Vaccinia Virus B1 Kinase: Phenotypic Analysis of Temperature-Sensitive Mutants and Enzymatic Characterization of Recombinant Proteins

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The vaccinia virus B1 gene encodes a 34-kDa protein with homology to protein kinases. In L cells infected nonpermissively with mutants containing lesions in the B1 gene (*ts2* and *ts25*), the infectious cycle arrests prior to DNA replication. In this report, we demonstrate that DNA synthesis ceases when cultures infected with these mutants at 32°C are shifted to the nonpermissive temperature (39.5°C) in the midst of DNA replication. We also show that B1 protein is synthesized transiently during the early phase of infection, even when the progression to later stages of gene expression is prevented. Although wild-type (wt) B1 is stable, the *ts* B1 proteins are markedly labile in both L and BSC40 cells at both permissive and nonpermissive temperatures. These results suggest that the *ts* phenotype of the mutants is complex and may in part reflect a temperature-dependent requirement for kinase activity, an induction of temperature sensitivity in B1 substrates under nonpermissive conditions, and/or *ts* complementation by host factors. To facilitate biochemical analyses, recombinant wt B1, *ts2* B1, and *ts25* B1 were produced in *Escherichia coli*. The wt protein was able to phosphorylate serine and threonine residues on several exogenous substrates in vitro. The activity of *ts25* B1 was 3% that of the wt enzyme, and no detectable kinase activity was associated with *ts2* B1. In light of the inactivity of the *ts2* B1 protein in vitro and its extreme lability in vivo, we attempted to isolate a vaccinia virus B1 null mutant by targeted interruption of the B1 gene at 32°C. No null mutants were isolated. These results indicate that the B1 protein kinase provides a vital function which cannot be supplied by the host or circumvented by incubation at 32°C.

Recent studies of the regulation of biological processes have underscored the importance of posttranslational modification in modulating and coordinating cellular events. Phosphorylation of soluble and membrane-bound proteins on serine, threonine, or tyrosine residues has been found to mediate cell cycle transitions, transduce extracellular signals to the nucleus, and activate or silence components of the replication and transcriptional machinery. The number of protein kinases identified in higher eucaryotes and yeasts has multiplied rapidly, and identification of the relevant genes and preliminary biochemical characterization of the enzymes are well underway. Because identification of kinases in yeasts has most frequently been associated with a genetic assay, insight into the phenotypes associated with the inactivation of particular enzymes is available. However, we have at best an incomplete knowledge of the in vivo substrates of these kinases. Consequently, the manner in which transient phosphorylation directly alters biochemical activities, and as a consequence regulates more distal cellular events, is of pressing interest.

Although the plurality of protein kinases is intriguing, it is also an obstacle to precise analysis of a particular pathway. Elucidating the role of cellular kinases in initiating, maintaining, and completing the events of S phase has been hampered by this complexity. Alternative systems which possess reduced complexity and are amenable to genetic and biochemical dissection therefore offer significant advantages.

Vaccinia virus, which possesses a high degree of genetic and biochemical autonomy from the host cell, is such a system. The life cycle of this virus is characterized by temporally regulated phases of differential gene expression, genome replication, and virion assembly, all of which occur in the cytoplasmic compartment and are mediated by virally encoded enzymes (32, 43). To date, analysis of viral DNA replication has revealed that the virus encodes a DNA polymerase, DNA ligase, topoisomerase I, thymidine kinase, thymidylate kinase, ribonucleotide reductase, and two other proteins identified as essential for DNA replication by the study of temperature-sensitive (*ts*) DNA⁻ mutants (42, 44). The D5 protein is a 90-kDa protein whose mutagenesis induces a fast-stop DNA⁻ phenotype and a deficiency in some aspects of homologous recombination (11, 11a). The predicted sequence of this protein contains a motif associated with nucleotide binding (14) and several motifs identified as acceptor sites for phosphorylation. The predicted amino acid sequence of the 34-kDa B1 protein bears the hallmarks of a serine/threonine protein kinase (14, 18, 45). We have previously shown that mutants containing lesions in the B1 gene have a deficiency in DNA replication whose severity is host dependent (35). Significantly, the lesion in one *ts* allele affects an invariant residue conserved in the vast majority of eucaryotic protein kinases (16, 45).

In this report, we present further phenotypic analysis of vaccinia virus mutants bearing lesions in the B1 kinase. The bacterial overexpression of this protein and the kinase activity of the enzymes encoded by the wild-type (wt) and *ts* alleles are also described. Our results are consistent with a direct or indirect role for the kinase throughout the phase of genome replication. That a cellular activity may partially complement the defect in these mutants is suggested by the

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host range of these *ts* mutants and the minimal in vitro kinase activity retained by the enzymes they encode. Because of the striking homology of many vaccinia virus enzymes to their mammalian counterparts, the existence of an analogous cellular kinase with a potential role during S phase is plausible and worthy of consideration. Our data also suggest that, in the absence of a functional B1 kinase, the target protein(s) may show a temperature-dependent defect which contributes to the *ts* phenotype of the mutants.

MATERIALS AND METHODS

Materials. Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or New England Biolabs, Inc. (Beverly, Mass.). [³⁵S]methionine, ³²P_i, and ³²P-labeled nucleoside triphosphates were obtained from New England Nuclear Corp. (Boston, Mass.). Proteins (unlabeled, pre-stained, and ¹⁴C labeled) for use as molecular weight standards were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). *Hind*III-digested lambda phage DNA and *Hae*III-digested phage φX174 DNA size markers were from New England Biolabs. Protein A-Sepharose and antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.); nitrocellulose was obtained from Schleicher & Schuell (Keene, N.H.). Immunoblots were developed with reagents obtained from Bio-Rad (Richmond, Calif.); determination of DNA sequence by the dideoxy-chain termination method was performed with a United States Biochemical (Cleveland, Ohio) Sequenase kit, version 1.

Cells and virus. Mouse L cells and African green monkey BSC40 cells were provided by Richard Condit; BSC1 cells, the parental line of BSC40 cells, were obtained from the American Type Culture Collection. All cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, N.Y.). wt vaccinia virus (WR strain) was originally provided by Bryan Roberts. The *ts* DNA replication mutants *ts2* and *ts25* were obtained from Richard Condit (5, 6). Viral stocks were grown in L-monolayer or L-spinner cultures and titrated in BSC40 cells according to previously described procedures (11). For virological studies of the mutants, 32 and 39.5°C were used as the permissive and nonpermissive temperatures, respectively.

Quantitation of viral DNA replication. The dot blot filter hybridization technique was used to quantitate the accumulation of viral DNA sequences after infection, as described previously (25, 35). In temperature shift experiments, confluent 35-mm-diameter dishes of mouse L cells were infected with *ts2* and *ts25* at a multiplicity of infection (MOI) of 15 PFU per cell. Infections were initiated at 32°C, and after 30 min the inoculum was removed, the cells were rinsed, and the cultures were fed with DMEM-FCS prewarmed to 32°C. At indicated times, selected cultures were refed with medium prewarmed to 39.5°C and transferred rapidly to the 39.5°C incubator. All subsequent steps, including harvesting the cultures, performing dot blot hybridizations and quantitating the DNA accumulated, were performed exactly as described previously (35).

Preparation of the TrpE-B1 fusion protein. Much of the B1 open reading frame was cloned into a pATH vector to allow production of a TrpE-B1 fusion protein (24). The pATH vectors are derivatives of pBR322 that include 1 kb of the *Escherichia coli trpE* gene under the control of the inducible *trp* promoter. A fragment containing the majority of the B1

gene as well as some upstream sequence, extending from the *Dra*I site (nucleotide -33) to the *Bbv*I site (+794), was excised from a plasmid containing 5.1 kb of sequences from the left end of the genomic *Hind*III B fragment. The fragment was made blunt ended with the Klenow fragment of DNA polymerase I, resolved by agarose gel electrophoresis (cast and run in 1× TAE [40 mM Tris-acetate, 1 mM EDTA]), purified by binding to glass beads (46), and inserted into *Sma*I-digested pATH11 DNA. This construct was used to direct the synthesis of a fusion polypeptide of 67 kDa. The fusion polypeptide contained the amino-terminal 336 residues (37 kDa) of the bacterial TrpE protein, several residues encoded by nucleotides upstream of the B1 ATG, and amino acids 1 to 266 of the B1 protein (30 kDa).

Large-scale preparation and gel isolation of the insoluble fusion protein were accomplished as previously described (11). The fusion protein was then used as an antigen for the generation of a rabbit polyclonal antiserum. The presence of antibodies reactive to the B1 protein was assessed by immunoprecipitation of metabolically labeled extracts (22, 29) and immunoblotting (41).

Metabolic labeling of infected cells. The synthesis and stability of the B1 proteins were assessed by immunoprecipitation of extracts prepared from metabolically labeled cells. Sixty-millimeter dishes of L or BSC40 cells were adsorbed with DMEM containing wt or mutant virus at an MOI of 15 PFU per cell. After 30 min of adsorption, the inoculum was removed and the cultures were rinsed and fed with DMEM-FCS. At selected times, the infected cultures were pulsed with [³⁵S]methionine and then either immediately harvested (for synthesis studies) or chased with complete medium for various periods before being harvested (for pulse-chase studies). The media used for rinsing, feeding, and labeling the cultures were prewarmed to the appropriate temperature, 32 or 39.5°C. Cultures to be labeled were rinsed with methionine-free DMEM and then labeled for 30 min in methionine-free DMEM supplemented with 100 μCi of [³⁵S]methionine per ml. At the designated harvest times, the cultures were placed on ice, drained, rinsed with cold phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄ [pH 7.4]) and disrupted in phospholysis buffer (100 mM NaPO₄ [pH 7.4], 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate) (11). The lysates were clarified by microcentrifugation at 4°C for 20 min and stored at -20°C.

For pulse-chase studies, metabolic labeling was performed at the time of peak synthesis of the B1 proteins: at 32°C, labeling was performed from 2 to 2.5 h postinfection (hpi); at 39.5°C, labeling was performed from 0.75 to 1.25 hpi. At the conclusion of the labeling period, the radioactive medium was removed, the cultures were rinsed and refed with prewarmed DMEM-FCS, and the plates were returned to the appropriate incubator for the designated chase period.

Immunoprecipitation. The lysates described above were thawed and microcentrifuged at 4°C for 20 min to remove cryoprecipitates. Four hundred microliters of lysate, prepared from 0.5×10^6 cells, was diluted with an additional 800 μl of phospholysis buffer, and incubated with 5 μl of preimmune or anti-B1 serum on ice for 4 h. The immune complexes were recovered with protein A-Sepharose as previously described (29). The antigen was liberated from the complex by boiling in protein sample buffer (1% SDS, 1% 2-mercaptoethanol, 50 mM Tris [pH 6.8], 10% glycerol) and

then resolved by electrophoresis through SDS-12% polyacrylamide gels (26). Dried gels were visualized by fluorography (2), and fluorographs were scanned densitometrically (Quick Scan Jr. [Helena Laboratories, Beaumont, Tex.] or LKB Ultrascan XL Enhanced Laser Densitometer [Pharmacia, Piscataway, N.J.]). The relative area under each peak was determined by weight or by computer integration (LKB GelScan XL [Pharmacia]); data were plotted with Sigma Plot software (Jantel Scientific, Corte Madera, Calif.).

Preparation of bacterial overexpression clones. (i) **wt 34-kDa B1 clone.** The site of the initiating ATG codon was altered to an *NdeI* site by polymerase chain reaction (PCR)-directed mutagenesis. PCRs were performed with AmpliTaq recombinant *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.) by using a Bellco DNA Pacer (Bellco, Vineland, N.J.). The template was the 1,100-bp *XbaI* fragment that was used successfully in rescuing *ts2* to the temperature-insensitive phenotype (35). The primers were purchased from Research Genetics (Huntsville, Ala.). The 5' primer, a 32-mer, was 5'-TAGAATTCATATGAACCTTCAAGGACTTGTG-3'; the 3' primer, a 24-mer, was 5'-GAATCGATATTCACATTAATGGA-3'. By using these primers, a 300-bp product was synthesized. This fragment contained a 5' terminal *EcoRI* site (boldface) adjacent to the new *NdeI* site (underlined) and extended to the single *ClaI* site (boldface) found at position +287 within the B1 gene. This PCR product was gel purified, phosphorylated with polynucleotide kinase and ATP, and cloned into the *SmaI* site of linearized pUC19. The *EcoRI-ClaI* fragment was then excised and inserted into a recipient pUC19 clone containing the downstream portion of the B1 gene. This recipient clone contained sequences from the *ClaI* site to a *BstNI* site found 148 bp downstream of the translation termination site. The complete sequence of the cloned PCR insert was obtained by the chain termination method (37) and confirmed to be identical to the genomic sequence. The entire B1 gene, from the engineered *NdeI* site to a downstream *BamHI* site within the vector, was excised from the pUC19 vector, resolved by TAE-agarose gel electrophoresis, and purified on glass beads. This fragment was cloned into a purified pET3a backbone (39) prepared by restriction of the plasmid with *NdeI* and *BamHI*. This construct was used to direct the synthesis of the authentic B1 protein after transformation into competent *E. coli* HMS174.

(ii) ***ts2* and *ts25* mutant 34-kDa B1 clones.** Mutant/wt chimeras were constructed. They featured a minimal fragment containing one of the mutant lesions embedded within the wt gene. The *ts2* fragment extended from the most 5' *HpaI* site (position +21) to the *ScaI* site (+177); the *ts25* fragment extended from the *BglII* site (+635) to the 3' *HpaI* site (+751). The specific lesions were confirmed as follows: *ts2* by monitoring the acquisition of a diagnostic *BglII* site created by the lesion and *ts25* by DNA sequencing (45). All additional sequences derived from the mutants were verified as being the same as that of the wt allele.

Overexpression of the B1 34-kDa proteins. HMS174 cells, transformed with either wt or mutant pET clones, were grown to a density of 4×10^8 cells per ml in the presence of maltose (0.2%) and ampicillin (50 µg/ml). Cultures were then induced by infection with λCE6 at an MOI of 10 PFU per cell. Twenty minutes of adsorption at room temperature was followed by incubation at 37°C with agitation for 90 min. Uninduced cultures were grown under the same conditions but were not infected with λCE6. On occasion, cultures were metabolically labeled for 5 min at the end of the induction period; labeling was performed in M9 minimal medium

supplemented with [³⁵S]methionine (10 µCi/ml) and the remaining unlabeled amino acids. The cultures were centrifuged at $5,000 \times g$ for 5 min to collect bacteria. To prepare soluble extracts, the bacterial pellet was frozen, thawed, resuspended in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 10% sucrose) containing 0.2 mg of lysozyme per ml, and incubated on ice for 30 min. After the addition of Triton X-100 to 0.1%, the extract was clarified by centrifugation and the supernatant fluid was retained. Extracts (1 ml/10 ml of culture) were stored frozen at -20°C. Whole-cell extracts were obtained by resuspending the cell pellet in PBS and lysing the cells in protein sample buffer. Samples were analyzed by electrophoresis through SDS-12% polyacrylamide gels; the gels were stained with Coomassie brilliant blue and, if metabolically labeled, exposed to Kodak XAR5 film. For immunoblot analysis, whole-cell extracts were fractionated on SDS-12% polyacrylamide gels and electrophoretically transferred to nitrocellulose. The anti-B1 antiserum was used at a dilution of 1:500; the second antibody, affinity-purified goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, was used at a 1:3,000 dilution with 4-chloro-1-naphthol as the enzyme substrate.

Kinase assays using wt B1 34-kDa extracts. In vitro kinase assays were performed with the soluble fraction of crude bacterial extracts. Extract sources included uninduced cells containing pET-wt B1, and induced cells containing pET-wt B1, the pET vector, or pET-topoisomerase (44a). The substrates used were 5 µg of α_s-type casein (Sigma), histone H2B, H3, or H1 (Boehringer Mannheim Biochemicals), or 1 µl of rabbit serum. The assay components, in a final reaction volume of 50 µl, were 2 µl of soluble extract, 10 µCi of [γ-³²P]ATP (aqueous, 3,000 Ci/mmol), 5 µM ATP, and substrate in a mixture of 50 mM Tris-HCl [pH 7.4], 10 mM MgCl₂, and 1 mM dithiothreitol. For the determination of divalent cation requirements, the kinase assay conditions were varied first by the omission of any cation and then by the addition of various amounts of a selected cation: 2, 5, and 10 mM MgCl₂; 2 and 10 mM MnCl₂; 2 and 10 mM CaCl₂; or 2 and 10 mM ZnCl₂. To further investigate the nucleotide requirements for the kinase activity, the levels of unlabeled ATP were varied: assays were performed with 0, 5, or 50 µM cold ATP. In a parallel set of assays, ATP (labeled and unlabeled) was replaced by GTP. The reactions were carried out for 30 min at 20°C and stopped by the addition of protein sample buffer. Samples containing casein or histone were analyzed by electrophoresis through SDS-15% polyacrylamide gels; samples containing serum were resolved by electrophoresis through 7 to 15% polyacrylamide gradient or SDS-10% polyacrylamide gels. The gels were dried and examined by autoradiography.

One-dimensional phosphoamino acid analysis. Standard kinase reactions were performed with the wt B1 extract and serum or casein as the substrate. The reaction products were supplemented with bovine serum albumin to attain an estimated protein concentration of 100 µg/ml and then precipitated in 10% trichloroacetic acid. Samples were incubated on ice for 60 min and then microcentrifuged at 4°C for 15 min. The precipitated protein pellets were rinsed with 100% acetone (-20°C) and dried in vacuo. The samples were resuspended in 50 µl of constant-boil HCl (Pierce, Rockford, Ill.), heated at 110°C for >2 h, and lyophilized, and the pellets were resuspended in distilled H₂O. Hydrolyzed samples and phosphoamino acid markers (20 nmol) were spotted on thin-layer cellulose F chromatography plates (EM Separations, Gibbstown, N.J.). Electrophoresis was performed in

pyridine-glacial acetic acid-water (1:10:189) at 2,000 V for 15 min. The plates were dried, sprayed with ninhydrin (Sigma), developed at 110°C, and exposed for autoradiography.

Comparison of wt and mutant B1 kinase activity. In vitro kinase assays were performed to compare the activity recovered from transformants expressing the *ts* B1 alleles with that seen in wt extracts. (i) Extracts from metabolically labeled bacteria containing pET-wt B1, pET-*ts2* B1, or pET-*ts25* B1 were assayed with serum as the substrate at 20, 30, and 40°C. The reaction products were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. (ii) Unlabeled extracts, adjusted to have equivalent amounts of the B1 species (as judged by the accumulation of metabolically labeled protein in parallel inductions), were used in kinase assays with serum as a substrate. Assay temperatures ranged from 0 to 40°C; in selected assays, extracts were preincubated at 40°C for 15 min. The reaction products were fractionated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue and exposed to X-ray film without being dried. The serum substrate band (visualized by both Coomassie brilliant blue staining and autoradiography) was excised from the wet gel and mixed with Biofluor (Du Pont) scintillant, and the radioactivity was quantitated by scintillation counting.

Transformation assays comparing the toxicity of plasmids containing wild-type and mutant B1 genes. Competent *E. coli* HMS174 and BL21(DE3) were transformed with various pET plasmids: pET vector, pET-wt B1, pET-*ts2* B1, and pET-*ts25* B1. The transformants were allowed to grow overnight on Luria-Bertani broth-ampicillin (50 µg/ml) plates, and the colonies were counted manually.

Construction of a B1 null allele by replacing B1 with the Neo^r gene. A pUC9 plasmid containing 5.1 kb of sequences from the vaccinia virus genome, extending from the leftmost border of the genomic *HindIII* B fragment to a downstream *EcoRI* site (35), was cleaved with *HpaI* to release a 731-bp fragment representing 81% of the B1 gene. The neomycin resistance (Neo^r) gene under the direction of the vaccinia virus early/late p7.5 promoter was excised from pVV:NEO (12) by digestion with *SalI* and *XbaI*; the termini were then made blunt with the Klenow fragment of DNA polymerase I. The Neo^r cassette was inserted into the *HpaI* site of the pUC-*HindIII-EcoRI* plasmid in place of the B1 sequences. Two pB1:NEO constructs, in which the Neo^r cassette was oriented in either direction relative to the flanking sequences, were selected. These plasmids retained 367 bp of upstream and 4,029 bp of downstream flanking sequence to facilitate recombination into the vaccinia virus genome. Thirty-five-millimeter dishes of BSC40 cells were infected with wt WR virus at an MOI of 0.03 PFU per cell; at 3 hpi the replicate dishes were independently transfected with 3.5 µg of a calcium phosphate precipitate (11) of either supercoiled or linearized pB1:NEO DNA and shifted to 32°C. G418 (2 µg/ml) was added to the medium at 15 hpi; cultures were maintained at 32°C in the presence of drug until 3 days postinfection. Monolayers were harvested and subjected to two cycles of freeze-thawing to release cell-associated virus. Viral yields were then titrated, and each stock was independently passaged twice through BSC40 cells at 32°C in the presence of G418 to enrich for recombinant progeny. Two rounds of plaque purification were then performed to prepare Neo^r isolates from each of 11 original infections/transfections. Six of these had been transfected with supercoiled pB1:NEO (three in each orientation); five had received linearized pB1:NEO (two in one orientation and three in the other orientation). Stocks prepared from these isolates

were then titrated on BSC40 cells at 32 and 39.5°C, in the presence and absence of G418, to test for a *ts* phenotype. Portions of the stocks were also applied to Zetaprobe membranes (Bio-Rad) with a dot blot apparatus (Bio-Rad); the filters were then hybridized with several radioactive probes prepared by nick translation (11, 36). The probes represented an 8.1-kb fragment derived from the vaccinia virus *HindIII* D genomic fragment, the excised Neo^r gene (without any vaccinia virus sequences), the 731-bp internal *HpaI* fragment deleted from the B1 gene during the preparation of pB1:NEO, and pUC9.

RESULTS

The B1 gene of vaccinia virus is predicted to encode a 300-amino-acid protein whose sequence contains numerous conserved motifs associated with serine/threonine protein kinases (16, 45). Key residues associated with ATP binding and catalysis in other kinases are well conserved in the B1 protein. We have previously identified two codon changes in the B1 gene which confer a temperature-sensitive phenotype on the virus (45). At the nonpermissive temperature, mutant stocks (*ts2* and *ts25*) initiate infection and direct the synthesis of early viral mRNAs and proteins, but DNA replication and the subsequent phases of late gene expression and production of infectious progeny are severely impaired (5, 6, 35).

Characterization of the temporal role of the B1 protein during viral DNA replication. Crucial to an understanding of the role of the B1 protein in the replicative cycle is the determination of whether it is required only to initiate DNA replication or whether it plays an ongoing role during DNA synthesis. To address this question, the impact of shifting *ts2*- or *ts25*-infected cultures to the nonpermissive temperature in the midst of DNA synthesis was assessed. Extracts prepared from infected L cells shifted to the nonpermissive temperature at designated times were applied to filters and hybridized with a genomic probe in order to quantitate the accumulation of viral DNA. Parallel cultures were maintained at 32 or 39.5°C throughout infection; as expected, these cultures showed significant DNA synthesis from 3 to 12 hpi or no DNA accumulation, respectively (Fig. 1). For cultures infected with either *ts2* or *ts25*, a shift from 32 to 39.5°C at either 4 or 7 hpi blocked any further DNA accumulation. The execution point of both mutants thus extends well into the phase of DNA replication, consistent with the B1 protein playing a bona fide role throughout DNA synthesis rather than one limited to early events which regulate the onset of DNA replication. The two mutants differed modestly in their responses: DNA synthesis in *ts2*-infected cultures (Fig. 1A) halted immediately, while *ts25*-infected cultures (Fig. 1B) exhibited a transient increase in the rate of DNA accumulation before levels plateaued (within 30 min postshift). The basis of this phenotypic divergence could reflect differences in the residual activity and/or heat lability of the mutant B1 proteins; these features are addressed below.

The B1 protein is synthesized transiently and early during infections performed with wt virus, *ts2*, and *ts25*. An anti-B1 antiserum was generated in order to investigate the temporal regulation of B1 protein synthesis and to compare expression patterns in wt and *ts* mutant infections. As shown in Fig. 2A, the immune serum (but not the preimmune serum [data not shown]) recognized a 34-kDa polypeptide in extracts prepared from infected cells but not from uninfected cells. (An additional protein species migrating slightly more

A. TS2

B. TS25

FIG. 1. Viral DNA synthesis in *ts2*- and *ts25*-infected cultures shifted from the permissive to the nonpermissive temperature at various times postinfection. Confluent monolayers of L cells were infected at 32°C with *ts2* (A) or *ts25* (B) at an MOI of 15 PFU per cell. At 4 and 7 hpi, selected cultures were shifted to 39.5°C (arrows). At the indicated times postinfection, cells were harvested, and the level of viral DNA within cell lysates was determined by quantitative filter hybridization. The x axis represents the harvest time in hours postinfection; the y axis represents the amount of radiolabeled probe which hybridized to each sample.

slowly than B1 is seen in some immunoprecipitates shown in Fig. 2B. This viral protein is an abundant species which has a tendency to aggregate and can be recovered on protein A-Sepharose beads in the absence of any serum; the relatively long autoradiographic exposures required to adequately visualize B1 are responsible for the detection of this unrelated species.) To determine the temporal profile of B1 synthesis, L cells were infected with either wt virus, *ts2*, or *ts25* at an MOI of 15 PFU per cell and maintained at 32 or 39.5°C. At various times postinfection, cultures were radiolabeled with [³⁵S]methionine for 30 min and harvested immediately. Extracts were subjected to immunoprecipitation analysis with the anti-B1 serum; the recovered immune complexes were fractionated by SDS-PAGE and visualized by fluorography. The fluorographs (Fig. 2B) and densitometric quantitation of the 34-kDa protein species (Fig. 2C) revealed that the temporal pattern of B1 expression is that of a classic vaccinia virus early protein.

At 32°C, synthesis of the B1 protein was found to initiate by 1 hpi, peak at 2.5 hpi, and cease by 4 hpi, concurrent with the onset of DNA replication (data not shown). In time course experiments performed at 39.5°C, the synthesis

curves were displaced to somewhat earlier times, reflecting a faster course of infection. The transient expression of the B1 protein was maintained even in the *ts* mutant infections performed at the nonpermissive temperature, during which DNA replication and subsequent late gene expression fail to occur. This finding is consistent with our observation that the transient synthesis of two other early replication proteins, E9 (DNA polymerase) and D5, is also independent of the progression of infection to DNA replication and late gene expression (11, 29). There appears to be at least a subset of early proteins whose synthesis is regulated independently of the transition to DNA replication and later transcriptional stages, although initial studies of DNA⁻ mutants revealed that the shutoff of some early proteins was impaired at the nonpermissive temperature (5–7, 33).

The *ts2*-encoded 34-kDa protein was found to have an altered mobility, migrating more slowly than the proteins encoded by the wt and *ts25* alleles. Its aberrant mobility can be attributed to the replacement of a Gly residue by an Asp residue. This mutant residue must distort the conformation of the protein sufficiently to affect its migration even under the denaturing conditions of SDS-PAGE. As shown below, this altered mobility was also a characteristic of the recombinant *ts2* B1 protein expressed in *E. coli*.

The B1 proteins encoded by *ts2* and *ts25* are labile during infections of L cells at 39.5°C. One possible explanation for the temperature sensitivity of these mutants is enhanced lability of their B1 proteins at 39.5°C. Pulse-chase experiments were performed with L cells at the nonpermissive temperature to evaluate this possibility. After infection with wt virus, *ts2*, or *ts25*, cultures were pulse-labeled with [³⁵S]methionine for 30 min at the time of peak B1 synthesis (as determined by the time courses described above) and then chased for various times with complete medium. The B1 proteins were immunoprecipitated from cell extracts and analyzed by gel electrophoresis and fluorography. The stabilities of the B1 proteins in L cell infections are illustrated in Fig. 3A. The wt protein was stable over the entire 3-h chase period, whereas the mutant B1 species were unstable, with half-lives of approximately 40 min (Fig. 3A). Since DNA synthesis occurs from 3 to 12 hpi and the synthesis of these proteins ceases by 3 to 4 hpi, the mutant B1 proteins would be virtually absent by the later stages of DNA replication. This observation could offer at least a partial explanation for the temperature sensitivity of these mutants.

The B1 proteins encoded by *ts2* and *ts25* are also labile at 39.5°C in BSC40 cells. BSC40 cells had previously been found to be more permissive than L cells for the mutants at 39.5°C, allowing significant replication of viral DNA (30 to 65% of that seen under permissive conditions at an MOI of 15 PFU per cell) and substantial yields of progeny virus (13 to 15% of that obtained under permissive conditions) (35). It seemed plausible that the mutant B1 proteins might be more stable during nonpermissive infections of BSC40 cells than had been observed in L cells (Fig. 3).

Parallel shift-up experiments were therefore carried out with BSC40 cells. Contrary to our expectations, the mutant proteins were even more labile in BSC40 cells than they had been in L cells; as shown in Fig. 3A, they exhibited half-lives of 20 min. A difference in the stabilities of the mutant B1 proteins in the two cell lines is obviously not a reasonable explanation for the host-dependent severity of the mutants' temperature sensitivity.

The B1 proteins encoded by *ts2* and *ts25* are also labile at 32°C. The pulse-chase experiments were then repeated at 32°C with the expectation that the mutant B1 proteins would

FIG. 2. Temporal pattern of B1 protein synthesis as determined by immunoprecipitation analysis. (A) Specificity of the anti-B1 antiserum. Mouse L cells incubated at 37°C were left uninfected (M) or infected with wt vaccinia virus at an MOI of 15 PFU per cell (VV). Uninfected cells and cells at 1 hpi were metabolically labeled with [35 S]methionine for 60 min and then harvested, and extracts were analyzed by immunoprecipitation with anti-B1 serum (α B1). The total extracts (left panel) and immunoprecipitates (I.P.) (right panel) were fractionated by SDS-PAGE and visualized by fluorography. The relative migration of molecular weight markers is shown in the lane marked MW. (B) The temporal profile of B1 protein synthesis was determined in infections with wt virus, *ts2*, or *ts25* at 32 and 39.5°C. Confluent monolayers of L cells were infected with the appropriate virus at an MOI of 15 PFU per cell. At successive times, cultures were metabolically labeled with [35 S]methionine for 30 min, harvested, and analyzed by immunoprecipitation with the anti-B1 serum. The immune complexes were resolved by SDS-PAGE (samples from wt and *ts2* infections were fractionated on the same gel; those from *ts25* infections were fractionated on a parallel gel) and visualized by fluorography. Only the relevant portion of the gel is shown; the migration of the 29-kDa molecular mass marker is shown at the left (lane MW). The numbers beneath each lane indicate the time (hours postinfection) at which the cultures were harvested. (C) Densitometric analysis of the fluorograph depicted in panel B is shown graphically. The x axis represents hours postinfection; the y axis represents a quantitation of the density of the radiolabeled signal in arbitrary units.

be significantly more stable under permissive conditions. As shown in Fig. 3B, the mutant B1 proteins were quite labile under permissive conditions in both cell lines. The *ts2*-encoded protein was as labile at 32°C as it was at 39.5°C. The stability of the *ts25*-encoded protein increased to an intermediate half-life of 2 h. At 32°C, reasonable amounts of the *ts25*-encoded protein could well be present several hours into DNA replication. This could account for the shape of the DNA accumulation curves observed in the shift-up experiments with *ts25* as described above (Fig. 1B). Upon shifting to 39.5°C, the residual *ts25* kinase may remain functional until it disappears because of accelerated degradation, accounting for the lag in the shutoff of DNA synthesis. That the *ts2* B1 protein and, to a lesser degree, the *ts25* B1 protein, proved to be labile at 32°C was unexpected. Clearly, the temperature-sensitive phenotype of these mutants cannot be ascribed to thermolability of their B1 proteins.

These pulse-chase studies suggest that the mutant B1 proteins would be effectively absent at the later stages of DNA replication in both cell lines under permissive (*ts2*) as well as nonpermissive (*ts2* and *ts25*) conditions. By this definition, *ts2* (and perhaps *ts25*) is closer to being a null allele of the B1 gene than a classical *ts* allele. One explanation for the observed phenotype could be that complementation by a putative host factor would be temperature sensitive. In L cells complementation would be effective only at 32°C, whereas in BSC40 cells complementation would be effective at 32°C and partial at 39.5°C. An alternative explanation for the temperature sensitivity of *ts2* and *ts25* is that the kinase activity is not required at 32°C but is required at 39.5°C. The stability or activity of a putative

substrate could be dependent on phosphorylation at 39.5 but not at 32°C, either because of intrinsic thermodynamic considerations or because of the interference of an antagonizing factor at 39.5 but not at 32°C. In this case, the biological ramifications of the B1 mutations would be indirectly temperature dependent.

Expression of the wt, *ts2*, and *ts25* alleles of B1 in *E. coli*. Given that the sequence of the B1 protein shows strong homology to protein kinases (45), direct investigation of B1-associated kinase activity was of obvious interest. Furthermore, assessing the kinase activity of the B1 proteins encoded by the two *ts* alleles was likely to help in resolving their phenotypes. However, the B1 protein is expressed at low levels for only a brief period during the early phase of viral infection (Fig. 2). As discussed above (Fig. 3), while the wt protein is stable in vivo, the mutant B1 proteins are very labile. These constraints posed a significant hindrance to biochemical analysis of the B1 protein. Attempts to demonstrate B1-specific kinase activity in immune complexes were not successful. Although preimmune serum did not precipitate any detectable kinase activity from cell extracts, kinase activity was recovered when the anti-B1 serum was used in immunoprecipitation assays of either uninfected or infected cell extracts (data not shown). The anti-B1 serum, prepared against a B1 fusion protein containing all of the conserved catalytic domains, presumably recognized abundant host kinases. The low levels of B1 protein might well have been insignificant against this background.

Expression of the B1 proteins in bacteria was chosen as a more favorable strategy for the production of material for biochemical analysis. wt and mutant alleles of the B1 gene were expressed in *E. coli* under the regulation of the T7

FIG. 3. Pulse-chase analysis of wt, *ts2*, and *ts25* B1 proteins. Confluent monolayers of L or BSC40 cells were infected with wt virus, *ts2*, or *ts25* at an MOI of 15 PFU per cell at 39.5°C (A) or 32°C (B). At the predetermined time of peak B1 synthesis (Fig. 2B), cultures were metabolically labeled with [³⁵S]methionine for 30 min and chased with complete medium for 0 to 180 min. At selected times, cultures were harvested, and the amount of radiolabeled B1 protein was determined by immunoprecipitation with anti-B1 antiserum. The immunoprecipitates were resolved by SDS-PAGE, visualized by fluorography, and quantitated densitometrically. A graphic representation of the densitometric analysis is shown. The x axis represents the minutes of chase following the pulse (P); the y axis represents the percentage of B1 protein remaining relative to that measured at the end of the pulse. The vertical arrows indicate the minutes of chase at which half (horizontal arrows) of each pulse-labeled B1 pool remains.

promoter on the pET3a plasmid (39). An *Nde*I site was engineered at the initiating ATG of the B1 genes to enable inducible expression of authentic B1 proteins. The bacterial strain HMS174 was transformed with the various plasmid constructs; the T7 promoters were induced by infection with λ CE6, a phage which directs expression of T7 RNA polymerase while shutting off host gene expression. Cultures were pulsed with [³⁵S]methionine at the end of a 90-min induction period, prior to the preparation of soluble extracts. A representative autoradiogram of fractionated extracts is shown in Fig. 4A. Upon induction of the pET-B1 clones (lanes 3 to 6), a novel band of 34 kDa appeared. The *ts2* protein, shown in lane 5, had the same altered mobility as it did when it was immunoprecipitated from lysates of *ts2*-infected cells (Fig. 2B). As shown by the immunoblot in Fig. 4B, induced pET-wt B1 extracts contained a 34-kDa species reactive with the polyclonal antibody generated to an *E. coli* TrpE-B1 fusion protein. As demonstrated by this immunoblot, the recombinant B1 protein was stable; it accumulated

during the induction, and no breakdown products were detected. By 90 min postinfection, the B1 protein was one of the most abundant bacterial proteins, as assessed by Coomassie brilliant blue staining of fractionated extracts (data not shown). Unfortunately, the B1 proteins were largely insoluble. Nevertheless, the levels of soluble B1 (easily detected in metabolically labeled extracts) were ample for biochemical analysis.

The wt B1 protein is a protein kinase. To determine whether the B1 protein is indeed a protein kinase, as predicted from analysis of its deduced sequence, *in vitro* kinase assays were performed on the soluble fraction of crude bacterial extracts. Extracts were incubated with a test substrate and [γ -³²P]ATP in an appropriate kinase buffer; reaction products were then fractionated on gels and examined by autoradiography. As seen in Fig. 5A, lane 5, a 180-kDa component of rabbit serum was phosphorylated exclusively by the extract prepared from an induced pET-wt B1 strain. This serum protein was a fortuitous substrate discovered when testing whether our previous inability to detect B1-specific kinase activity in immunoprecipitates of infected-cell extracts was due to blocking of activity by the anti-B1 antibody. Rather than blocking the *in vitro* phosphorylation of other substrates, inclusion of serum led to the phosphorylation of this 180-kDa species. The abundant serum protein α -2-macroglobulin, an inhibitor of proteases within the coagulation cascade, is the strongest candidate for this substrate; purified human α -2-macroglobulin (Sigma) comigrated with the serum substrate and was phosphorylated by the B1 kinase *in vitro* (data not shown).

No kinase activity was seen in reaction mixtures containing no extract (Fig. 5A, lane 1), extract from uninduced cultures (lane 4), or extracts from induced bacteria transformed with the pET vector (lane 2) or pET-topoisomerase (lane 3). Casein (Fig. 5B), histone H2B (Fig. 5C), and histone H3 (Fig. 5D) were all *in vitro* substrates for the B1 kinase (lanes 5). (Phosphorylation of casein by the B1 kinase [Fig. 5B, lane 5] is evident above the background level of radioisotope trapped by this substrate in the absence of an exogenous source of enzyme or divalent cation.) Histone H1 was not phosphorylated by the pET-wt B1 extracts (data not shown). Some kinases with serine/threonine specificity, such as Raf-1 (48), Pim-1 (17), and Mos (47), exhibit autophosphorylation, a phenomenon more commonly associated with tyrosine kinases. No autophosphorylation of the B1 protein was observed, although the conditions of our assay may not have favored the detection of such a reaction (approximately 5 μ g of test substrate was used, whereas only nanogram levels of B1 kinase were present in these assays).

To assess the divalent cation and nucleotide requirements for B1 kinase activity, assays were performed in which these constituents were varied. Figure 6A, illustrates results of those assays in which various divalent cations were tested for their ability to support B1 kinase activity. No activity was detected when divalent cations were omitted or Ca²⁺ was utilized. Mg²⁺ and Mn²⁺ supported approximately equivalent levels of kinase activity. Different effects were seen when Zn²⁺ was tested. At the lower concentration of 2 mM Zn²⁺, some B1 kinase activity was observed, albeit less than with Mg²⁺ or Mn²⁺. At the higher concentration (10 mM), although minimal phosphorylation of the 180-kDa serum substrate was seen, a novel species of a lower molecular mass was phosphorylated. Labeling of this smaller protein, however, was retained when the pET-wt B1 extract was replaced by a pET-topoisomerase extract (Fig. 6A, lane 10, *topo) but not when the serum was replaced by

FIG. 4. Expression of the vaccinia virus B1 protein in *E. coli* under the regulation of an inducible T7 promoter. wt and mutant alleles of the B1 gene were cloned into the pET3a vector. HMS174 bacteria bearing these plasmids were infected with λ CE6 to induce production of the authentic 34-kDa B1 proteins. (A) After 20 min of adsorption and 90 min of induction, cultures were pulse-labeled with [35 S]methionine, and soluble extracts were fractionated on an SDS-12% polyacrylamide gel and examined by autoradiography. The lanes contain extract from uninduced cells containing pET-wt B1 (lane 1) or extract from induced cells containing the pET vector (lane 2), pET-wt B1 (lanes 3 and 4), pET-ts2 B1 (lane 5), or pET-ts25 B1 (lane 6). Arrows indicate the induced 34-kDa B1 proteins; the bottom arrow marks the wt and ts25 B1 proteins; the top arrow indicates the altered mobility of the ts2 protein. The migration of the molecular weight standards is shown in the lanes marked MW; their molecular masses are indicated at the left. (B) Whole-cell extracts, prepared from uninduced and induced cultures, were fractionated on an SDS-12% polyacrylamide gel and electrophoretically transferred to nitrocellulose. The filter was incubated with the anti-B1 polyclonal antiserum (α -B1) and developed with a horseradish peroxidase-reactive second antibody. Lanes 7 to 9, extracts from cells containing the pET vector prepared at 0, 45, and 90 min postinduction, respectively; lanes 10 to 12, extracts from cells containing pET-wt B1 prepared at 0, 45, and 90 min postinduction, respectively. The arrow indicates the 34-kDa B1 protein. The migration of the prestained molecular weight standards is shown in lane MW.

casein (data not shown). These data suggest the presence of a bacterial (or serum) kinase which requires high levels of Zn^{2+} for activity and has specificity for a particular component of serum.

The effects of varying nucleotide concentrations and replacing ATP with GTP are illustrated in Fig. 6B. In several assays, the ratio of radiolabeled to cold nucleotide was varied. The B1 enzyme was most active when ATP levels were increased by the addition of 5 μM cold ATP to the assay, despite the accompanying dilution of the radiolabeled ATP species. When a higher level of cold ATP (50 μM) was added, however, reduced ^{32}P labeling of the serum substrate was observed. Presumably, as the nucleotide concentration rose above the K_m of the enzyme-substrate complex for ATP, dilution of the radiolabeled ATP with cold ATP led to a decrease in signal. In a parallel set of assays, the B1 kinase was found to utilize GTP as a phosphate donor, but peak activity was less than that seen for ATP. Activity with GTP was optimal when no cold GTP was added to the reaction, indicating either that the kinase-substrate complex has a lower K_m for GTP than for ATP or that the endogenous GTP level in the extract was higher.

The B1 kinase has specificity for serine and threonine residues. The B1 protein was predicted to be a kinase with serine/threonine specificity (45). Phosphoamino acid analysis was performed on the products of standard in vitro kinase

assays. As shown in Fig. 7, casein was phosphorylated on serine and threonine residues, whereas the serum component (α -2-macroglobulin) was phosphorylated predominantly on serine residues.

The kinase activity of the B1 proteins encoded by ts2 and ts25 is severely impaired but not temperature sensitive. The recombinant B1 proteins encoded by the ts2 and ts25 alleles were analyzed with the hope of illuminating the defects in the ts mutants. During infections of mammalian cells by ts2 and ts25, the mutant B1 proteins were extremely labile, as gauged by pulse-chase analysis (Fig. 3). When expressed in bacteria, however, the mutant B1 proteins were stable. As measured by Coomassie brilliant blue staining of gel-fractionated extracts, the proteins encoded by the wt, ts2, and ts25 alleles accumulated to similar levels (data not shown). When kinase reactions performed with [35 S]methionine-labeled extracts were analyzed by SDS-PAGE and autoradiography, it was observed that the wt and mutant B1 proteins were equally stable under assay conditions (data not shown). However, the ts2 extract had no detectable kinase activity, while the ts25 extract had minimal but reproducible activity, as shown in Fig. 8. The autoradiogram in Fig. 8A shows the phosphorylation of the serum substrate (α -2-macroglobulin) after standard kinase assays were performed with soluble extracts containing equivalent amounts of B1 protein. Substrate bands were excised from a parallel gel and

FIG. 5. pET-wt B1 extracts possess a specific kinase activity which can phosphorylate a variety of substrates in vitro. The products of standard kinase reactions using a variety of extracts and substrates were fractionated by SDS-PAGE and visualized by autoradiography. The substrates used were rabbit serum (A), casein (B), histone H2B (C), and histone H3 (D). Lanes 1, no extract; lane 2, induced pET vector extract; lanes 3, induced pET-topoisomerase extract; lane 4, uninduced pET-wt B1 extract; lanes 5, induced pET-wt B1 extract. In each panel, the arrow indicates the phosphorylated substrate. The molecular weight markers for the gel shown in panel A and the gel shown in panels B to D are shown in lanes MW; their molecular masses are shown at the left.

subjected to scintillation counting. A graphic representation of the radioactivity covalently associated with the substrate is provided in Fig. 8B. The *ts2* extract exhibited no kinase activity above background (<0.5% of wt activity); the *ts25* extract showed approximately 3% of the activity of the wt extract. The *ts25* B1 kinase appeared to be no more temperature sensitive than the wt enzyme; both showed peak activity in assays performed at 20°C. Both kinase activities

were heat labile; wt enzyme function, when assayed at either 20°C or 40°C, was reduced sixfold by preincubation at 40°C, whereas *ts25* B1 function was reduced to background levels by preincubation at 40°C.

The impaired kinase activity of the *ts2*- and *ts25*-encoded enzymes was not a reflection of an increased K_m for ATP, because the addition of higher levels of ATP (up to 50 μ M) in assays performed with extracts expressing the mutant pro-

FIG. 6. B1-specific kinase activity: divalent cation and nucleotide requirements. (A) Various divalent cations were tested for their ability to support B1 kinase activity (induced pET-wt B1 extract) with serum as the substrate source. In the lane marked *topo, induced bacteria carrying the pET-topoisomerase plasmid were used as the extract source. (B) In kinase reactions using pET-wt B1 extract, serum as the substrate source, and 10 mM MgCl₂ as the divalent cation, ATP and GTP were compared as nucleotide cofactors. For each nucleotide, the concentration of cold nucleotide added to the reaction was varied, while the amount (10 μ Ci; 3,000 Ci/mmol) of [γ -³²P]ATP or [γ -³²P]GTP remained constant. The products of kinase reactions were fractionated by SDS-PAGE and visualized by autoradiography; the positions of molecular mass markers are shown at the left of each panel. Only the relevant portion of the autoradiograph is shown.

FIG. 7. Phosphoamino acid analysis of substrates phosphorylated by the B1 kinase. Kinase assays were performed with induced pET-wt B1 extract and the substrate casein (lane 1) or rabbit serum (lane 2) or without substrate (lane 3). The products were precipitated with trichloroacetic acid and then hydrolyzed with hot HCl. The hydrolysates were mixed with phosphoamino acid markers and fractionated by high-voltage electrophoresis on a thin-layer cellulose plate. The markers were visualized after color development with ninhydrin; radiolabeled species were visualized by autoradiography. The dotted wickets indicate the migration of the phosphoserine [ser(P)], phosphothreonine [thr(P)], and phosphotyrosine [tyr(P)] markers. The radiolabeled phosphoserine and phosphothreonine products are indicated by arrows.

teins failed to stimulate their kinase activities (data not shown).

Constitutive expression of the wt B1 protein, but not that encoded by *ts2* or *ts25*, is toxic to *E. coli*. The two bacterial hosts frequently used with the T7/pET overexpression system are the HMS174 and BL21(DE3) strains. Induction of target genes in the HMS174 strain requires infection with λ CE6 and is thus tightly regulated. An alternative approach relies on the BL21(DE3) strain, a strain carrying an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible T7 RNA polymerase gene within an integrated lysogen. Addition of IPTG to the culture medium stimulates expression of the T7 RNA polymerase from the *lacUV5* promoter, which in turn activates the target gene on the resident pET plasmid. Repression of T7 RNA polymerase synthesis in the absence of IPTG is incomplete in BL21(DE3) cells, and as a result there is a basal level of constitutive expression of the target gene. Several genes are of sufficient toxicity when introduced into BL21(DE3) to prevent the survival of any transformants. The topoisomerase gene of vaccinia virus is toxic when cloned for expression into a pET plasmid and transformed into BL21(DE3) cells; mutant alleles of the topoisomerase vary as to how detrimental they are to the host bacterium (31, 44a). With the wt and mutant kinases having

FIG. 8. Quantitation of the activity of the mutant B1 kinases. (A) A portion of an autoradiogram of an SDS-polyacrylamide gel depicts the phosphorylation of the 180-kDa serum substrate after in vitro kinase assays were performed with four extract types (pET-wt B1 [wt], pET-*ts2* B1 [ts2], pET-*ts25* B1 [ts25], and pET-topoisomerase [topo]) at the indicated temperatures. The effect of preincubation of extracts at 40°C on subsequent activity at 20 or 40°C is demonstrated for pET-wt B1 and pET-*ts25* B1 extracts. (B) Graphic representation, from a parallel experiment, of the radioactivity covalently associated with the 180-kDa substrate.

been compared in vitro, BL21(DE3) cells presented an opportunity for in vivo comparison. The effect of the wt and mutant alleles of the B1 gene on the viability of BL21(DE3) transformants was tested. Transformation of the control strain HMS174 by either wt or mutant B1 clones led to viable colonies. The pET-*ts2* B1 and pET-*ts25* B1 constructs could be established in BL21(DE3) bacteria, but the wt pET-B1 plasmid proved toxic (data not shown). Therefore, the toxicities of the B1 expressor plasmids in vivo correlated with the kinase activities of their respective B1 proteins in vitro.

Attempted generation of a B1 null mutant of vaccinia virus at 32°C. Within vaccinia virus-infected cells, the *ts2*-encoded B1 protein was shown to be as labile at the permissive temperature as at the nonpermissive temperature (Fig. 3). This observation stimulated our proposal that the B1 allele of *ts2* was essentially a null mutation. The inability to detect any kinase activity associated with recombinant *ts2* B1 protein strengthened this hypothesis. As a direct test, we attempted to construct a true null mutant—a virus in which the B1 gene had been deleted and replaced by a selectable marker. Were such a mutant viable, it would be expected to have a *ts* phenotype. All manipulations aimed at generating such a virus were therefore performed at 32°C.

BSC40 cells infected with wt vaccinia virus were transfected with a pUC9 construct containing modified *HindIII* B sequences; 81% of the B1 gene was deleted and replaced with the Neo^r gene under the control of a constitutive vaccinia virus promoter (12). Infected cells were transfected with either supercoiled or linearized plasmid DNA and maintained at 32°C under G418 selection until 3 days post-infection. Under these conditions, recombination between homologous sequences on the plasmid and viral genome

should occur. The viral yields from 11 independent infections/transfections were passaged twice in the presence of G418 to enrich for recombinant progeny.

Doubly plaque-purified viral stocks were prepared from each original infection and subjected to titration at 32 and 39.5°C and to genomic analysis by dot blot hybridization. None of the 11 stocks were temperature sensitive for plaque formation. All of the stocks contained the *Neo^r* gene and an intact B1 gene. In the case of transfections performed with supercoiled plasmid DNA, the presence of integrated pUC9 sequences was consistent with a single crossover event leading to a genome containing tandem copies of the B1 gene, one intact and one largely replaced by the *Neo^r* gene. In the case of transfections performed with linearized plasmid DNA, some isolates had a similar structure, suggesting that minor amounts of circular DNA were present in the preparation or that plasmid recircularization had occurred after transfection and prior to recombination. Other isolates had indeed integrated the *Neo^r* gene but did not contain any sequences from the pUC9 backbone, suggesting that some form of nonhomologous recombination had occurred. Taken together, these studies strongly suggest that the B1 gene is essential, even at 32°C in BSC40 cells.

DISCUSSION

The B1 gene of vaccinia virus encodes a 300-amino-acid protein whose sequence contains numerous motifs associated with the catalytic domains of serine/threonine protein kinases (16, 18, 45). Attention was first drawn to this gene because lesions within its sequence were responsible for the *ts* DNA⁻ phenotype of vaccinia virus mutants *ts2* and *ts25* (35, 45). At the nonpermissive temperature (39.5°C), the infectious cycle directed by these mutants in mouse L cells progresses through the transcription and translation of early gene products but arrests prior to DNA synthesis and, as a consequence, fails to activate ensuing phases of gene expression. Using an antiserum directed against the B1 protein, we have shown that the wt and *ts* B1 proteins are synthesized transiently and at low levels during the early phase of the vaccinia virus life cycle. Although synthesis of the protein has essentially ceased when DNA replication commences, the wt protein is stable and would endure throughout the replicative phase. That the function of the B1 protein is required throughout DNA replication and is not restricted to early events which regulate the onset of the replicative phase was suggested by shift-up experiments. *ts2*- or *ts25*-infected L cells cease to accumulate additional viral DNA sequences when they are shifted to 39.5°C in the midst of DNA replication. The impact of B1 on DNA synthesis might be direct or indirect; were it direct, B1 could affect chain elongation and/or the initiation of synthesis on daughter templates. Given the current replication model, in which a single DNA polymerase replicates the entire 192-kb genome from an initiating nick (42, 44), and assuming that the replication fork progresses at 50 to 100 nucleotides per second, one can estimate that approximately 30 to 60 min would be required for synthesis of one-half of the dimeric intermediate. The 30-min lag seen before the cessation of synthesis in the case of *ts25* could therefore be compatible with either aforementioned role. Were the role of B1 in DNA replication indirect, it might affect another ongoing process whose interruption upon the shift to the nonpermissive temperature would in turn depress DNA synthesis.

However, simple interpretation of these results is precluded by pulse-chase studies which probed the lability of

the wt and mutant B1 proteins under various conditions. In infected L cells, the *ts25*-encoded protein was found to have a half-life of 40 min at 39.5°C, the nonpermissive temperature, and 120 min at 32°C, the permissive temperature. The *ts2*-encoded B1 protein exhibited a half-life of 40 min at 39.5°C and 30 min at 32°C. The B1 proteins encoded by these mutants are far more labile under all conditions than the wt protein. The *ts2*-encoded protein, in fact, is as unstable at the permissive temperature as at the nonpermissive temperature. Not only do these studies not provide an explanation for the temperature sensitivity of the mutants, they demand a reinterpretation of the shift-up experiments. Given that synthesis of the B1 protein is maximal at 2.5 hpi and has ceased by 4 hpi at 32°C, and given the 30-min half-life of the *ts2*-encoded B1 protein under these conditions, a maximum of 0.5% of the wt levels of B1 protein would remain at 7 hpi. However, DNA synthesis proceeds efficiently at 32°C until at least 12 hpi, and when cultures are shifted to 39.5°C at 7 hpi, an abrupt cessation of synthesis is observed.

It seems highly unlikely, therefore, that the phenotype observed upon shift-up at 7 hpi reflects an inactivation of a continuously required B1 kinase activity. Other working hypotheses emerge, including temperature sensitivity of unphosphorylated substrates, temperature-dependent antagonism by phosphatases, and temperature sensitivity of complementing host kinases. B1 kinase activity might be unnecessary at 32°C but required at 39.5°C. One might imagine that phosphorylation of a substrate(s) might not be mandatory for its basic activity but might affect its stability, localization (30), or interaction with other proteins (3, 23, 27, 28). An unphosphorylated (or poorly phosphorylated) substrate might function adequately at 32°C but might be incompetent under the altered thermodynamic conditions encountered at 39.5°C. In this case, the cessation of DNA synthesis observed after the shift to 39.5°C would reflect the inactivation of the substrate(s). The apparent requirement for the B1 kinase throughout the phase of DNA replication might be deceptive, reflecting downstream events mediated by its substrate(s).

If the kinase activity is indeed needed transiently, and the impact of late shifts to 39.5°C reflects a cascade phenomenon, then the role of antagonistic phosphatases could play a role in mediating the phenotype of the mutants. If the mutants retain the ability to catalyze some early phosphorylation events, their temperature sensitivity could reflect an increased level of antagonistic phosphatase(s) at the higher temperature. Such a model is an alternative to the one described above in which the substrate(s) displays temperature sensitivity as a result of underphosphorylation. However, we were unable to relieve the temperature sensitivity of *ts2* or *ts25* by inclusion of okadaic acid in the culture medium (data not shown). Okadaic acid acts rapidly to inhibit the cellular protein phosphatases P1 and P2A and has been reported to exert potent effects on cells which include enhancement of nuclear protein phosphorylation, inhibition of mitotic progression, and tumor promotion (4). Neither the severe DNA⁻ phenotype characteristic of *ts2* or *ts25* infections performed at 39.5°C nor the cessation of synthesis observed when cultures were shifted to 39.5°C in the midst of DNA replication was moderated by this treatment. If diminished levels of B1 kinase activity are insufficient to overcome an antagonistic phosphatase, the relevant phosphatase may not be inhibited by the particular drug used. In this regard, it is worth noting that vaccinia virus encodes a serine/tyrosine phosphatase (H1) that is resistant to okadaic acid (15).

A third hypothesis invokes the participation of a complementing host factor. There is precedent for temperature-sensitive complementation of viral mutants by host functions. A *ts* mutant of herpes simplex virus has been shown to bear a point mutation that abolishes ribonucleotide reductase activity; the cellular enzyme complements this defect at 31°C but not at 39.5°C (34). The severity of the phenotypes of both *ts2* and *ts25* at 39.5°C is dependent on the host cell, with the inhibition of both DNA synthesis and viral yield being more complete in L cells than in BSC40 cells (35) or BSC1 cells (data not shown). (BSC1 is the parental line from which the BSC40 subclone was originally chosen for supporting growth and viral infection at 40°C; the similar behavior of the two lines with respect to *ts2* and *ts25* suggests that their primate or epithelial origin is more important than their particular culture history.) Because the lability of the B1 proteins is not diminished in BSC40 cells, this host dependency is more likely to reflect the cellular environment than the intrinsic behavior of the B1 protein. Primate cells have been shown elsewhere to be more thermostable than rodent cells (e.g., reference 19), and BSC40 cells have been found to be somewhat more permissive than L cells for *ts* mutants of vaccinia virus with lesions in an encapsidated DNA-dependent ATPase (20). However, the *ts* phenotype of a mutant defective in the DNA polymerase gene was as severe in BSC40 cells as in L cells (35), suggesting that there is specificity to these host-virus interactions. Eucaryotic cells contain numerous cytoplasmic serine/threonine protein kinases, and complementation by one of these is certainly plausible. In this regard, it is interesting that a human cDNA encoding a putative kinase with significant homology to the B1 gene has been isolated (12a).

Any complementing factor(s) provided by BSC40 cells, whether a kinase or not, might be enriched during certain stages of the cell cycle. To test this hypothesis, cells were deprived of serum to induce quiescence and maintained in serum-free conditions or refed with serum 12 or 6 h or immediately before infection with wt virus or *ts25*. Neither DNA accumulation nor viral yield varied significantly as a result of these manipulations (data not shown). These findings suggest that the abundance or activity of complementing host proteins is unlikely to be tightly associated with a particular stage of the cell cycle.

Because vaccinia virus infection abrogates RNA polymerase II-mediated transcription and because translation of host mRNAs is diminished upon infection (32), complementation would rely on residual levels of a stable cellular protein. The phenotype of the mutants would demand that complementation be effective at 32°C but partial (BSC40 cells) or negligible (L cells) at 39.5°C. Temperature lability, temperature-sensitive activity, or temperature-dependent recognition of the viral substrate(s) of or by the complementing factor could account for the observed phenotype.

To facilitate biochemical analysis of wt B1 and elucidate the properties of the mutant B1 species, recombinant B1 protein was produced in *E. coli* from an inducible T7 promoter. Induction of the wt B1 protein was specifically correlated with the appearance of a novel protein kinase activity within induced cultures. In vitro kinase assays performed with soluble bacterial extracts demonstrated that casein, histone H2B, histone H3, and a 180-kDa component of serum (most probably α -2-macroglobulin) are acceptable substrates for the B1 kinase. Kinase activity relies on a divalent cation and can utilize ATP or GTP as a phosphate donor. The optimum temperature for the activity of the B1 kinase in crude bacterial extracts is 20°C. As predicted by

analysis of the deduced amino acid sequence, the B1 kinase phosphorylates both serine and threonine residues.

The B1 proteins encoded by the *ts2* and *ts25* alleles were also produced in *E. coli*; the mutant proteins were synthesized at the same rate and accumulated to the same level as the wt protein, demonstrating no lability. Either the lability of the proteins in vaccinia virus-infected cells is triggered by their inability to function properly, or, if proteolysis is triggered solely by an altered conformation, the appropriate protease is lacking in *E. coli*. No kinase activity could be detected at any assay temperature in extracts producing *ts2* B1 protein; if the B1 protein does retain enzymatic activity, its relative specific activity is <0.5% of that seen for the wt protein. Although the lesion in the *ts2* allele was not originally identified as affecting a highly conserved domain, the altered amino acid can be considered to be adjacent to the conserved L/I/V residue diagnostic of subdomain 4 (16). Directed mutagenesis of this subdomain in the yeast cyclic AMP-dependent protein kinase was shown to impair kinase activity (13). In *ts2*, a Gly residue is changed to a charged Asp residue, an amino acid which is not found in this position in any of the numerous kinase sequences which have been compiled (16).

The lesion in *ts25* changes an invariant Gly residue in subdomain 9 (16) to a Ser. It is therefore not surprising that the relative activity of the recombinant *ts25* B1 enzyme is only 3% of that observed for the wt kinase. Raising the concentration of ATP in in vitro reactions failed to stimulate the kinase activity of either the *ts2* or *ts25* B1 protein, suggesting that their impairment does not reflect an altered K_m for the nucleotide cofactor. Interestingly, the kinase activity of the *ts25* B1 protein, although greatly reduced, showed no increase in temperature sensitivity relative to the wt enzyme. Previously, *ts* alleles of the transforming tyrosine kinases *src* and *abl* were also found to have reduced kinase activities in vitro which were not temperature sensitive (8, 10). The *ts* transformation seen with these alleles in vivo was ascribed to temperature-sensitive interactions with endogenous host proteins in conjunction with diminished enzymatic activity.

That the results of in vitro kinase reactions were a valid reflection of how the B1 proteins might function in vivo was suggested by investigations of B1 toxicity in *E. coli*. Under conditions in which constitutive expression of the B1 proteins would be expected to occur at a basal level, the wt allele, but neither of the *ts* alleles, was extremely toxic. Because the same plasmid constructs direct the inducible synthesis of equal levels of the three B1 proteins in another host in which basal expression is tightly repressed (HMS174), it follows that toxicity in BL21(DE3) is correlated with kinase activity and not with the synthesis and/or accumulation of high levels of B1 protein.

The inability to associate the *ts2* B1 protein with any kinase activity, coupled with its extreme lability under permissive conditions in vivo, lent credence to the hypothesis that *ts2* represented a null allele of the B1 gene. The inference from such a hypothesis would be that the B1 gene was indeed nonessential at 32°C. However, our unsuccessful efforts to construct a viable virus at 32°C which lacked the B1 gene refuted this hypothesis. We were unable to recover any viral progeny in which the endogenous B1 gene had been interrupted by the targeted insertion of a vaccinia virus-promoted Neo^r gene. All of the progeny recovered had inserted the Neo^r gene either via a single crossover event, which spares the endogenous B1 gene and inserts the entire transfected plasmid at an adjacent site, or by illegitimate

recombination. This strategy for gene interruption has been used repeatedly to isolate vaccinia virus recombinants with functional deletions in nonessential genes (1, 21). The inability to recover deletion mutants has been taken as evidence of the essential nature of a gene (38); this inference has been validated when subsequent studies with inducible constructs have been performed (9).

If the B1 gene is indispensable at 32°C, then the *ts2* allele must retain some vital function. Because of the extreme lability of the *ts2*-encoded protein, this function almost certainly occurs early within the viral life cycle. The *in vitro* kinase studies suggest that the *ts* B1 protein retains no kinase activity. Nevertheless, the *ts2* B1 protein might possess minimal enzymatic activity in infected cells, either at a level below the limits of our detection or with a specificity detected only *in vivo*. Alternatively, the critical role of the B1 protein may not require kinase activity but may involve another property such as the actuation of protein complex formation. Regardless of whether the *ts2* B1 acts early, transduction of the signal it transmits to downstream events may affect any stage of the infectious cycle.

We previously reported that, upon infection of BSC40 cells at 39.5°C, *ts2* and *ts25* directed the synthesis of as much as 60% of control levels of DNA but only 15% of control levels of viable progeny (35). This disparity is in contrast to our studies of viral isolates which contain mutations rendering the viral DNA polymerase resistant to pharmacological inhibitors. In these studies (40, 40a), parallel reduction of viral DNA synthesis and viral progeny have been seen. Moreover, macroscopic plaques which approach normal size are seen when DNA synthesis is reduced to $\geq 15\%$ of control levels by inclusion of drugs to which the virus is largely resistant. In contrast, *ts2* forms pinpoint plaques and *ts25* forms no observable plaques in monolayers of infected BSC40 cells maintained at 39.5°C (data not shown). Taken together, these results suggest that B1 inactivation may impair facets of the viral life cycle distinct from DNA synthesis. If indeed the BSC40 cells provide a factor which complements the defect in DNA synthesis, they may not relieve the obstacle(s) which B1 inactivation erects to other aspects of the infectious cycle.

In summary, these studies demonstrate that the B1 protein of vaccinia virus is a bona fide protein kinase with serine/threonine specificity. They also demonstrate that alteration of amino acid 79 of the protein leads to the loss of any detectable *in vitro* kinase activity and that alteration of amino acid 227 spares only 3% of the wt levels of kinase activity. The phenotype of the *ts* mutants *in vivo* is complex. The mutant B1 proteins are extremely labile *in vivo* under both permissive and nonpermissive conditions and possess minimal or no kinase activity *in vitro*. Nevertheless, a virus containing a true null mutation in the B1 gene could not be isolated at 32°C, suggesting that the proposed complementation by host proteins is not sufficient to allow the production of infectious viral progeny. Multiple substrates for the B1 protein, some of which play a role in DNA synthesis and others of which may participate in other facets of the life cycle, are suggested. The *ts* phenotype of the mutants may only indirectly reflect the inactivation of the B1 gene and more proximally reflect a temperature sensitivity of underphosphorylated substrates. Identification of these substrates should help to unravel the role of the B1 kinase in the vaccinia virus life cycle and to shed light on how cellular kinases may coordinate overlapping but distinct biological processes.

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