propose that members of this protein kinase family have overlapping specificity for viral and/or cellular substrates and consequently may play analogous roles in regulating the metabolism of the cellular genome.

MATERIALS AND METHODS

Reagents. Restriction endonucleases, *Escherichia coli* polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, calf intestinal phosphatase (CIP), pancreatic RNase, PCR-grade deoxynucleoside triphosphates, high-fidelity *Taq* polymerase, *Taq* polymerase, and DNA molecular weight standards were purchased from Roche Applied Science (Indianapolis, Ind.) and were used as specified by the manufacturer. [^{32}P]nucleoside triphosphates, [^{32}P]orthophosphate, and [^{35}S]methionine were purchased from Perkin Elmer Life Sciences (Boston, Mass.). 5-Bromo-2′-deoxyuridine (BrdU) was obtained from Sigma (St. Louis, Mo.). Lipofectamine Plus and ^{14}C-labeled protein molecular weight markers were acquired from Invitrogen (Carlsbad, Calif.) and used as specified. Oligonucleotides were purchased from IDT (Coralville, Iowa)

Cells and virus. Monolayer cultures of African green monkey BSC40 cells, mouse L929 cells, or human thymidine kinase-minus (TK$^-$) 143 cells, were maintained in Dulbecco modified Eagle medium (DMEM; Invitrogen) containing 5% fetal calf serum (FCS). wt vaccinia virus (WR strain) and the B1 *ts* mutant *ts2* (kindly provided by Richard Condit, University of Florida, Gainesville) were grown in BSC40 cells or L cells. Viral stocks were prepared from cytoplasmic lysates of infected cells by ultracentrifugation through 36% sucrose; titers were determined on BSC40 cells. For virological studies, 31.5 and 39.7°C were used as the permissive and nonpermissive temperatures, respectively.

Preparation of anti-hVRK1 and anti-mVRK1 antisera. Amino acid alignment of the mammalian VRK1 kinases demonstrates divergence in the carboxyl termini; therefore, sequences found within the C-terminal region outside of the conserved kinase domain were used to generate the immunogen.

(i) Construction of pATH:hVRK1. A fragment encoding the C-terminal 128 amino acid residues of the hVRK1 ORF (GenBank accession no. AB000449) was amplified from a cDNA clone template (kindly provided by Jun-ichi Nezu, Chugai Research Institute for Molecular Medicine, Inc., Nagai, Niihari, Ibaraki, Japan). The upstream primer (5′-CG**GGATCC**AAATATGTTAGAGATTC-3′) introduced a *Bam*HI site (bold); the downstream primer (5′-GG**ATCGAT**TTA CTTCTGGACTCTC-3′) introduced a *Cla*I site (bold) directly after the termination codon (underlined). The 380-bp PCR product was digested with *Bam*HI and *Cla*I and inserted into pATH11 DNA (19) that had been similarly digested and treated with CIP. The resultant plasmid (pATH:hVRK1) fused a portion of the hVRK1 ORF downstream of, and in frame with, the *E. coli trpE* gene.

(ii) Construction of pATH:mVRK1. A fragment encoding the C-terminal 100 amino acid residues of the mVRK1 (GenBank accession no. AF080253) ORF was amplified by PCR with a cDNA clone as a template (kindly provided by Masahiko Negishi, National Institutes of Health [NIH], Research Triangle Park, N.C.). The upstream primer (5′-CG**GGATCC**TTTAGTGCTGTGGAG-3′) introduced a *Bam*HI site (bold); the downstream primer (5′-GG**ATCGAT**TTAC TTCTGGGCTTTC-3′) introduced a *Cla*I site (bold) directly after the termination codon (underlined). The 300-bp PCR product was digested with *Bam*HI and *Cla*I and inserted into pATH11 DNA (19) that had been similarly digested and treated with CIP. The resultant plasmid (pATH:mVRK1) fused a portion of the mVRK1 ORF downstream of, and in frame with, the *E. coli trpE* gene.

(iii) Expression of trpE:hVRK1 and trpE:mVRK1. *E. coli* transformants containing the pATH:hVRK1 construct directed synthesis of a 48-kDa fusion protein containing 34 kDa of *E. coli trpE* and 14 kDa of hVRK1; pATH:mVRK1 transformants expressed a 45-kDa fusion protein comprising 34 kDa of *E. coli trpE* and 11 kDa of mVRK1. These fusion proteins were induced by tryptophan starvation and the addition of indoleacrylic acid (19), and lysates from induced cultures were fractionated on a sodium dodecyl sulfate (SDS)–10% acrylamide gel. The fusion proteins were visualized by soaking the acrylamide gel in 0.3 M $CuCl_2$; the band of interest was excised and processed for the immunization of rabbits. The reactivity of the resultant polyclonal antisera against hVRK1 and mVRK1 was confirmed by immunoblot analysis.

Preparation and isolation of recombinant B1 *ts* viruses containing mammalian kinase ORFs. (i) Preparation of transfer vectors. Each of the alleles described below were cloned into the transfer vector pGS53 (kindly provided by Bernard Moss, NIH) after it had been digested with *Bam*HI and treated with CIP. The alleles were generated as follows. (a) The hVRK1 ORF was subcloned from a cDNA clone (provided by J. Nezu) by digesting pcDNA3/VRK1-MYC with *Bam*HI to excise the entire 1,190-bp ORF plus 30 bp encoding a carboxyl-terminal c-MYC epitope tag. (b) The $hVRK1_{K71A,D197G}$ allele was generated from hVRK1 by overlap PCR (8). First, $hVRK1_{K71A}$ was generated. Two separate PCRs were performed to produce products that overlapped by 17 bp. The primers used were upstream primer (UP) (5′-GAGGATCCATGCCTCGTGT AAAAGC-3′) and $hVRK1_{K71A}$-3′ (5′-GTTCCACT**GC**TACAACACAAGGTG CAT-3′) and $hVRK1_{K71A}$-5′ (5′-GTGTTGTA**GC**AGTGGAACCCAGTGAC A-3′) and the downstream primer (DP) (5′-CAGGATCCTTACTTCTGGACT C-3′). The *Bam*HI sights are indicated by the double underline, the initiating and terminating codons are shown by the single underline, and the bold sequences mark the altered nucleotides. A mixture of the two purified PCR products served as the template for a third PCR, which was performed with the UP and DP primers. The final PCR product was sequenced, and an hVRK1 clone containing

the K71A mutation without any spurious changes was used as a template for the next round of PCRs designed to incorporate the nucleotides encoding the D197G substitution. Two separate PCRs were performed with the primers UP and hVRK1$_{D197G}$-3′ (5′-GGCCATAA**C**CTACCAAGTACACCTGG-3′) and hVRK1$_{D197G}$-5′ (5′-CTTGGTAG**G**TTATGGCCTTGCTTATC-3′) and DP. Again, a mixture of the two purified PCR products served as the template for a third PCR, which was performed with UP and an hVRK1 primer that introduces the c-MYC epitope tag (single underline) hVRK1$_{cMYC}$ (5′-TC CCCCCGGGGATCCTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCC TTCTGGACTCTCTTTCTGG-3′). The restriction endonuclease sites for *Sma*I and *Bam*HI are indicated by italics and a double underline, respectively. (c) The mVRK1 ORF was amplified from a cDNA template (provided by M. Negishi) with mVRK1-specific primers 5′-GGGATCCCAT**ATG**CCCCGTGTAAAAG-3′ and 5′-CAGGATCC**TTA**CAGATCCTCTTCTGAGATGAGTTTTTGTTCCT TCTGGGCTTTC-3′, which incorporated *Bam*HI sites (double underline), initiating and terminating codons (boldface type), and a c-MYC epitope (single underline). The mVRK1ΔΔ and mVRK1Δ ORFs were obtained via reverse transcription-PCR with total RNA isolated from L929 cells as a template (29a) and the above-mentioned mVRK1-specific primers. (d) A wt copy of the vaccinia virus B1 ORF was amplified with the specific primers 5′-CAGGATCC**ATG**AA CTTTCAAGGAC-3′ and 5′-CAGGATCC**TTA**CAGATCCTCTTCTGAGATG AGTTTTTGTTCATAATATACACCCTGC-3′, which incorporated *Bam*HI sites (double underline), initiating and terminating codons (boldface type), and a c-MYC epitope (single underline). (e) An N-terminal hemagglutinin (HA) epitope-tagged hCK1α2 was subcloned by digesting the v367 hCK1α2 cDNA clone (kindly provided by David Virshup, University of Utah, Salt Lake City) with *Hin*dIII to excise the entire 1,143-bp ORF. The 3′ overhang was filled in with the Klenow fragment of *E. coli* DNA polymerase I. The gel-purified hCK1α2 fragment was ligated to pGS53, which was previously digested with *Sma*I and treated with CIP. All clones used to generate the recombinant viruses were sequenced by utilizing the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.) per the manufacturer's directions on an ABI Prism 310 genetic analyzer (PE Applied Biosystems).

(ii) Generation and isolation of recombinant viruses. Thirty-five-millimeter-diameter dishes of BSC40 cells were infected with *ts2* at an MOI of 0.03 PFU per cell and incubated at 31.5°C. At 3 h postinfection (hpi), cells were transfected with 3.5 μg of linearized plasmid by either calcium phosphate precipitation or Lipofectamine Plus reagent. At 3 days postinfection, the infected cell monolayer was harvested by scraping and subjected to repeated freeze-thaw cycles. Two rounds of plaque purification were carried out at 31.5°C on human TK$^-$ cells in the presence of 25 μg of BrdU per ml to select for the inactivation of the endogenous viral TK gene. To identify those plaques in which this inactivation reflected the insertion of the targeted genes, rather than spontaneous mutation, BrdU-resistant plaques were screened by PCR analysis with primers specific for the genes carried within the targeting vectors. The recombinant viruses were expanded, and viral stocks were prepared by propagation at 31.5°C.

Characterization of the B1 *ts* recombinant viruses. (i) Retention of the endogenous *ts2* lesion. The 900-bp endogenous B1 ORF was amplified from plaque-purified viral stocks with primers specific for the flanking ORFs (A57, 5′-GAC AGATAGATCACAC-3′, and B2, 5′-CGACCAAACACCACAC-3′). Retention of the *ts2* lesion was confirmed by utilizing the BigDye terminator cycle sequencing ready reaction kit per the manufacturer's directions on an ABI Prism 310 genetic analyzer.

(ii) Confirmation of in vivo expression of the inserted mammalian kinases. BSC40 cells were infected at an MOI of 10 and incubated at 31.5°C for 17 h. Cells were harvested by scraping with a rubber policeman and collected by centrifugation. After washing with phosphate-buffered saline (PBS) (140 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4 [pH 7.4]), cells were lysed by the addition of protein sample buffer (final concentrations, 1% SDS, 1% β-mercaptoethanol, 50 mM Tris [pH 6.8], 10% glycerol). Proteins were resolved by electrophoresis on SDS–12% acrylamide gels and transferred electrophoretically to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.). The blots were analyzed by incubation with polyclonal sera directed against either hVRK1 or mVRK1 (described in this report), B1 (34), HA (Santa Cruz Biotech, Santa Cruz, Calif.), or c-MYC (Santa Cruz Biotech) (all used at a 1:1,000 dilution, except for the c-MYC antibody, which was used at a 1:300 dilution) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G serum (1:10,000; Bio-Rad). Immunoreactive proteins were visualized on Kodak MR film after chemiluminescent development with the Super Signal WestPico reagents (Pierce, Rockford, Ill.).

Dot blot hybridization to assess viral DNA accumulation. Confluent 35-mm-diameter dishes of BSC40 cells were infected at an MOI of 2 and incubated at 31.5 or 39.7°C. Cells were harvested at the indicated time points (3, 4.5, 6, 7.5, 9, and 12 hpi) by scraping, collected by centrifugation, washed once with PBS, and resuspended in 500 μl of loading buffer (10× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0], 1 M ammonium acetate). The cells were disrupted by three cycles of freeze-thawing. After vortexing, 25 μl of each sample was spotted in duplicate onto a ZetaProbe blotting membrane (Bio-Rad) with the Bio-Dot microfiltration dot blot apparatus (Bio-Rad). DNA was denatured in situ with 0.5 M NaOH and 1.5 M NaCl for 10 min and washed twice with 10× SSC for 5 min. The membrane was air-dried, and viral DNA was detected by hybridization with a radiolabeled nick-translated probe representing the *Hin*dIII E and F fragments of the vaccinia virus genome. Data were analyzed on a phosphorimager.

Immunodetection analyses of viral proteins. Rabbit polyclonal antiserum directed towards E9 (25), A20 (18), D5 (11), I3 (35), H5 (8; U. Sankar and P. Traktman, unpublished data), A17 (45), A14 (45), L4 (22), and F18 (22) have all been developed and described by our laboratory. Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred electrophoretically to nitrocellulose, and incubated with primary and secondary antibodies as originally described. Blots were developed with colorimetric or chemiluminescent reagents.

Determination of 24-h virus yield. Confluent 35-mm-diameter dishes of BSC40 cells were infected at an MOI of 2 and maintained at 31.5 or 39.7°C. Cells were harvested at 24 hpi, collected by centrifugation, and resuspended in 1 mM Tris (pH 9.0). The cells were disrupted by three cycles of freeze-thawing and two 15-s bursts of sonication. Virus yields were assessed by titer determination on BSC40 cells at 31.5°C. Three separate experiments were performed, and the titers of each were determined in duplicate. A ratio was calculated [(PFU obtained at 39.7°C)/(PFU obtained at 31.5°C)] for each virus, and the results were graphed as percentages, with standard errors indicated.

Immunoprecipitation of H5 after metabolic labeling with ^{32}PPi. Confluent 35-mm-diameter dishes of BSC40 cells were infected at an MOI of 15 and maintained at 39.7°C. At 3 hpi, cells were incubated with warmed phosphate-free DMEM (ICN Biomedicals Inc, Costa Mesa, Calif.) supplemented with L-glutamine and 100 μCi of $^{32}PP_i$ per ml. After 2 h of metabolic labeling, cells were harvested, rinsed once with PBS, and lysed in 1× PLB (0.1 M $NaPO_4$ [pH 7.4], 0.1 M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate [1.2 ml per 3×10^6 cells]) containing 1 mM sodium orthovanadate. Clarified lysates were incubated with primary anti-H5 serum (8; Sankar and Traktman, unpublished) for 4 h followed by incubation with protein A-Sepharose (Sigma) for 1.5 h with end-over-end mixing; immunoprecipitates were then retrieved, washed extensively, and analyzed by SDS-PAGE and autoradiography.

Proteolytic processing of virion proteins. Confluent 35-mm-diameter dishes of BSC40 cells were infected with wt, *ts2*, *ts2*/hVRK1, or *ts2*/mVRK1ΔΔ at an MOI of 2 and maintained at 31.5 or 39.7°C. As a control, cells were infected with wt virus in the presence of 100 μg of rifampin per ml. At 7 hpi, the cells were rinsed with warmed methionine-free DMEM and incubated for 60 min with warmed methionine-free DMEM supplemented with 100 μCi of [^{35}S]methionine per ml. The cells were either placed on ice and harvested immediately (pulse) or subjected to a 5-h chase protocol in which the medium was aspirated, and cells were rinsed and fed with warmed DMEM supplemented with 5% FCS and maintained at the original temperature until 12 hpi. The cells were scraped, collected by centrifugation, resuspended in PBS, and lysed with SDS sample buffer. Proteins were resolved by electrophoresis on an SDS–10% acrylamide gel and visualized by autoradiography on Kodak MR film.

Preparation of digital figures. Sequences were retrieved from GenBank and aligned by the Clustal V method and Lasergene software (DNASTAR Inc., Madison, Wis.). Original data were scanned on a SAPHIR scanner (Linotype-Hell Co., Hauppauge, N.Y.) and were adjusted with Photoshop software (Adobe Systems Inc., San Jose, Calif.). Data from the dot blot hybridization were acquired on a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and quantitated with ImageQuant software (Molecular Dynamics). Graphs were plotted with SigmaPlot (SPSS Science, Chicago, Ill.). Plaque assays were photographed with an AlphaImager documentation system (Alpha Innotech, San Leandro, Calif.). Final figures were assembled and labeled with Canvas software (Deneba Systems, Miami, Fla.).

RESULTS

The vaccinia virus B1 protein, although it contains all the motifs predictive of protein kinases, possesses sequence variations within catalytic signatures that distinguish it from the majority of cellular Ser/Thr protein kinases (43). Members of

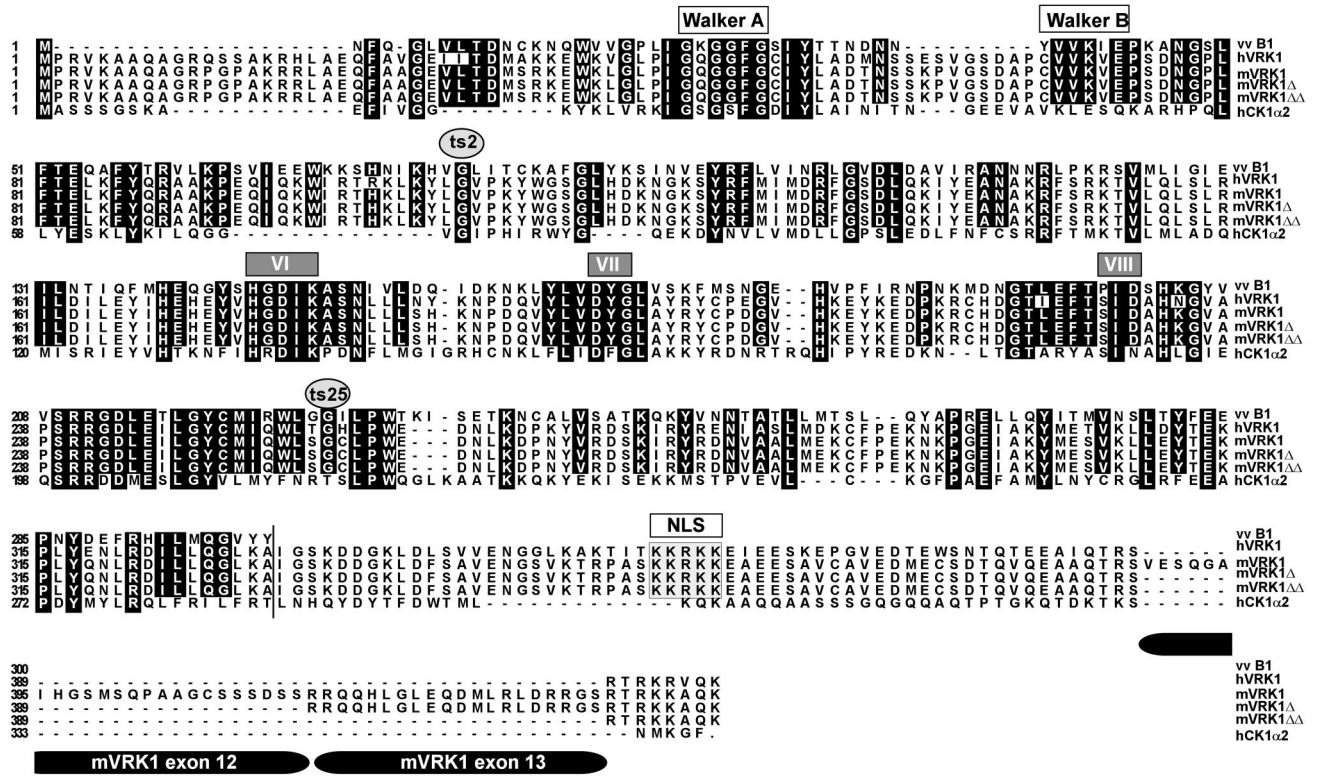

FIG. 1. Alignment of B1 kinase with human and mouse VRK1s: conservation of distinctive catalytic motifs throughout the family. Predicted amino acid sequences for vaccinia virus (vv) B1 (Western Reserve strain, nucleotide database entry J05178), hVRK1 (NP_003375), mVRK1 (NP_035835) and its splice variants mVRK1Δ and mVRK1ΔΔ (29a), and hCK1α (P48729) were aligned by the Clustal V method within the MegAlign program of DNAStar (Lasergene, Inc.). Residues shown in white type within black boxes indicate those that are found in vvB1 and are shared by the majority of the VRK sequences. Essential conserved domains with known catalytic functions are noted with boxes above the relevant sequences: the Walker A and Walker B bipartite ATP binding domains, the catalytic loop (VI), active site (VII), and substrate recognition (VIII) domains. The Gly residues altered in *ts2* and *ts25* (gray circles) are conserved in all VRK1 sequences. A vertical line marks the C terminus of the 300-amino-acid vvB1 sequence and highlights the presence of C-terminal extensions of 60 to 120 amino acids in the mammalian VRK1 proteins. The putative NLS found within the hVRK1 and mVRK1 sequences is also marked. mVRK1 sequences that are absent in the alternatively spliced mVRK1Δ (missing exon 12) and mVRK1ΔΔ (missing exons 12 and 13) variants are also noted. Because B1 and the VRK1 families have been placed within the CK1 superfamily, hCK1α is included in the alignment. Although key portions of the catalytic domains are retained in hCK1α, there is much less overall homology, and several of the distinguishing sequence variants found in B1 and the VRK1 proteins are absent in hCK1α.

the CK1 family were shown to resemble B1 most closely but still shared only 20% sequence identity. However, we and others have recently identified a novel family of mammalian Ser/Thr protein kinases with significant homology to the vaccinia virus B1 protein (29a, 46). This new family of protein kinases comprises VRK1, VRK2, and VRK3. Three genes encoding these proteins are found in the human and mouse genomes, and orthologs of some of the family members are found in the genomes of diverse eukaryotes such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio*. More-distantly related genes are also found in budding and fission yeast. For simplicity, we have aligned the predicted amino acid sequence for the hVRK1 and mVRK1 proteins along with B1 and hCK1α (Fig. 1). hVRK1 and mVRK1 show ~40% identity to B1, whereas the sequence for hCK1α is more distantly related. The homology is highest within the enzymatic domains of the proteins. The catalytic domain (subdomain VI), active site (VII), and substrate recognition (VIII) domains show the greatest degree of conservation, showing 100, 100, and 71% identity, respectively. Because the VRK proteins possess the same variant residues within these distinctive motifs that distinguish B1, they have been grouped into a family named for their relatedness to B1. It is worth noting that the glycine residues altered in the *ts2*- and *ts25*-encoded alleles are present in all members of this family, suggesting that they might play an important and conserved role. With regard to mVRK1, we have found that differential splicing leads to the expression of three isoforms: a full-length protein (mVRK1) and variants in which exon 12 (mVRK1Δ) or exons 12 and 13 (mVRK1ΔΔ) are excluded (29a). Furthermore, both m- and hVRK1 proteins contain a basic nuclear localization signal (NLS) that appears to be functional (23, 29a, 47). Lastly, although B1 represents a 34-kDa minimal catalytic domain, the VRK1 proteins are larger in size and contain divergent carboxy termini.

The high degree of sequence similarity between the VRK1 proteins and B1 led us to propose that these proteins might also display overlapping substrate specificities. If this were true, we hypothesized that expression of the VRK1 proteins during *ts*B1 infections might complement for the loss of B1

function. We therefore generated a panel of *ts2* recombinants expressing h- or mVRK1, wt B1, or CK1α from within the nonessential TK locus.

Generation of *ts2* viruses expressing the various mammalian kinases. cDNA inserts encoding C-terminally MYC-tagged hVRK1, mVRK1, mVRK1Δ, mVRK1ΔΔ, or wt B1 or N-terminally HA-tagged hCK1α were generated and inserted into the transfer vector pGS53. In these constructs, the genes are placed under the regulation of the constitutive viral p7.5 promoter and are flanked by the left and right halves of the viral TK gene. By performing infections and transfections at permissive temperature and selecting for TK^- viruses, we were able to isolate a panel of recombinant viruses in which each of these genes had been individually inserted into the TK locus. A summary of the viruses generated and their predicted genotypes is diagramed in Fig. 2. In each case, retention of the *ts2* lesion in the endogenous B1 gene was confirmed by sequence analysis (data not shown). To verify that the mammalian proteins were indeed being stably expressed during infections with these viruses, lysates were prepared from BSC40 cells infected at 31.5°C with the various recombinants and subjected to SDS-PAGE and immunoblot analysis. As shown in Fig. 3, infection with each of the recombinant viruses did lead to the expression of the appropriate VRK1, CK, or B1 proteins, as detected by either antigen-specific (anti-B1, anti-hVRK1, or anti-mVRK1) or epitope-specific (anti-MYC or anti-HA) antibodies. In this experiment, the endogenous VRK1 proteins are not evident in the immunoblot analysis, and the B1 proteins expressed from the endogenous loci are also below the limits of detection. The anti-hVRK1 antibody shows some cross-reactivity with the mVRK1 protein, due to the high degree of conservation between these proteins. The mVRK1 antibody does not cross-react with hVRK1, since much of the immunogen was derived from exon 12 and 13 sequences which have no cognates in hVRK1. The anti-mVRK1 serum, however, recognizes two additional proteins, one of cellular (square) and one of viral (circle) origin.

Viral DNA synthesis is rescued by expression of the VRK1s but not hCK1α. Since DNA replication is compromised during nonpermissive infections with the *ts* B1 mutants, the first question we addressed was whether expression of the mammalian kinases could restore DNA synthesis at 39.7°C. BSC40 cells were infected at 31.5 or 39.7°C with wt virus, *ts2*, or the *ts2* recombinant viruses. At 3, 4.5, 6, 7.5, 9, and 12 hpi, cultures were harvested and the levels of viral DNA were quantitated by Southern dot blot analysis. The experiment was performed in triplicate, and each result was spotted in duplicate. The data shown in Fig. 4 are from a representative experiment. Figure 4A illustrates the accumulation of DNA during infections performed with wt virus, *ts2*, *ts2*/B1, and *ts2*/hCK1α at 31.5 and 39.7°C. In all the infections performed at 31.5°C, the profiles of viral DNA synthesis were similar, with the accumulation of DNA being detected as early as 6 hpi. However, dramatic differences were observed for the infections performed at 39.7°C. During wt infections performed at 39.7°C, viral DNA began to accumulate earlier (4.5 hpi) and reached higher levels than had been seen at 31.5°C. In contrast, cells infected with *ts2* at 39.7°C exhibited a reproducible 3-h delay in viral DNA accumulation. DNA accumulation was not detected until 7.5 hpi and never reached the levels observed during wt infections.

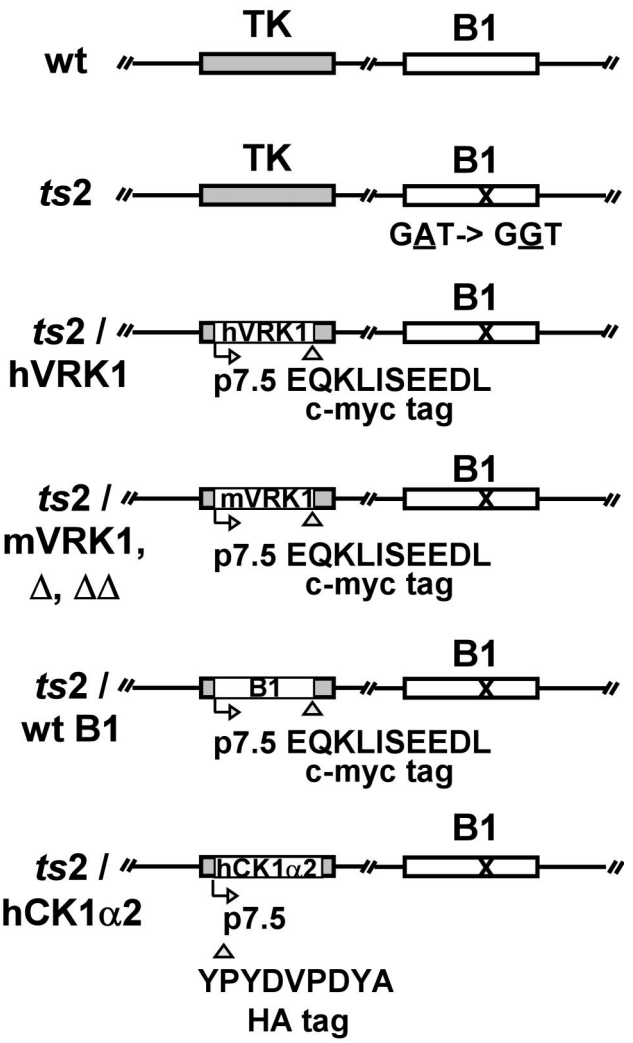

FIG. 2. Schematic representation of the genotypes of the *ts2* recombinants containing ectopic kinase alleles. wt vaccinia virus (WR strain) was used as a reference for the phenotypic analysis, since it encodes wt B1. In the *ts2* virus, the lesion resulting in the *ts* phenotype (GAT→GGT) is designated by an X within the B1 gene (open box). The hVRK1, mVRK1 (and the Δ and ΔΔ variants), wt B1, and hCK1α2 ORFs were placed under the control of the constitutive p7.5 promoter and inserted into the nonessential TK locus of the *ts2* genome. hVRK1, mVRK1s, and wt B1 were epitope tagged with C-terminal c-MYC tags (EQKLISEEDL), whereas an HA tag (YPYDVPDYA) was fused to the N terminus of hCK1α.

Cells infected with *ts2*/hCK1α at 39.7°C also demonstrated a comparable 3-h delay and an overall reduction in DNA accumulation. However, introduction of a wt copy of B1 into the *ts2* genome restored the early onset of viral DNA synthesis. During *ts2*/B1 infections at 39.7°C, DNA accumulation from 4.5 to 7.5 hpi was comparable to that seen during the wt infection, although DNA accumulation diminished thereafter.

In Fig. 4B, the data obtained for wt and *ts2* infections are compared to those obtained for infections performed with *ts2* recombinants expressing the mammalian VRK1s. For all infections performed at the permissive temperature, viral DNA was detectable by 6 hpi and continued to accumulate steadily

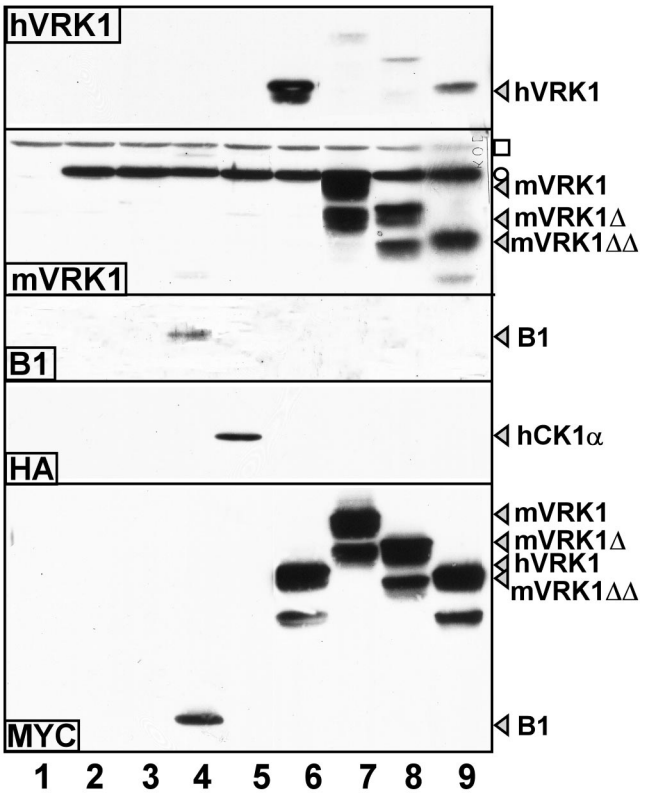

FIG. 3. hVRK1, mVRK1s, hCK1α, and wt B1 are expressed during infections performed with the *ts 2* recombinant viruses. BSC40 cells were either left uninfected (lane 1) or infected with wt virus (lane 2), *ts2* (lane 3), *ts2*/B1 (lane 4), *ts2*/hCK1α (lane 5), *ts2*/hVRK1 (lane 6), *ts2*/mVRK1 (lane 7), *ts2*/mVRK1Δ (lane 8), or *ts2*/mVRK1ΔΔ (lane 9) at 31.5°C for 17 h (MOI of 10). Cell lysates were subjected to immunoblot analysis with antisera directed against hVRK1, mVRK1, B1, HA, or c-MYC followed by incubation with an HRP-conjugated secondary antibody and chemiluminescent development. In the hVRK1 panel, a strong signal was seen in cells infected with the *ts2*/hVRK1 recombinant; in addition, the sera show a moderate level of cross-reactivity with the mVRK1 proteins but do not detect endogenous hVRK1 levels. Two cross-reactive proteins (one viral, ○, and one cellular, □) are seen in the mVRK1 panel. For the blot developed with anti-B1 serum, endogenous levels of B1 are not detected, but the B1 protein overexpressed in the *ts2*/B1 recombinant is easily seen.

throughout the time course. When infections were performed at 39.7°C, expression of any of the VRK1 proteins restored the early onset of DNA replication and augmented the levels of viral DNA that accumulated even beyond those seen with wt virus. At 12 hpi, the amount of viral DNA observed was 4- to 6-fold higher than that seen in *ts2* infections and ~2-fold higher than that seen in wt infections. These data clearly demonstrate that the mammalian VRK1 proteins can complement a B1 deficiency and restore viral DNA synthesis.

Impact of B1 and the VRK1s on the levels of known replication proteins. We were interested in exploring the mechanism(s) behind the ability of the VRK1 proteins to rescue viral DNA synthesis. The most obvious hypothesis would be that VRK1 restores the phosphorylation of a B1 substrate whose activity is dependent upon this posttranslational modification. Despite performing numerous immunoprecipitation analyses of ^{32}P-labeled infected cell extracts, we have been unable to detect phosphorylation of any of the known key replication proteins such as E9 (the catalytic DNA polymerase), A20 (stoichiometric component of the processive polymerase), D5 (NTPase), or D4 (UDG) (unpublished data). I3, the putative replicative SSB, is phosphorylated, but not by a viral kinase (P. Traktman, unpublished data). The next logical hypothesis is that, either directly or indirectly, the loss of B1 function leads to reduced levels of one or more of the replication proteins. To explore this hypothesis, BSC40 cells were infected with wt virus, *ts2*, *ts2*/hVRK1, or *ts2*/B1 at an MOI of 15 and incubated at 39.7°C for 4 h. Cell lysates were fractionated by SDS-PAGE and subjected to immunoblot analysis with sera directed against the E9, A20, D5, I3, or H5 protein. H5 is an abundant, constitutively expressed protein known to associate with A20 (24) and to be a substrate of the B1 kinase (4). The levels of all five of these proteins appeared to be unchanged when B1 is defective or when VRK1 is expressed (Fig. 5A, compare lanes 1 to 4). A noteworthy difference in the electrophoretic mobility of the H5 protein was observed. In extracts prepared from cells infected at the nonpermissive temperature, higher-molecular-weight forms of H5 were seen in wt and *ts2*/B1 infections (Fig. 5A, lanes 1 and 4), but not in *ts2* or *ts2*/VRK1 infections (Fig. 5A, lanes 2 and 3). Previous analyses of the H5 proteins (Sankar and Traktman, unpublished) indicated that these forms correspond to the highly phosphorylated forms of H5 distinguishable by two-dimensional gel analysis (2, 3).

To directly examine the phosphorylation status of H5 during these various infections, BSC40 cells were infected at 39.7°C with wt virus, *ts2*, *ts2*/hVRK1, or *ts2*/B1 and labeled with $^{32}PP_i$ from 3 to 5 hpi. Cell lysates were then prepared and subjected to immunoprecipitation with an anti-H5 serum. As shown in Fig. 5B, H5 was strongly phosphorylated in samples prepared from wt and *ts2*/B1 infections (lanes 5 and 8) but not in those prepared from *ts2* or *ts2*/hVRK1 infections (lanes 6 and 7). Two important points emerge from these findings. First, full phosphorylation of H5 is B1 dependent and cannot be restored by hVRK1 or the viral F10 kinase, which is expressed as a late protein after the onset of DNA replication. Second, the VRK1 proteins are able to rescue viral DNA synthesis without restoring H5 phosphorylation, which indicates that the latter modification must be unimportant for this viral process.

Expression of the VRK1s restores *ts2*'s ability to complete a productive infectious cycle. Having shown that expression of the VRK1 proteins was able to fully restore viral DNA synthesis to nonpermissive *ts2* infections, we wanted to determine whether the ability to complete a productive infection was also restored. Therefore, the 24-h virus yield from a single infectious cycle was determined. BSC40 cells were infected with the various recombinant viruses at 31.5 or 39.7°C (MOI, 2), and the total yield of cell-associated virus was determined by titration at 31.5°C. The ratio of the virus yield produced at 39.7°C to that obtained at 31.5°C was expressed as a percentage; the data are depicted in the bar graph shown in Fig. 6. The ratios for wt virus and *ts2* were 125 and 7%, respectively. These data are comparable to those obtained in the initial characterization of *ts2* (33). Expression of hVRK1 is able to boost virus production significantly at 39.7°C, albeit not to wt levels, yielding a 39.7°C/31.5°C ratio of 50%. Somewhat lower restoration of virus production is seen upon expression of any of the mVRK1 isoforms (ratios of 17 to 31%). In this regard, it is worth noting

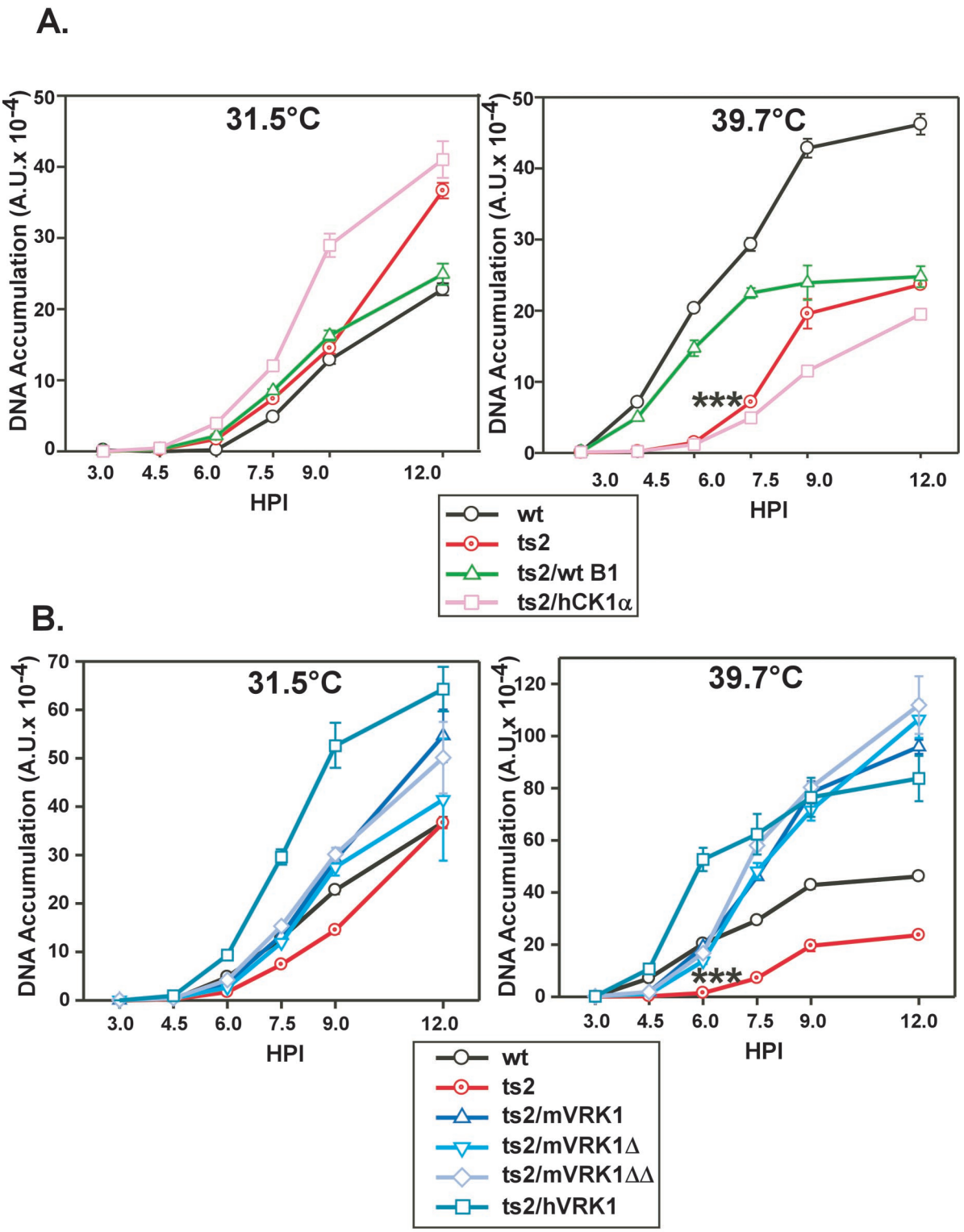

FIG. 4. Expression of the mammalian VRK1s, but not hCK1α, rescues the *ts2* DNA synthesis defect. Confluent monolayers of BSC40 cells were infected with wt virus, *ts2*, or the panel of *ts2* recombinant viruses (MOI of 2) and maintained at 31.5 or 39.7°C. Individual cultures were harvested at 3, 4.5, 6, 7.5, 9, or 12 hpi, and the levels of accumulated viral DNA were determined by dot blot hybridization, which was performed in duplicate. Data were acquired on a Storm Phosphoimager, quantified with Image-Quant software, and graphed with SigmaPlot software. Panel A presents the data obtained for cells infected with wt virus, *ts2*, *ts2*/B1, and *ts2*/hCK1α at either the permissive (31.5°C) or nonpermissive (39.7°C) temperature. Panel B presents the data obtained for cells infected with wt virus, *ts2*, *ts2*/mVRK1, *ts2*/mVRK1Δ, *ts2*/mVRK1ΔΔ, and *ts2*/hVRK1 at either permissive (31.5°C) or nonpermissive (39.7°C) temperature. The asterisks emphasize that the 6- and 7.5-h time points demonstrate the maximal difference in DNA accumulation between wt and *ts2* infections and, hence, represent the most informative point at which to judge whether expression of the ectopic kinase genes inserted into the recombinants rescues the replication defect. Note the different scales used for the ordinates in the four panels.

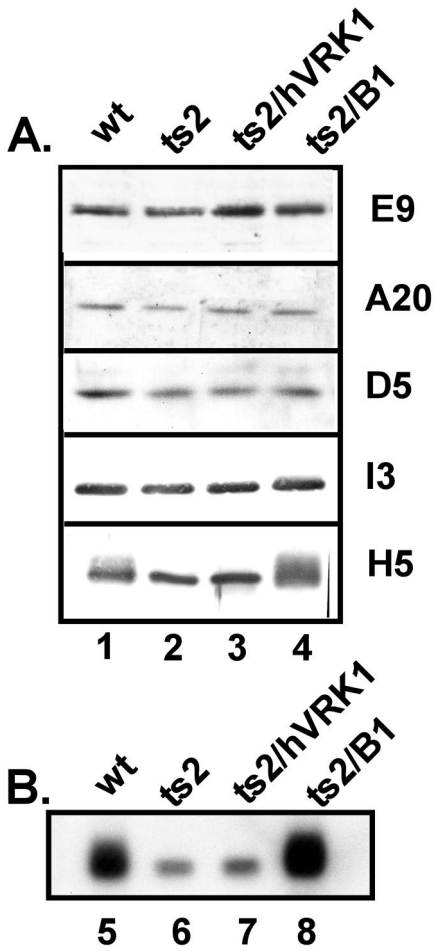

FIG. 5. Does the *ts2* defect or its rescue affect the accumulation or phosphorylation of viral replication factors? (A) Levels of replication proteins are not affected by the presence or absence of active B1. Confluent monolayers of BSC40 cells were infected with wt virus (lane 1), *ts2* (lane 2), *ts2*/hVRK1 (lane 3), or *ts2*/B1 (lane 4) (MOI of 15) and incubated at 39.7°C for 4 h prior to harvesting. Cell lysates were fractionated on SDS–12% polyacrylamide gels, and proteins were transferred to nitrocellulose filters, which were cut appropriately and probed with antibodies directed against E9, A20, D5, H5, or I3. After incubation with HRP-conjugated secondary antisera, immunoreactive proteins were visualized either by colorimetric (H5 and I3) or chemiluminescent (E9, A20, and D5) development. Equivalent levels of all five proteins are seen in the four samples; note, however, that the H5 protein seen in lanes 1 and 4 is heterogeneous, containing additional species with reduced mobility. (B) Immunoprecipitation of ^{32}P-labeled H5 protein: H5 hyperphosphorylation is B1 dependent and is not required for the rescue of DNA replication. Confluent 35-mm-diameter dishes of BSC40 cells were infected with wt virus (lane 5), *ts2* (lane 6), *ts2*/hVRK1 (lane 7), or *ts2*/B1 (lane 8) (MOI of 15) and incubated at 39.7°C for 3 h. Cells were then incubated in phosphate-free medium supplemented with 100 μCi of $^{32}PP_i$ per ml at 39.7°C for 2 h prior to harvesting. Radiolabeled cell lysates were immunoprecipitated with anti-H5 serum, and immunocomplexes were fractionated on an SDS–12% polyacrylamide gel and visualized by autoradiography.

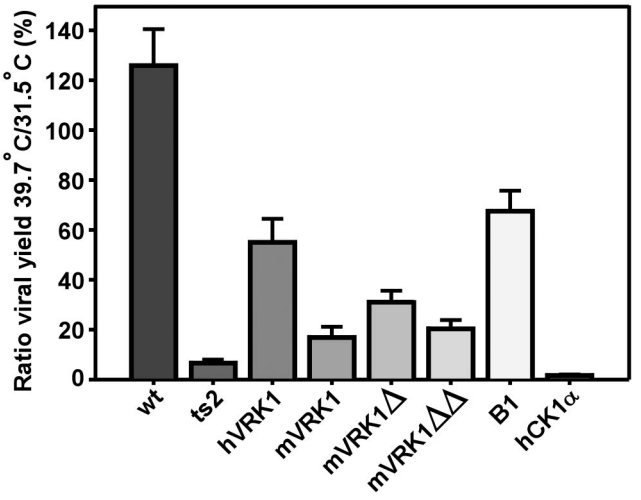

FIG. 6. Biological complementation by VRK1: analysis of 24-h virus yield. Confluent 35-mm-diameter dishes of BSC40 cells were infected with wt virus, *ts2*, or the panel of *ts2* recombinant viruses (MOI of 2) and incubated for 24 h at either 31.5 or 39.7°C. Cells were harvested and disrupted, and the virus yield was quantitated by titration on BSC40 cells at 31.5°C. Three separate experiments were performed, and each was titrated in duplicate. The percent of virus yield obtained at 39.7°C relative to that obtained at 31.5°C was calculated; the average values were plotted with the standard errors shown.

that mVRK1ΔΔ possesses 86% identity to hVRK1 and can restore viral DNA synthesis to comparable levels but yet is not as effective at restoring virus production. Expression of wt B1 rescues virus production, but again, levels do not reach that seen with wt virus (ratio of 68%). This somewhat surprising result suggests that the mutant B1 protein expressed from the endogenous *ts2* allele may exert an inhibitory effect; alternatively, expression of the ectopic B1 allele under the regulation of a constitutive promoter may not be optimal. Finally, as would have been predicted from its inability to restore DNA synthesis, expression of hCK1α does not rescue virus production at all (ratio of ~2%). We have confirmed that the purified recombinant hCK1α encoded by this allele possesses enzymatic activity in vitro (K. A. Boyle and P. Traktman, unpublished data).

As an additional measure of biological function, we performed plaque assays at both 31.5 and 39.7°C to compare the size and morphology of the plaques formed by the various viruses. In all cases, BSC40 cells were infected with 200 PFU per dish; after 48 h, the dishes were stained with crystal violet. As seen in Fig. 7, wt virus forms plaques at both temperatures, with the 39.7°C plaques being of larger size. In contrast, *ts2* forms microscopic, but not macroscopic, plaques at 39.7°C. The *ts2*/B1 recombinant virus forms plaques of wt size at 39.7°C, whereas the *ts2*/hCK1α virus did not form any macroscopic plaques at this temperature. The *ts2*/hVRK1 and *ts2*/mVRK1 recombinants formed plaques at 39.7°C, although these were somewhat smaller than those seen with the wt virus. In sum, these data parallel the more quantitative assessment obtained from titrating the 24-h yield from a single infectious cycle (see above) (Fig. 6).

Complementation by hVRK1 requires enzymatic activity. To determine whether hVRK1 was complementing the defect in *ts2* by serving as a protein kinase or whether it was serving a nonenzymatic role in the replication complex, we generated an allele of hVRK1 that contained mutations in two key catalytic domains. We mutated the Walker B box by changing the invariant $lysine_{71}$ to alanine ($K_{71}A$), disrupting the residue that

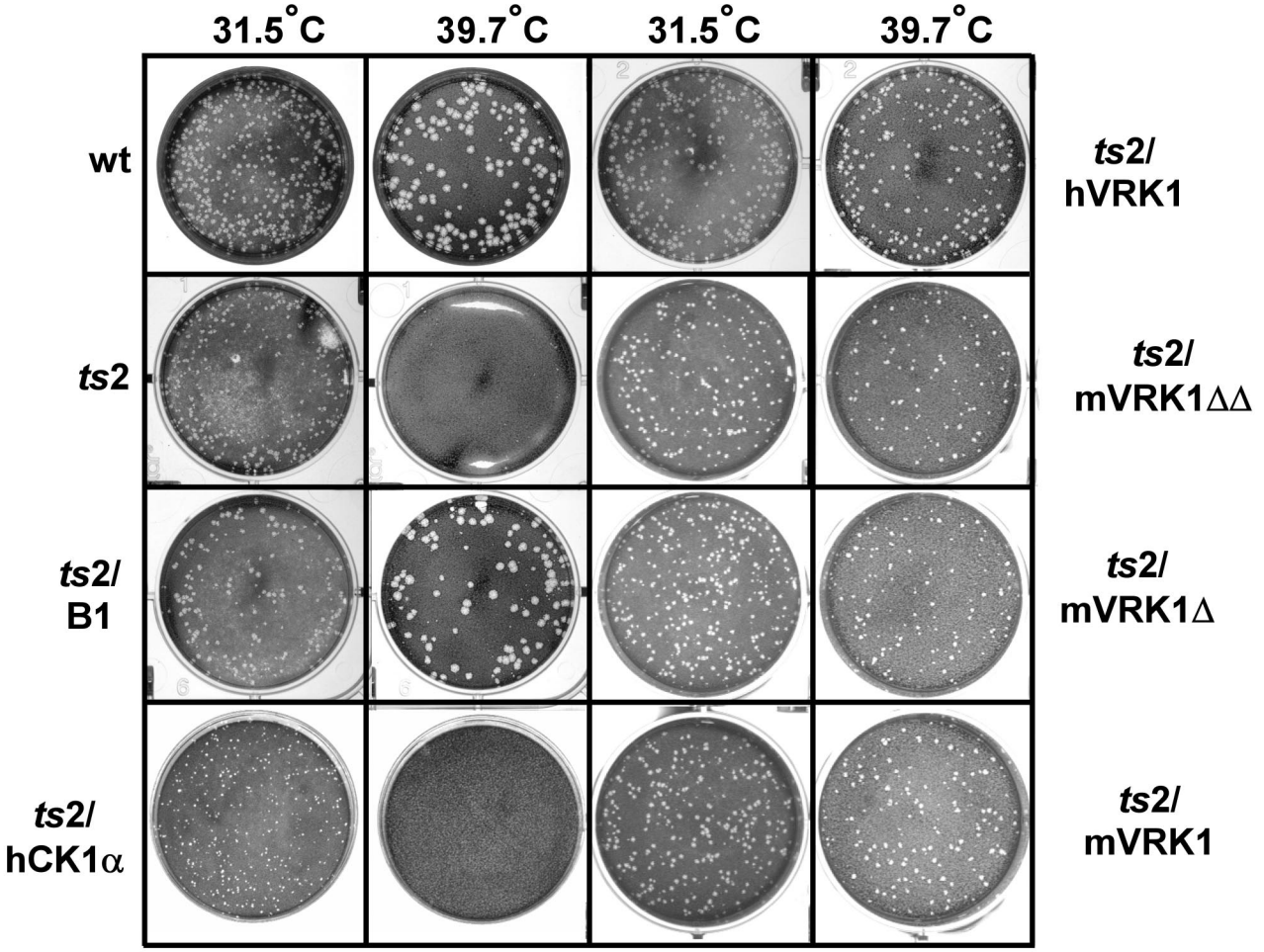

FIG. 7. Plaque formation of *ts2* at 39.7°C is restored by the expression of hVRK1, mVRK1, and wt B1, but not hCK1α. Confluent 35-mm-diameter dishes of BSC40 cells were infected with 200 PFU of the viruses indicated/plate and incubated at either 31.5 or 39.7°C for 2 days. Cells were then fixed and stained with 3.7% formaldehyde–0.1% crystal violet to visualize the viral plaques.

anchors and orients ATP by forming a salt bridge with a glutamic acid residue within kinase subdomain III (15). We also mutated kinase subdomain VII by changing the invariant aspartic acid$_{197}$ to glycine ($D_{197}G$); this residue interacts with the phosphate groups on ATP via an Mg^{2+} salt bridge (15). This $hVRK1_{K71A,D197G}$ allele was transferred into the *ts2* genome as described above for the other recombinant viruses. Although the $hVRK1_{K71A,D197G}$ protein was synthesized during infection (Fig. 8), it was unable to restore virus production to *ts*2 infections, as assessed in plaque assays (data not shown) and by determinations of 24-h virus yield from single infectious cycles (Fig. 8). Furthermore, the $hVRK1_{K71A,D197G}$ protein cannot restore viral DNA synthesis to *ts2* at high temperature (data not shown). These data provide compelling evidence that the ability of hVRK1 to complement a *ts2* infection is dependent upon its ability to serve as an active protein kinase.

Examination of the later stages of *ts2* infection. Although expression of the VRK1 proteins fully restores the accumulation of viral DNA, only a partial restoration of virus production was observed. We postulated that the loss of B1 might have a direct impact on late protein expression that could not be fully complemented by VRK expression. Although we had previously observed that cells infected with *ts2* at 39.7°C retained the ability to direct transcription of a plasmid-borne reporter gene from an intermediate promoter (Traktman, unpublished), Kovacs et al. (21) reported that inactivation of B1 affected intermediate gene expression. We therefore infected BSC40 cells with wt virus, *ts2*, and our panel of recombinants. At 7.5 hpi, cells were harvested and lysates were subjected to SDS-PAGE and immunoblot analysis. Filters were probed with sera directed against a variety of proteins: I3, H5, F18, L4, A14, and A17. As seen in Fig. 9A, viruses that were unable to replicate their DNA (*ts2* and *ts2*/hCK1α) (lanes 2 and 6) were unable to progress to intermediate and late gene expression. The early-intermediate protein I3 and the early-late H5 protein were easily detected, but the true late proteins (F18, L4, A14, and A17) did not accumulate. When cells were infected with wt virus, *ts2*/hVRK1, or *ts2*/B1 (lanes 1, 3, and 5, respectively), all proteins (I3, H5, F18, L4, A14, and A17) were detected and expressed to comparable levels in the different infections. These data indicate that expression of hVRK1 and wt B1 fully restored intermediate and late gene expression. Expression of mVRK1ΔΔ, in contrast, only restored the accumulation of intermediate levels of the late proteins F18, L4,

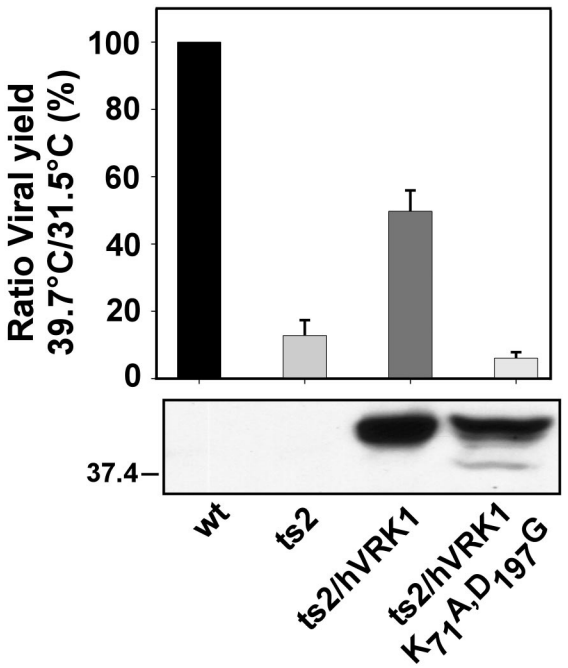

FIG. 8. Complementation activity of hVRK1 requires catalytic activity. A similar experiment to that described for Fig. 6 was performed with wt virus, *ts2*, *ts2*/hVRK1, and *ts2*/hVRK1$_{K71A,\ D197G}$. Cells were infected at an MOI of 2 for 24 h at both 31.5 and 39.7°C. The percentage of virus produced at high versus low temperature is shown graphically; the immunoblot shown below the graph (developed with anti-hVRK1) confirms that both the wt hVRK1 and the catalytically inert mutant were expressed in the appropriate infections.

A14, and A17 (lane 4). As we showed above, the hyperphosphorylated species of H5 was only detected when a wt B1 allele was present (lanes 1 and 5).

The data described above suggest that although hVRK1 restores wt levels of DNA and late protein synthesis, the levels of virus produced are slightly compromised. To address the possibility that the loss of B1 might lead to a block in virion morphogenesis only partially relieved by hVRK1, we examined the proteolytic processing of the major structural components of the virion core (Fig. 9B). This processing occurs during the final stages of virion assembly. Cells were infected at either 31.5 or 39.7°C and metabolically labeled with [^{35}S]methionine at 7 hpi for 60 min. Dishes were either harvested immediately (pulse) or fed with fresh media and harvested at 12 hpi (chase). In wt infections performed at either temperature, nearly complete processing of the p4a, p4b, and pre-L4 precursors (Fig. 9B, lanes 1 and 3) to their mature forms (lanes 2 and 4) was observed. As a negative control, wt infections were performed in the presence of rifampin (lanes 5 and 6), which arrests virion morphogenesis (and hence proteolytic processing) by preventing the correct localization of the D13 protein (12, 42). In cells infected with *ts2*, proteolytic processing of p4a, p4b, and pre-L4 occurred at the permissive temperature, as expected (lane 8); at the nonpermissive temperature, the precursors are not even synthesized due to the block at the prior stage of DNA synthesis (lane 9). Expression of either hVRK1 or mVRK1ΔΔ from the *ts2* genome at 39.7°C restored not only the expression but also the processing of p4a, p4b, and preL4 (lanes 14 and 18). Therefore, the reduced virus yield seen in *ts2*/hVRK1 infections relative to that seen with wt virus does not appear to reflect a block in morphogenesis. Secondly, although late protein synthesis and virus production are somewhat compromised in *ts2*/mVRK1ΔΔ infections, it is unlikely that there is any significant defect in the progression through morphogenesis.

DISCUSSION

Our lab has recently described the classification and properties of a novel family of mammalian protein kinases (VRK1, VRK2, and VRK3) distinguished by their high degree of similarity to the vaccinia virus B1 protein kinase (29a). A natural extension of our work on the structural similarities between these kinases was an inquiry into the functional relatedness of the mammalian and viral kinases. To this end, we generated recombinant viruses in which the m- or hVRK1 genes were introduced into a nonessential locus within the *ts2* genome. *ts2* is a vaccinia virus mutant bearing a defined lesion in the B1 gene that engenders a conditionally lethal defect in DNA synthesis (33). The mammalian kinases were placed under the regulation of an early-late viral promoter and were fused in frame to an epitope tag that facilitated their detection.

Our data showed clearly that ectopic expression of either hVRK1 or any of three mVRK1 isoforms was able to restore viral DNA replication to nonpermissive *ts2* infections (Fig. 4). In fact, the amount of DNA synthesized was comparable to or greater than observed with wt virus. This restoration is specific for B1-like kinases, since expression of a more distantly related cellular kinase, hCK1α, was unable to do so. Overexpression of wt B1 within *ts2* infections led to a more modest restoration of viral DNA synthesis. This partial complementation might reflect the use of an early-late promoter rather than the endogenous early promoter. An alternative explanation is stimulated by the somewhat unusual properties noted earlier for *ts2*. Unlike most *ts* mutants, the severity of the *ts2* phenotype increases at high MOIs and is milder at low MOI (33). This finding suggests that the accumulation of defective B1 may have an inhibitory effect on the viral life cycle. In the *ts2*/B1 recombinant that we generated, it is possible that the *ts2* allele exerts a dominant inhibitory effect that dampens the ability of wt B1 activity to complement the *ts* defect. The mammalian VRK1s appear to be refractory to the inhibitory effects of the *ts2* B1 protein, since VRK1-expressing viruses show accelerated and enhanced DNA synthesis compared to wt infection (Fig. 4B).

We were next interested in defining the mechanism(s) by which VRK1 expression restored viral DNA synthesis. Because we do not know how B1 itself functions during replication, addressing this question was not straightforward. A likely pathway would be the regulation of either the stability or function of known replication proteins by VRK1- or B1-mediated phosphorylation. We have been unable to observe phosphorylation of any of the known replication proteins (E9, A20, D4, and D5) in vivo (Traktman, unpublished) during wt infections and thus could not productively address the question of whether these proteins might be VRK1 substrates. I3, the single-stranded DNA binding protein, is known to be phosphorylated in vivo (35), but its phosphorylation is not compromised in *ts2*-in-

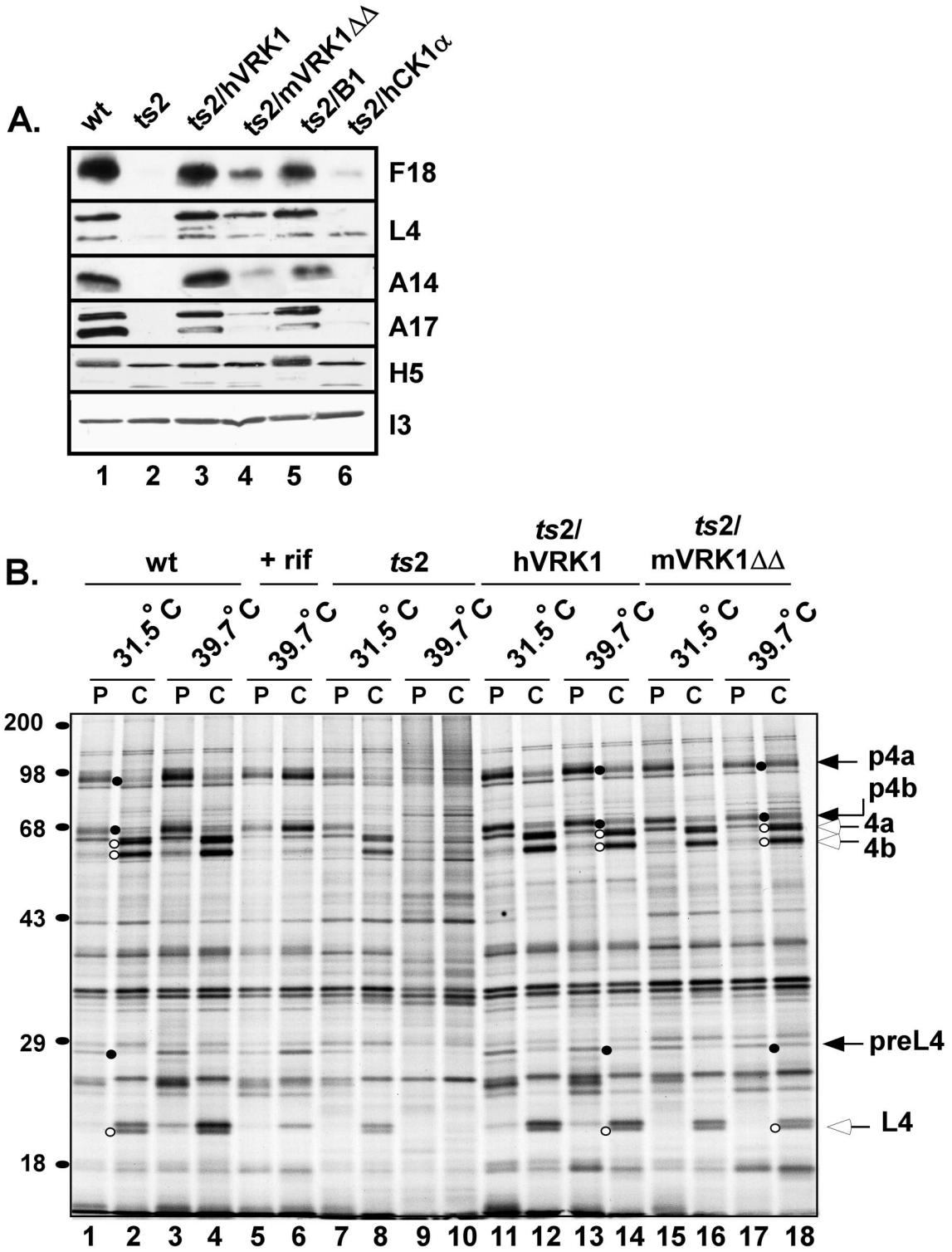

FIG. 9. Analysis of late protein synthesis and processing during infections performed with wt virus, *ts2*, and the *ts2*/VRK1 recombinants. (A) Immunoblot quantitation of select late proteins. BSC40 cells were infected with the viruses indicated (MOI of 5) and incubated at 39.7°C for 7.5 h. Cell lysates were fractionated on SDS–17% polyacrylamide gels; proteins were transferred to nitrocellulose filters which were probed with antisera specific for the viral F18, L4, A14, A17, H5, and I3 proteins. Filters were incubated with the appropriate alkaline phosphatase- or HRP-conjugated secondary antisera, and immunoreactive species were visualized by either colorimetric or chemiluminescent development. (B) Visualization of the profile of late protein synthesis and processing by [^{35}S]Met pulse-chase analysis of infected cells. Confluent 35-mm-diameter dishes of BSC40 were infected with the indicated viruses at the temperatures shown (MOI of 2) and incubated at either 31.5 or 39.7°C. As a control, cells were infected with wt virus and treated with 100 μg of rifampin per ml (+ rif) throughout infection. At 7 hpi, the cells were metabolically labeled with [^{35}S]methionine for 60 min. The cells were either harvested immediately (pulse [P]) or refed with DMEM supplemented with 5% FCS and harvested at 12 hpi (chase [C]). Samples were resolved by electrophoresis on an SDS–10% acrylamide gel and exposed for autoradiography. Solid arrows and circles indicate the precursor forms of major core proteins (p4a, p4b, and preL4); open arrows and circles indicate the proteolytically processed forms (4a, 4b, and L4). Protein standards are indicated on the left, with their masses shown in kilodaltons.

fected cells (Sankar and Traktman, unpublished). In terms of the stability of the replication apparatus, we have not observed diminished levels of any of the known replication proteins under conditions of B1 deficiency (Fig. 5A), suggesting that loss of stability of a key replication component is an unlikely explanation for the *ts2* phenotype. Furthermore, we can conclude that wt B1 is not essential for the assembly of a functional DNA polymerase, since extracts of *ts2*-infected cells retain the ability to direct processive replication of a primed, single-stranded template (32). H5 is the only viral protein for which we can show clearly altered properties when B1 is defective. H5 is an abundant, 34-kDa protein that is expressed at both early and late times postinfection, has been shown to stimulate late transcription in vitro (20), and interacts in two-hybrid analyses with the processivity factors of both the transcriptional and replication apparatus (5, 24). Characterization of a *ts* H5 mutant revealed an unanticipated defect in the early stages of virion morphogenesis (8). H5 colocalizes with known replication proteins (I3 and B1) and nascent viral DNA in vivo (9) and has been shown to be a bona fide substrate for B1 both in vivo and in vitro (3, 4). Phosphorylation of H5 by the viral F10 kinase and by cellular kinases has also been observed (Sankar and Traktman, unpublished). H5 exists in multiple phosphorylation states (2, 4), whose isoelectric points can be distinguished by two-dimensional gel analysis. Some of the modifications cause mobility shifts in one-dimensional gel analysis. Indeed, we observed highly phosphorylated forms of H5 in wt-infected or *ts2*/B1-infected cells (Fig. 5B, lanes 5 and 8) that could not be seen in the absence of functional B1 (Fig. 5B, lane 6). Notably, expression of hVRK1 did not restore the accumulation of the hyperphosphorylated forms of H5 (Fig. 5B, lane 7). These data are important in that they uncouple H5 hyperphosphorylation from the process of efficient viral DNA replication. However VRK1 is restoring viral DNA replication, it is not doing so by affecting the phosphorylation status of H5.

Ectopic expression of wt B1 or the VRK1 proteins does not restore virus production to wt levels. Although *ts2*/B1 forms wt-sized plaques at high temperature, *ts2*/hVRK1 and *ts2*/mVRK1 do not. In all cases, the 24-h yields from single infectious cycles are reduced compared to that seen with wt infections. This impairment may reflect the altered temporal profile of protein expression, i.e., use of a constitutive promoter rather than the endogenous early promoter. Alternatively, these data may reflect additional roles for the B1 kinase, distinct from DNA synthesis per se, that are less well complemented by hVRK1 or mVRK1. Indeed, when cell extracts were prepared at 7 hpi, we observed that late proteins were less abundant after infection with *ts2*/mVRK1 than with either *ts2*/hVRK1 or *ts2*/B1 (Fig. 9A). Kovacs et al. (21) have shown that the B1 protein plays a role in regulating intermediate gene expression, although our studies have indicated that *ts2*-infected cells are not impaired in their ability to direct transcription from a transfected plasmid bearing an intermediate promoter (Traktman, unpublished). Further studies are needed to resolve how B1 participates in intermediate and/or late gene expression, at the level of either transcription or translation. Although hVRK1 and mVRK1 share 86% sequence identity, ectopic expression of hVRK1 leads to the expression of wt levels of late proteins and to the production of significantly more virus.

It is also possible that the inability of *ts2*/hVRK1 and *ts2*/mVRK1 to produce wt levels of infectious virus reflects in a deficiency in viral morphogenesis. As seen in Fig. 9B, the maturation-dependent proteolytic processing of the major structural proteins appeared to occur normally in all of the recombinant viruses tested. Although these data suggest that virion morphogenesis is efficient, it would be difficult to see minor differences that could engender the two- to sixfold reduction in virus production seen with these recombinants.

Given our demonstration of VRK1's ability to complement a B1 deficiency when expressed from within the *ts2* genome, it is worth asking why the endogenous cellular protein is not sufficient to support *ts2* replication. Several explanations are forthcoming. It may be that the levels of endogenous VRK1 are insufficient given the shutdown of host transcription and the diminution of host translation that accompanies infection (31). Intracellular compartmentalization may be a more important factor. Normally, cellular VRK1 localizes to the nucleus, where it is presumably unable to rescue the cytoplasmic replication of vaccinia. However, when the VRK1s are expressed from the viral genome, nascent cytoplasmic transcripts should permit the coordinated translation of VRK1 along with the other components of the viral replication apparatus. Such coupling would facilitate protein-protein and enzyme-substrate interactions prior to the NLS-mediated trafficking of VRK1 to the nucleus.

Previous studies have not clarified whether the key roles for B1 in the viral life cycle are structural or enzymatic, i.e., whether B1 is an integral part of a replication complex or whether it acts transiently by phosphorylating cellular and/or viral proteins which mediate viral DNA synthesis. To address the issue of whether the catalytic activity of B1 is essential, we first attempted to generate a *ts2* recombinant expressing a catalytically null allele of B1 from the TK locus. The allele we chose contains a $D_{167}G$ substitution, which affects an invariant aspartic acid residue predicted to play a key catalytic role by forming a salt bridge with Mg^{2+} ions that serves to orient the γ phosphate of the bound ATP residue (15). Our lab has previously shown that recombinant protein expressed from this allele appears to fold properly but is enzymatically inactive (Sankar and Traktman, unpublished). However, when we attempted to produce the *ts2* $B1_{D167G}$ virus, we found that the endogenous B1 locus had reverted to the wt sequence in all of the isolates that were recovered (data not shown). We concluded that expression of the $B1_{D167G}$ protein was lethal in the context of the *ts2* allele but not in the context of wt B1. Perhaps substrate sequestration by the $B1_{D167G}$ protein cannot be overcome by the impaired and labile *ts 2* B1 but can be overcome by wt B1.

As an alternative approach, we generated a catalytically null allele of hVRK1, placed it within the *ts2* genome (*ts2*/$hVRK1_{K79A,D197G}$), and monitored its ability to complement the conditional lethality of *ts2*. Although we could verify the synthesis and accumulation of this protein, we found that it could not restore viral DNA synthesis, virus production, or plaque formation (Fig. 8 and data not shown). We conclude from these data that the enzymatic activity of hVRK1, and by extrapolation of B1, is essential for its biological function.

It is tempting to speculate that the observed rescue of B1-deficient *ts2* infections by the VRK1 proteins is due to overlapping substrate specificity between the viral and cellular ki-

nases. Either the VRK1s are fortuitously able to phosphorylate virus-encoded protein(s) that function in DNA replication and/or the later stages of the virus replication cycle or both B1 and the VRK1s recognize and phosphorylate cellular proteins which participate in these processes. To date, we have not shown that any of the repertoire of viral proteins involved in viral DNA synthesis are substrates for B1 phosphorylation. An expanded search for relevant B1 substrates is therefore an area of importance for future studies. The VRK1 proteins show robust autophosphorylation activity, unlike the B1 kinase (29a and data not shown). Using heat-killed extracts prepared from either uninfected or vaccinia-infected BSC40 cells as a source of substrates for in vitro kinase assays, B1 and hVRK1 do share the ability to phosphorylate several common cellular and viral proteins (Boyle and Traktman, unpublished). There have also been reports that hVRK1 can phosphorylate fragments of murine p53 (1, 23); however, the functional role of this modification remains to be determined. While we have been unable to confirm the phosphorylation of intact p53 by hVRK1, p53 does indeed appear to be a B1 substrate in vitro (Boyle and Traktman, unpublished). Further analysis of whether VRK1 or B1 intersect with the p53 pathway in vivo and what roles these kinases play in DNA replication or repair deserves attention. Genetic data from other organisms suggest that such a role for VRK1 is quite possible. In *C. elegans*, RNA interference-mediated depletion of a VRK1-related kinase (WormBase ID F28B12.3) causes embryonic lethality characterized by a number of defects in cell division (http://rnai.org). Indeed, this functional relatedness is conserved across species, since a recombinant *ts2* virus ectopically expressing a *D. melanogaster* VRK homolog (gene CG6386, also known as BcDNA: LD09009) can form plaques and produce a robust 24-h virus yield at 39.7°C (data not shown). In *Saccharomyces cerevisiae*, mutation of the gene encoding the most closely related protein kinase (Hrr25) leads to increased sensitivity to HO endonuclease and methyl methanesulfonate as a result of diminished repair (16).

It is worth noting that B1 is highly conserved among all members of the *Poxvirus* family, except Molluscum contagiosum virus (MCV), from which it is conspicuously absent (36, 37). This is particularly intriguing in light of MCV's restricted host range and its inability to replicate in tissue culture. It will be worth addressing the question of whether the absence of B1 plays a role in restricting MCV replication to human keratinocytes and whether the availability of VRK1 in this particular cell population might render them supportive of infection.

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