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Expression and Functional Analysis of a Baculovirus Gene Encoding a Truncated Protein Kinase Homolog

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Autographa californica nuclear polyhedrosis virus (Ac*M*NPV) potentially encodes a 215-amino acid polypeptide containing 6 out of 11 motifs conserved among eukaryotic protein kinases (Morris *et al., Virology* 200, 360–369, 1994). We examined the expression of this gene, named *pk2*, at the transcriptional and translational levels and the possible role of the gene during baculovirus replication in cell culture and insect larvae. Northern (RNA) blot analysis revealed that *pk2* was transcribed primarily as an early 1.2-kb RNA. Western blot analysis showed that *pk2* was expressed as a 25-kDa protein, PK2, which was present both early and late during virus infection. To examine the function(s) of *pk2*, we constructed a mutant baculovirus, vKINdel, in which one-third of the PK2-coding region was deleted and then compared the characteristics of vKINdel with wild-type Ac*M*NPV and a marker-rescued revertant. The *pk2* deletion mutation had no discernable effect on the number, size, or appearance of plaques, the kinetics of protein synthesis or protein phosphorylation profiles during virus infection of cultured SF-21 cells. Deletion of *pk2* also had no significant influence on the infectivity or virulence of the baculovirus in larval bioassays and the level of occluded virus production was normal. Thus, *pk2* does not appear to have a significant influence on virus replication in the host systems examined.

INTRODUCTION

Phosphorylation is a common mechanism for regulating the activity of enzymes and/or intracellular distribution of proteins. Phosphorylation is involved in the control of a variety of cellular functions including general metabolism, cell cycle progression, hormone response, ion transport, transcription, and translation (for reviews, see Edelman *et al.,* 1987; Hunter, 1987; Hunter and Karin, 1992). The basis of this regulatory mechanism is reversable phosphorylation which is achieved by the interplay of protein kinases and phosphatases. These enzymes usually act on serine and threonine or tyrosine residues and are thus generally grouped into serine/threonine- or tyrosine-specific enzymes (Hunter, 1987). A few, however, are able to phosphorylate or dephosphorylate all three hydroxyamino acids (Featherstone and Russell, 1991; Guan *et al.,* 1991; Millar *et al.,* 1991; Potts *et al.,* 1993; Seger *et al.,* 1991; Sheng and Charbonneau, 1993; Stern *et al.,* 1991) and are separately classified.

Phosphorylation is also known to regulate the replication of some viruses (for a review, see Leader and Katan, 1988) and several lines of evidence suggest that protein phosphorylation has regulatory or structural significance during baculovirus replication. Numerous proteins are phosphorylated during baculovirus infection (Funk and Consigli, 1993; Kelly and Lescott, 1984; Maruniak and Summers, 1981). Among these are viral phosphoproteins such as pp31 associated with the virogenic stroma (Guarino *et al.,* 1992) and a nucleocapsid-associated protein (Vialard and Richardson, 1993); both appear to be essential for virus replication (Guarino *et al.,* 1992; Possee *et al.,* 1992) but the significance of their phosphorylation remains to be established. Phosphorylation of host proteins may also have a role in regulating virus infection (Burma *et al.,* 1994). Protein kinase activities are associated with both the nonoccluded and occluded forms of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) (Miller *et al.,* 1983) and are specifically associated with capsids of *Plodia interpunctella* granulosis virus (Wilson and Consigli, 1985). These kinases are capable of phosphorylating serine and threonine residues and possibly arginine (Miller *et al.,* 1983; Wilson and Consigli, 1985). A novel protein kinase is found in silkworms infected with *Bombyx mori* nuclear polyhedrosis virus (Zemskov *et al.,* 1992). In none of these cases, however, has the protein kinase activity been linked to a specific viral gene product.

Beculoviral genes with the potential to encode proteins involved in phosphorylation or dephosphorylation are known and increase the likelihood that phosphorylation plays a role in viral replication. Two open reading frames (ORFs) in the genome of Ac*M*NPV are predicted to encode protein kinase homologs based on the presence of conserved motifs found among eukaryotic protein kinases. One of these ORFs, *pk1*, located at 4.9 to 6.5 m.u.

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in the Ac*M*NPV genome (Possee *et al.,* 1991) contains all 11 catalytic subdomains of kinases defined by Hanks *et al.* (1988) based on the corrected sequence data (Li and Miller, 1994) and is predicted to be serine- and threonine-specific with a catalytic domain but not a regulatory domain. A homolog of the Ac*M*NPV *pk1* gene is present in the genome of *Lymantria dispar* nuclear polyhedrosis virus and encodes a kinase which can phosphorylate histone 2A *in vitro* (Bischoff and Slavicek, 1994).

A second Ac*M*NPV ORF is predicted to encode a protein with several motifs of eukaryotic protein kinases. This ORF, ORF-215 (Morris *et al.,* 1994) or *pk2* (Ayres *et al.,* 1994), is located at 76.2 to 78.1 m.u. of the Ac*M*NPV genome over 35 kb from the other protein kinase-related ORF. The predicted protein, PK2, contains six signature motifs of serine- and threonine-specific protein kinases. Unless splicing occurs, PK2 is unlikely to have kinase activity because it lacks the first five catalytic subdomains, including the putative nucleotide binding site and catalytic site. An outstanding feature of PK2 is the notable sequence identity to eIF2α kinases which inhibit translation by phosphorylating initiation factor eIF2α.

Ac*M*NPV also contains a gene which encodes a protein tyrosine phosphatase (Hakes *et al.,* 1993; Kim and Weaver, 1993; Possee *et al.,* 1991; Sheng and Charbonneau, 1993). The enzyme belongs to the family of phosphatases which dephosphorylates not only tyrosine residues but also serine/threonine residues *in vitro* (Hakes *et al.,* 1993; Kim and Weaver, 1993; Sheng and Charbonneau, 1993).

Although genes encoding proteins predicted to be involved in protein phosphorylation and dephosphorylation are present in baculoviruses (Bischoff and Slavicek, 1994; Hakes *et al.,* 1993; Kim and Weaver, 1993; Morris *et al.,* 1994; Possee *et al.,* 1991; Sheng and Charbonneau, 1993), none of them have been characterized in terms of their function in viral replication. We were interested in *pk2* of Ac*M*NPV because of its potential role in translational control. In this study, we have characterized the expression of *pk2* by Northern (RNA) blot and Western blot analyses. To determine the function of the gene, we constructed a *pk2*-deletion virus and compared the characteristics of the deletion virus with wild-type Ac*M*NPV and a marker-rescued revertant both in cell culture and in insect larvae.

MATERIALS AND METHODS

Cells, viruses, and insects

Spodoptera frugiperda IPLB-SF21 (SF-21) cells (Vaughn *et al.,* 1977) were grown in TC-100 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. Ac*M*NPV (L1 strain) (Lee and Miller, 1978) and recombinant viruses were prepared and titered on SF-21 cells for infection studies (O'Reilley *et al.,* 1992). *S. Frugiperda* eggs were provided by W. D. Perkins (USDA-ARS, Tifton, GA), and the insects were reared on artificial diet at 27° under a 14:10 hr light–dark cycle.

Construction of vKINZ, vKINZR, and vKINdel

For construction of an Ac*M*NPV mutant with *pk2* disrupted, we first constructed a plasmid containing *Escherichia coli lacZ* within *pk2*. pC4-5, a pBluescript-based deletion plasmid generated for sequencing of *lef-7* (Morris *et al.,* 1994) and containing Ac*M*NPV DNA from approximately 76.2 to 78.2 m.u., was linearized with *Sst*II at a site (at 77.1 m.u.) within the PK2 ORF (nucleotide 1936 in the sequence reported in Morris *et al.* (1994)), filled in with the large fragment of DNA polymerase I (Klenow), and ligated to an HSP70 promoter-driven lacZ cassette generated from plasmid pHS70lacZ (McLachlin and Miller, 1994) by *Bam*HI digestion and then filling in. The resultant plasmid, pKIN2AZ, has 1.4-kb viral sequences flanking each side of the lacZ cassette; the lacZ is in the same orientation as *pk2*. Plasmid pKIN2AZ and wild-type Ac*M*NPV DNA were cotransfected into SF-21 cells (O'Reilly *et al.,* 1992). Supernatant fluids harvested 4 days after transfection were then screened for viruses that formed blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Recombinant virus vKINZ, resulting from a double crossing-over recombination between Ac*M*NPV and pKIN2AZ, was identified by restriction analysis of the viral DNA among those viruses that formed blue plaques.

vKINZ was used to construct a revertant virus, vKINZR, and a deletion virus, vKINdel, in the locus of *pk2*. The *Pst*I-L fragment of Ac*M*NPV was cloned into pUC19 at the *Pst*I site, giving rise to plasmid pPstI-L which contains a wt copy of *pk2*. To create a deletion within *pk2*, pPstI-L was then digested with *Sst*II and *Apa*I; the large fragment (5.5 kb) was isolated, blunt-ended with Klenow, and self-ligated. The resultant plasmid pKIN2AD contains a deletion of 34% of the PK2-coding region. vKINZ was cotransfected with pPstI-L or pKIN2AD into SF-21 cells for construction of vKINZR, the revertant, or vKINdel, the deletion mutant. vKINZR and vKINdel were screened as viruses which formed white plaques in the X-gal plates. The structures of vKINZR and vKINdel were confirmed by restriction analyses of the viral DNA. Structures of recombinant viruses were also verified by Southern (DNA) blot analysis using *Pst*I-digested viral DNA and a nick-translated pPstI-L probe.

Metabolic labeling and SDS–polyacrylamide gel electrophoresis

In vivo radiolabeling of proteins with [^{35}S]methionine and [^{35}S]cysteine was carried out in SF-21 monolayers (10^6 cells/35-mm tissue culture dish) mock-infected or infected with virus at a multiplicity of infection (m.o.i.) of 20 plaque forming units (PFU) per cell. After virus

adsorption for 1 hr, the inoculum was replaced with fresh TC-100 complete medium. The infected cells were then incubated at 27°; the beginning of the incubation was considered as zero time p.i. Two hours before each time point, the medium was replaced with methionine- and cysteine-free TC-100 medium. One hour before the time point, the medium was replaced with 0.5 ml fresh methionine- and cysteine-free TC-100 supplemented with 25 μCi Tran[^{35}S] label (ICM Biomedicals, Irvine, CA). The labeling medium was removed 1 hr later; the monolayers were washed three times with PBS (1 m*M* Na_2HPO_4, 10.5 m*M* KH_2PO_4, 140 m*M* NaCl, 40 m*M* KCl, pH 6.2) and harvested with 50 μl lysis buffer (50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 1% NP-40, 1 m*M* phenylmethylsulfonyl fluoride, 40 μ*M* pepstatin, 20 μ*M* leupeptin). Protein samples (5 μl) were separated by electrophoresis on 12% SDS–polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue R-250 and the radiolabeled proteins were detected by fluorography (Bonner and Laskey, 1974).

For *in vivo* radiolabeling of phosphoproteins, SF-21 monolayers were similarly infected with viruses as described above in 24-well tissue culture plates (1.5×10^5 cell/well). Cells were starved for phosphate by incubation in phosphate-free TC-100 medium for 1 hr and then incubated in 0.2 ml phosphate-free TC-100 medium supplemented with 20 μCi and 10 μCi [^{33}P]orthophosphoric acid (NEN, Du Pont, Wilmington, DE) for 1 and 6 hr, respectively. At the end of the 1- or 6-hr labeling period, cells were washed with cold PBS and harvested in 50 μl lysis buffer containing 50 m*M* EDTA. A portion of the lysates was run on 12% SDS–polyacrylamide gels for 1-hr labeling samples and 10 and 14% SDS–polyacrylamide gels for 6-hr labeling samples, and the gels were subjected to fluorography.

RNA isolation, Northern blotting, and primer extension analysis

Total RNA was isolated by the guanidinium isothiocyanate method (David *et al.*, 1986) from SF-21 cells as previously described (Li *et al.*, 1993).

For Northern blot analysis, 10 μg total RNA was denatured by glyoxalation (McMaster and Carmichael, 1977), loaded onto a 1.0% agarose gel, and transferred to nylon blotting membranes. The blot was hybridized to a strand-specific RNA probe using the same conditions as previously described (Li *et al.*, 1993). For construction of a *pk2*-specific probe, the full-length PK2-coding fragments were amplified from a *pk2*-containing plasmid pBSXBglII by polymerase chain reaction (PCR) with primers 2AKIN5 and 2AKIN3. 2AKIN5 (5′-GCCGGATCCAACATGAAACCCGAACAATTGG-3′) and 2AKIN3 (5′-CGCGAATTCGGTCGCTTCTAAATCACC-3′) contain sequences, as underlined in parentheses, corresponding to those near the N- and C-terminal PK2-coding regions (Morris *et al.*, 1994). The amplified fragments were cleaved with *Bam*HI and *Eco*RI, and ligated to *Bam*HI- and *Eco*RI-digested pBluescript KS vector (Stratagene, La Jolla, CA). The resultant plasmid, pKS-KIN2A, was then linearized with *Bam*HI and transcribed in the presence of [^{32}P]UTP with T3 RNA polymerase.

Primer extension analysis was carried out as described (Li *et al.*, 1993) using total RNAs described above and a radiolabeled oligonucleotide (5′-CTCGTTTAGAGGCGACG-3′). A DNA sequencing ladder was generated from a *pk2*-containing plasmid using the same radiolabeled oligonucleotide primer.

Preparation of GST-PK2 fusion protein, antibody production, and Western blot analysis

PK2-coding fragment was amplified from a plasmid containing *pk2* by PCR with primers 2AKIN5 and 2AKIN3, and cloned to the glutathione S-transferase (GST) fusion vector pGEXK-2TK (Pharmacia, Piscataway, NJ) at *Bam*HI and *Eco*RI sites to give rise to plasmid pGEXK-KIN. The plasmid was transformed into XL-1 blue *E. coli* (Stratagene), and expression of the GST-PK2 fusion gene was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) as described (Smith and Johnson, 1988). Cell lysates were separated on 10% polyacrylamide gels containing sodium dodecyl sulfate (Laemmli, 1970); the band containing overexpressed GST-PK2 fusion proteins was excised from the gel and used to immunize two rabbits according to standard procedures (Harlow and Lane, 1988). For Western blot analysis, extracts of cells infected with wild-type Ac*M*NPV or vKINdel were prepared as described (O'Reilly *et al.*, 1992), separated on a 10% SDS–polyacrylamide gel, and electrotransferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA). The membrane was probed first with polyclonal antibodies against GST-PK2 at a dilution of 1:5,000 and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobin G secondary antibody at a dilution of 1:25,000. The antibody conjugates were detected using an enhanced chemiluminescence Western blot kit (Amersham, Buckinghamshire, England).

Production of polyhedral inclusion bodies (PIBs) and larval oral bioassays

For preparation of PIBs, fifth instar *S. frugiperda* were injected with 2×10^5 PFU of wt Ac*M*NPV, vKINZR, or vKINdel. Larvae were collected upon death and homogenized in distilled H_2O. The homogenate was filtered through a 114-μm PE macrofilter (Spectrum Medical Industries, Inc., Los Angeles, CA) and centrifuged at 5000 *g* for 10 min. The pellets were sequentially washed with 0.5% SDS and 0.5 *M* NaCl and then resuspended in distilled H_2O. The yield of PIB was determined based on total PIBs from 24 to 27 larvae pooled for each virus.

For oral bioassays, neonate *S. frugiperda* were al-

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  1 MKPEQLVYLNPRQHRIYFASPLNEYMLSDYLKQRNLQTFAKTNIKVPADFGFYI
                              VI                    VII
 55 SKFVDLVSAVKAIHSVNIVHHNINPEDIFMTGPDFDLYVGGMFGSLYKTFIKNN

109 PQNITLYAAPEQIKKVYTPKNDMYSLGIVLFELIMPFKTALERETTLTNFRNNV
      VIII              IX                  X
163 QQMPASLSQGHPKLTEIVCKLIQHDYSQRPDAEWLLKEMEQLLLEYTTCSKKL
                        XI
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FIG. 1. The predicted amino acid sequence of PK2 (Morris *et al.*, 1994). Kinase subdomains VI to XI, as defined in Hanks *et al.* (1988), are indicated. Residues that are conserved in kinases are in bold type. Note that there is an extra amino acid residue between two conserved residues D and N in subdomain VI of PK2 as compared to other kinases. Subdomain X of protein kinases has no rigorously conserved residues and the approximate position is indicated by dots.

lowed to feed for 24 hr on artificial diet containing PIBs at various concentrations. The larvae were then transferred to uncontaminated diet, incubated at 27° in a 14:10 hr light–dark cycle, and monitored for mortality at 12-hr intervals. LC_{50} and LT_{50} were determined by probit analysis (Daum, 1970) from mortality data of 60 larvae per dose, which ranged from 5×10^4 to 2×10^7 PIBs/ml diet.

RESULTS

Based on its nucleotide sequence, *pk2* was predicted to encode a truncated eIF2α kinase homolog (Morris *et al.*, 1994). Database searches using the BLAST program (Altschul *et al.*, 1990) revealed that PK2 was most closely related to heme-regulated eIF2α kinase of rabbit reticulocytes (Chen *et al.*, 1991) and *S. cerevisae* protein kinase GCN2 (Roussou *et al.*, 1988), which is also an eIF2α kinase (Dever *et al.*, 1992), with Poisson probabilities of 1.2×10^{-12} and 1.2×10^{-8}, respectively. PK2 is considerably smaller than other protein kinases; it contains only a portion of the conserved kinase domain (i,e., it contains only subdomains VI to XI (Hanks *et al.*, 1988)) (Fig. 1) and, unlike most protein kinases, has no regulatory domain. PK2 has a predicted molecular mass of 24,979 Da.

Construction of mutant baculoviruses deficient in PK2 production

To determine if *pk2* was essential for baculovirus replication, we constructed a mutant virus, vKINZ, in which *pk2* was disrupted by a 4.5-kb HSP70 promoter-driven lacZ cassette. It was expected that an allelic replacement between Ac*M*NPV DNA and pKIN2AZ, a plasmid containing a lacZ-disrupted *pk2*, would result in an insertion of the lacZ cassette in the Ac*M*NPV genome at the *Sst*II site (77.1 mu) within the PK2 ORF (Fig. 2A). *Pst*I digestion of viral DNAs followed by Southern (DNA) blot analysis using PstI-L as a probe showed a 3.1-kb fragment L in Ac*M*NPV and a 7.6-kb version as expected in vKINZ (Figs. 2B and 2C). The ability to generate vKINZ suggested that the *pk2* was not essential for virus replication in SF-21 cell culture.

We also constructed a *pk2*-deletion virus, vKINdel, which contains a 222-bp deletion in the *pk2* coding region, encompassing the catalytic subdomains VI and VII of the putative protein kinase (Fig. 2A), to eliminate any possible influence lacZ expression might have on virus replication. To control for the possibility that a second site mutation might have occurred during the generation of vKINZ, we also constructed its revertant form, vKINZR (see Materials and Methods). The structures of vKINdel and vKINZR were confirmed by restriction mapping (Fig. 2B) and Southern (DNA) blot analysis (Fig. 2C) which showed a 3.1-kb fragment L in vKINZR and a 2.9-kb ver-

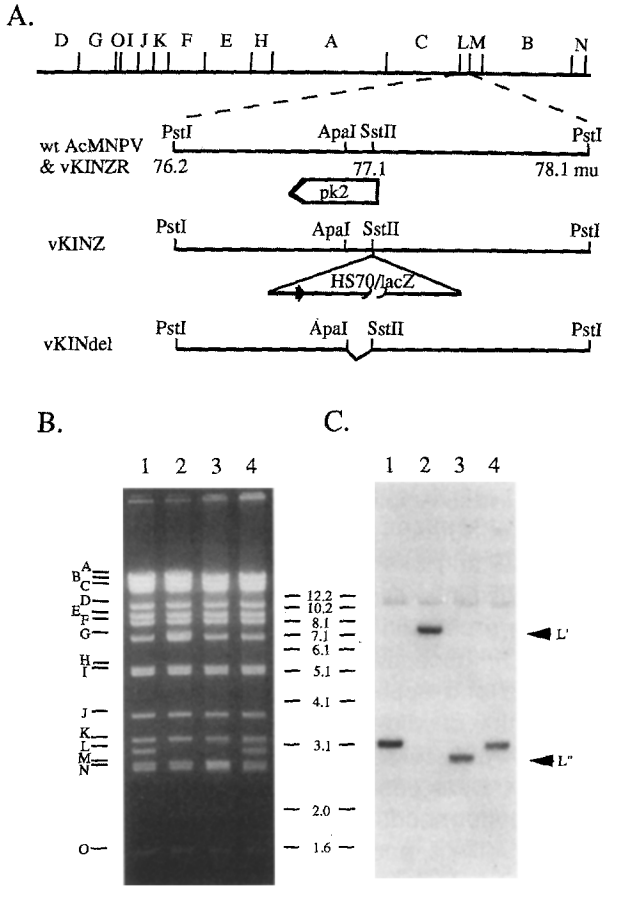

FIG. 2. Organization and characterization of wild-type Ac*M*NPV and recombinant virus genomes. (A) The *Pst*I restriction map of Ac*M*NPV genome is at the top. The dashed lines indicate the enlargement of *Pst*I-L fragment with the position of *pk2* indicated by an open arrow. The two mutant virus genomes are diagrammatically shown below. Map units are given for the wild-type Ac*M*NPV fragment and key restriction sites are indicated. Compared to wild-type Ac*M*NPV, vKINZ had a *lacZ* (directed by an hsp70 promoter designated with an arrowhead) insertion in *Sst*II site and vKINdel had a deletion in *pk2* from *Apa*I to *Sst*II. (B) Restriction analysis of viral DNAs. DNAs isolated from Ac*M*NPV (lane 1), vKINZ (lane 2), vKINdel (lane 3), and the revertant vKINZR (lane 4) were digested with *Pst*I, electrophoretically separated, and stained with ethidium bromide. *Pst*I fragments are indicated with letters to the left. (C) Southern (DNA) blot analysis of viral DNAs. The samples in (B) were blotted and hybridized to radiolabeled, nick-translated pPstI-L. Lanes 1 to 4 are the same as those in panel B. The band designated L′ resulted from insertion of the lacZ cassette into *Pst*I-L; the band designated L″ resulted from a deletion in *Pst*I-L. The DNA markers are indicated in kilobases.

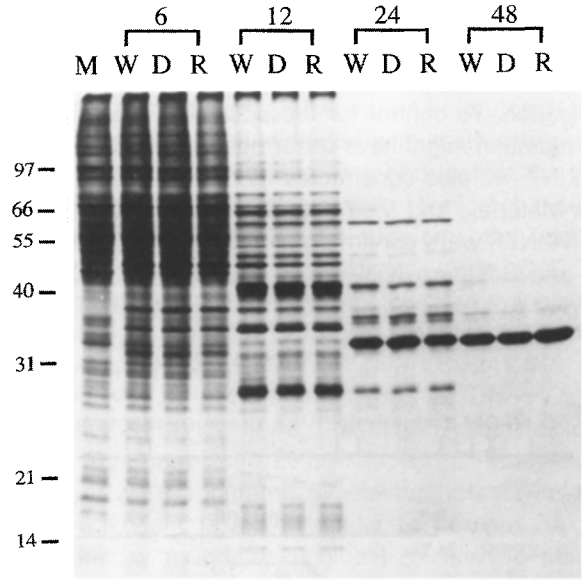

FIG. 3. Kinetics of protein synthesis in Ac*M*NPV-, vKINdel-, and vKINZR- infected cells. SF-21 cells were mock-infected (lanes with M on the top) or infected with the viruses indicated at the top (W for wt Ac*M*NPV, D for vKINdel, and R for vKINZR). The cells were radiolabeled with methionine and cysteine for 1 hr and then harvested at the time shown at the top of lanes. Equal volumes of cell lysates were electrophoresed and the gel was subjected to fluorography. The sizes of protein markers are indicated in kilodaltons to the left.

sion in vKINdel. Amplification of *pk2* locus by PCR with primers 2AKIN5 and 2AKIN3 generated a 0.7-kb product from vKINZR and wild-type Ac*M*NPV DNA and only a 0.5-kb product from vKINdel DNA (data not shown), further confirming a deletion and restoration of *pk2* in vKINdel and vKINZR, respectively.

The size and appearance of vKINdel plaques were similar to that of wild-type Ac*M*NPV plaques both in SF-21 and TN-368 cells (data not shown). No significant differences were observed in the yields of budded viruses and polyhedrin occlusion bodies (PIBs) among Ac*M*NPV, vKINZR, and vKINdel from SF-21 cell cultures (data not shown).

Effect of *pk2*-deletion on protein synthesis and phosphorylation during virus infection in cell culture

To examine if deletion of *pk2* from Ac*M*NPV would affect protein synthesis and/or phosphorylation, we studied the kinetics of protein synthesis and phosphorylation during viral infection. To examine protein synthesis, SF-21 cells were mock-infected or infected with Ac*M*NPV, vKINdel, and vKINZR, and pulse-labeled with radiolabeled methionine and cysteine for 1 hr at various times p.i. As shown in Fig. 3, no significant difference could be discerned among these three viruses as far as temporal appearance of virus-induced proteins and shut-off of host proteins. In addition, most, if not all, rates of protein synthesis are comparable among the viruses according to intensities of radiolabeled protein bands. Similarly, no significant difference in protein synthesis kinetics was consistently observed in TN-368 cells infected with those viruses (data not shown).

To examine protein phosphorylation, we radiolabeled cells with [^{33}P]orthophosphoric acid. Infected-SF-21 cells were pulse-labeled at various times for 1 hr (Fig. 4A) or 6 hr (Figs. 4B and 4C), respectively, in order to detect transient and stable phosphoproteins of different classes. Phosphoprotein profiles of radiolabeled cell lysates are shown in Fig. 4. The patterns of phosphoproteins were indistinguishable between wild-type Ac*M*NPV-infected cells and vKINdel-infected cells (quantitation of the gels using a phosphoimager revealed no significant differences between wild-type Ac*M*NPV and vKINdel in the overexposed protein bands shown in Fig. 4 (data not shown)).

Northern (RNA) blot and primer extension analysis of *pk2* expression

To determine if and when the truncated eIF2α kinase-like gene is transcribed, we carried out a Northern blot analysis using a *pk2*-specific riboprobe (see Materials and Methods). The probe detected a major transcript of 1.2 kb, with a maximum level at 6 hr p.i., which was not present in mock-infected cells (Fig. 5). The level of the 1.2-kb transcript decreased dramatically at 12 hr p.i. and was barely visible at 24 hr p.i. (Fig. 5, lanes 12, 24). Treatment for 12 hr with aphidicolin, a DNA polymerase inhibitor, slightly increased the level of the transcript at 12 hr p.i. (Fig. 5, compare lanes 12 and A) while treatment for 12 hr with cycloheximide, a protein synthesis inhibitor, dramatically increased the level of the transcript (Fig. 5, compare lanes 12 and C).

The temporal pattern of the 1.2-kb RNA observed by Northern blot analysis is consistent with primer extension analysis which revealed that the 5′ end of transcription started at a site 29 bp upstream the initiation codon of *pk2* and 23 bp downstream of a putative TATA box (Fig. 6). The size of the 1.2-kb transcript is consistent with a predicted transcript covering a 932-bp region extending from this 5′ end to a putative polyadenylation signal (AATAAA) 252 nucleotides downstream the PK2 ORF. Thus, *pk2* appears to be transcribed primarily as an early 1.2-kb RNA. The nature of the larger late transcripts of 1.6 and 2.0 kb detected at 12 and 24 hr p.i. (Fig. 5, lane 12, 24) was not determined.

Western blot analysis of *pk2* expression

To study expression of *pk2* at the protein level, we obtained an antibody which recognized PK2 for use in Western blot analysis. The PK2-coding region was PCR amplified and cloned into a bacterial expression vector to direct PK2 synthesis as a fusion protein to glutathione-S-transferase (GST-PK2). The overexpressed GST-PK2,

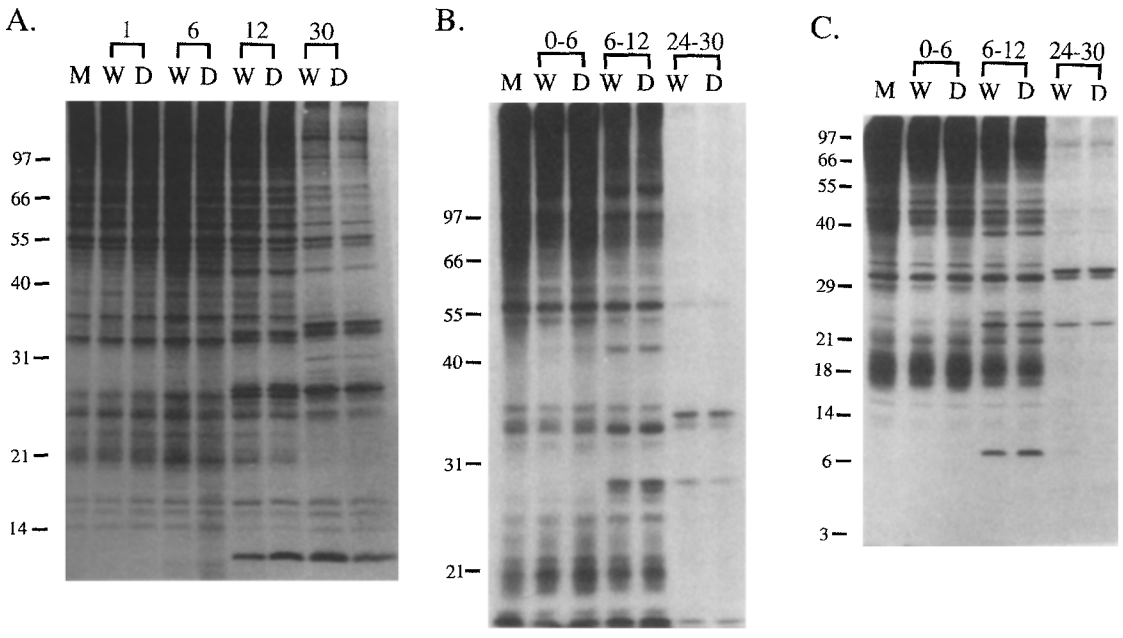

FIG. 4. Phosphoprotein profiles of Ac*M*NPV- and vKINdel-infected cells. SF-21 monolayers were mock-infected (lane M) or infected with Ac*M*NPV (lanes under W) or vKINdel (lanes under D). At selected times, the cells were labeled with [^{33}P]orthophosphoric acid for 1 hr (A) or 6 hr (B and C). For the shorter pulse labeling, cells were starved of phosphate for 1 hr before labeling and were collected at the time indicated on the top of lanes (A). For the longer pulse labeling, cells were starved of phosphate for 1 hr followed by labeling for the period indicated on the top of lanes (B and C) and then harvested. Equal fractions of each cell lysate were analyzed by SDS–PAGE (12, 10, and 14% polyacrylamide for A, B, and C, respectively) and fluorography. The positions and sizes (in kilodaltons) of protein markers are indicated at the left.

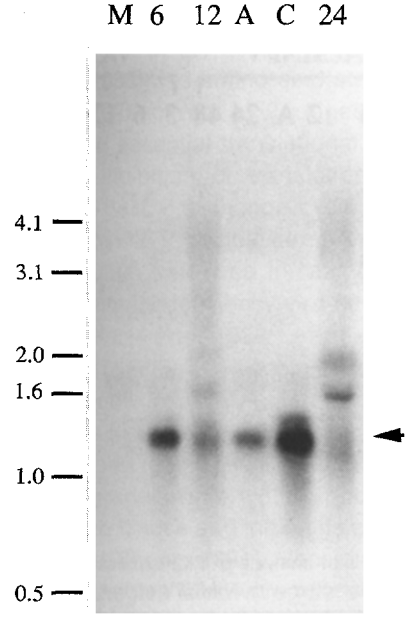

FIG. 5. Northern blot analysis of *pk2* transcripts. Mock-infected (lane M) or Ac*M*NPV-infected SF-21 cells were collected at 6, 12, 24, hr p.i. (lanes 6, 12, 24, respectively) and total RNA was isolated. The RNA was denatured with glyoxal, electrophoretically separated on 1.0% agarose gel, transferred to a nylon blotting membrane, and hybridized to a *pk2*-specific riboprobe as described under Materials and Methods. Lane A contains RNAs isolated from aphidicolin-treated cells at 12 hr p.i. while lane C contains RNAs isolated from cycloheximide-treated cells at 12 hr p.i. The major 1.2-kb transcript is indicated with an arrowhead. Size markers are given in kilobases at the left.

which was insoluble in bacteria, migrated in SDS–polyacrylamide gels to a position of about 52 kDa as expected for the fusion of 27-kDa GST and 25-kDa PK2 (data not shown). Rabbit anti-GST-PK2 antisera were obtained using the fusion protein and the antisera were used to detect PK2 on Western blots of lysates of SF-21 cells mock-infected or infected with wild-type Ac*M*NPV or vKINdel.

As shown in Fig. 7, a protein of about 25 kDa was detected in Ac*M*NPV-infected but not mock-infected cells with the PK2 antisera. The size of the protein is in good agreement with the predicted size of PK2. The 25 kDa protein was not present in vKINdel-infected cells (Fig. 7, lanes under vKINdel), strongly supporting the view that the 25-kDa protein is the product of PK2. The 25-kDa protein was detected as early as 3 hr p.i. (when immunoblots are overexposed (data not shown)) and was maximal at 12 hr p.i. Compared to the Northern blot data (Fig. 5), the maximal level of PK2 appears to be delayed several hours after maximal levels of *pk2* transcripts are observed. Treatment of SF-21 with aphidicolin followed by viral infection showed no inhibitory effect on the level of 25-kDa protein, reflecting again the "early" nature of *pk2* transcription despite the relatively "late" time that maximal levels of PK2 are observed.

Several other proteins appear to cross-react with the antisera to GST-PK2. Two of these, approximate 31 and 43 kDa, are also present in mock-infected cells. Two other cross-reacting products, approximate 61 and 32

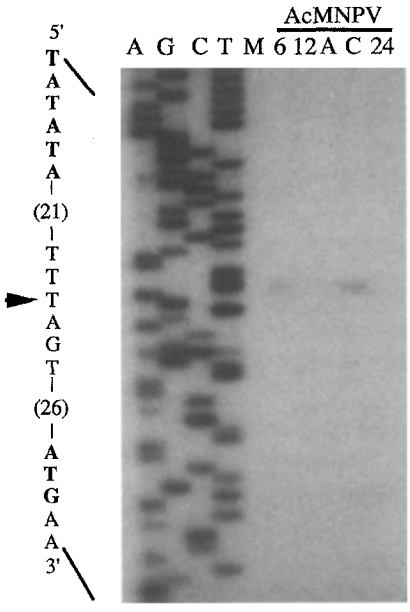

FIG. 6. Primer extension analysis of the 5′ end of pk2 mRNA. A radiolabeled oligonucleotide (see Materials and Methods) was hybridized to total RNAs from mock-infected cells (lane M) or Ac*M*NPV-infected cells (lanes under Ac*M*NPV are same as those described in legend to Fig. 5) and reverse transcribed. The DNA ladder was generated from a plasmid using the same oligonucleotide primer in the presence of dideoxyribonucleotides ddTTP (lane A), ddCTP (lane G), ddGTP (lane C), or ddATP (lane T). The transcriptional start site is indicated with an arrowhead in a region specified by the nucleotide sequence of DNA strand in the same sense as pk2 mRNA. On the left, key sequences including a putative TATA box and the first ATG codon of *pk2* are in bold type; the nucleotide spacing between key sequences is indicated between the dashes.

kDa, appear around 24 hr p.i. The 61-kDa polypeptide is present in both wild-type Ac*M*NPV- and vKINdel-infected cells. The 32-kDa protein is likely to be polyhedrin, which tends to cross react with many different antisera on Western blot analysis (general observation). Other proteins of 23 and 22 kDa are also observed in the 48-hr lane of wild-type Ac*M*NPV-infected cells. We think these may be polyhedrin-related polypeptides; their levels tend to vary among preparations, but we cannot exclude the possibility that they are PK2-related proteins at this time. A 35-kDa polypeptide appeared to cross react with the secondary antibodies (horseradish peroxidase-conjugated anti-rabbit Ig) supplied with ECL Western blot kit from Amersham (Fig. 7) but did not cross react with the secondary antibody (alkaline phosphatase-conjugated anti-rabbit Ig) supplied by Promega (data not shown); thus this polypeptide is probably not related to the *pk2* product.

The antibody we obtained was also able to immunoprecipitate the 25-kDa protein from extracts of ^{35}S-labeled cells infected with Ac*M*NPV (data not shown). However, the 25-kDa protein was not detected when extracts of ^{33}P-labeled infected cells were subjected to immunoprecipitation with the antibody (data not shown). Therefore, the 25-kDa protein does not appear to be autophosphorylated or phosphorylated by other kinases during virus replication.

Effect of *pk2*-deletion on virus replication and biological activities in insect larvae

Although there was no obvious phenotype associated with *pk2* mutants in cell culture, it was clear that *pk2* was expressed during infection and the possibility remained that *pk2* was involved in the organismal infection cycle. We first determined the effect of *pk2*-deletion on occluded virus production in insect larvae by injecting *S. frugiperda* larvae (at a late instar stage) with wild-type Ac*M*NPV or *pk2* mutant viruses at lethal doses and determining the average yield of PIBs produced following larval death. vKINdel produced levels of PIBs which were similar to those produced by Ac*M*NPV in the species tested; wt Ac*M*NPV produced (6.20 ± 0.02) × 10^8 PIB/larva while vKINdel produced (6.50 ± 0.03) × 10^8 PIB/larva (the average yield and standard error were based on three independent counts of each of the pooled samples obtained as described under Materials and Methods).

To address the possibility that deletion of *pk2* from Ac*M*NPV affects the infectivity and/or virulence of the virus, we bioassayed vKINdel as well as Ac*M*NPV and vKINZR as controls in *S. frugiperda* larvae. Briefly, neonate larvae were fed diet containing various concentra-

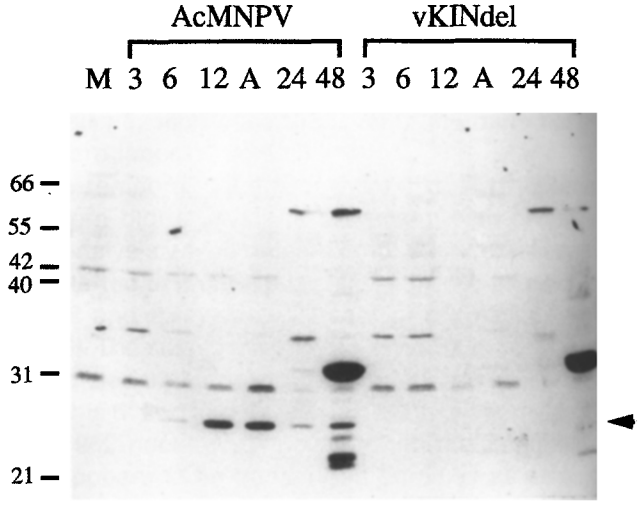

FIG. 7. Western blot analysis of PK2 expression. Cells were either mock-infected or infected with Ac*M*NPV and vKINdel, respectively. At the times indicated in hours on the top of each lane, cells were washed, collected, and lysed. After gel electrophoresis, the proteins were blotted and probed with antisera to GST-PK2 and then probed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobin G secondary antibody. The blot was developed with a chemiluminescence Western blot kit from Amersham. The protein size markers are given in kDa. Lanes marked as A contain samples collected at 12 hr p.i. from aphidicolin-treated infected cells. PK2 is noted with an arrowhead at the right. A 37-kDa polypeptide in the Ac*M*NPV-infected cells was consistently detected in other similar blots in the 3-hr lane but was not detected in this blot because of technical problems during protein transfer.

TABLE 1

BIOLOGICAL ACTIVITIES OF WT AcMNPV, vKINdel, AND vKINZR IN *Spodoptera frugiperda* LARVAE (TIME–MORTALITY RESPONSE)

Virus	LT_{50} (hr)	Fiducial limits (hr)		Slope
		Upper	Lower	
wt AcMNPV	108	116	105	19.0
vKINdel	105	109	103	18.7
vKINZR	112	116	108	18.7

tions of PIBs, which mimics the natural route of infection. The larvae were transferred to uncontaminated diet after 24 hr and monitored for mortality. As shown in Table 1, vKINdel had a LT_{50}, the time by which 50% of the larvae are killed , similar to that of AcMNPV or vKINZR, suggesting that there was no significant difference in virulence among those viruses. Likewise, the LC_{50}, the concentration of virus at which 50% of the larvae are killed, of vKINdel was also similar to that of AcMNPV of vKINZR (Table 2), indicating that vKINdel is comparable to AcMNPV and vKINZR in terms of virus infectivity.

DISCUSSION

eIF2α kinase is a key enzyme in eukaryotic translational control pathways responding to a variety of stress conditions. Viral infection or heme-deprivation in mammalian cells (Chen *et al.,* 1991; Mathews and Shenk, 1991; Samuel, 1993) and amino acid starvation in yeast (Dever *et al.,* 1992) activate eIF2α kinase. Phosphorylation of eIF2α, an essential translational initiation factor, results in the inhibition of translational initiation by blocking the GTP/GDP exchange cycle required for regenerating eIF2α–GTP complexes. A variety of viruses such as adenovirus (reviewed in Mathews and Shenk, 1991), human immunodeficiency virus (Roy *et al.,* 1990), influenza virus (Katze *et al.,* 1988; Lee *et al.,* 1990), poliovirus (Black *et al.,* 1989), and vaccinia virus (reviewed in Smith 1993) are able to downregulate the activity of eIF2α kinase, which is induced by interferons and is activated by double-stranded (ds) RNAs. The best understood example of this is adenovirus, which encodes VAI RNAs that bind to eIF2α kinase and inhibit its activity (reviewed in Mathews and Shenk, 1991). Vaccinia virus employs two different strategies to downregulate eIF2α kinase. In one case dsRNA activators of eIF2α kinase are sequestered by the protein product of the viral E3L gene and, in the other case, an eIF2α homolog encoded by the viral K3L gene competes with eIF2α for interaction with eIF2α kinase (reviewed in Smith, 1993). Although PK2 did not appear to be an active protein kinase, it was conceivable that a truncated form of eIF2α kinase such as the one encoded in the baculovirus genome might also be able to downregulate cellular eIF2α kinase by blocking the access of cellular eIF2α kinase to its activator or substrate through competition. However, our genetic data provided no support for this hypothesis even though our molecular data indicated that this gene was expressed as a 25-kDa protein.

The *pk2* gene is transcribed during the early phase of baculovirus infection and PK2 is present from early through late phases during viral infection. Although RNA splicing has been reported for a few baculoviral gene transcripts during baculovirus replication (Chisholm and Henner, 1988; Kovacs *et al.,* 1991), *pk2* transcripts do not appear to be spliced to other messengers that might encode N-terminal catalytic subdomains of kinases based on expression analysis data. That both the size of major *pk2* transcript detected by Northern blot analysis is 1.2 kb and the size of PK2 detected in Western blots is 25 kDa is consistent with the view that *pk2* is expressed as a short truncated kinase homolog. The larger late transcripts of 1.6 and 2.0 kb are not likely to encode proteins containing more catalytic subdomains of kinases, otherwise we would have detected a corresponding protein in AcMNPV-infected cells which would then disappear in Western blot analysis of vKINdel-infected cells.

Deletion of *pk2* from the AcMNPV genome did not noticeably alter protein synthesis and protein phosphorylation kinetics during virus infection in SF-21 cell culture. Phenotypically, the *pk2*-deletion mutant was indistinguishable from wild-type AcMNPV in both cell culture and insect larvae in all aspects examined, including plaque morphology, virus yield, infectivity, and virulence. Therefore, *pk2* probably plays an insignificant role if any in the cell line and insect species tested under the conditions used.

Our results are not necessarily contradictory to the hypothesis that *pk2* might participate in down-regulating cellular eIF2α kinase activity. In the case of vaccinia virus, deletion of the viral K3L gene shows significant effect on virus replication only in interferon-treated cells (Beattie *et al.,* 1991). It remains possible that a phenotypic effect could be observed under different conditions, such as a different insect species. An eIF2α kinase pathway might exist in insect cells (Barber *et al.,* 1992), although

TABLE 2

BIOLOGICAL ACTIVITIES OF WT AcMNPV, vKINdel, AND vKINZR IN *Spodoptera frugiperda* LARVAE (DOSE–MORTALITY RESPONSE)

Virus	LC_{50} (PIB/ml)	Fiducial limits (PIB/ml)		Slope
		Upper	Lower	
wt AcMNPV	7.7×10^5	1.5×10^6	3.9×10^5	1.28
vKINdel	5.9×10^5	1.5×10^6	2.2×10^5	1.16
vKINZR	8.3×10^5	1.8×10^6	3.9×10^5	1.22

an interferon response to virus infection has not been demonstrated in invertebrates.

It is also possible that *pk2* has lost function in the Ac*M*NPV genome, i.e., is an evolutionary vestige with no current function. Although *pk2* may have been transduced from the host genome, visual examination of sequences around the PK2 ORF revealed no signs of retrotransposition, such as long terminal repeats flanking *pk2* or an A-rich stretch following *pk2*. Evolution of *pk2* may be addressed by comparing Ac*M*NPV *pk2* with its counterparts in other baculoviruses.

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