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Characterization of a Unique OpMNPV-Specific Early Gene Not Required for Viral Infection in Tissue Culture

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opep-2 is an *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) early gene in the *ie1–ie2* gene region for which there is no homolog in either the archetype virus, *Autographa californica* MNPV, or *Bombyx mori* NPV. *opep-2* is transcribed immediately upon infection as three mRNAs which initiate from a early gene motif (TATA-N_{27}-CAGT). The expression of multiple transcripts at very early times postinfection has only been previously described for the baculovirus early gene *ie1,* which produces spliced mRNAs. However, distinct from *ie1,* the multiple mRNAs of *opep-2* are due to multiple termination sites and not splicing. Western blot analysis of steady-state levels of OPEP-2 showed that in OpMNPV-infected Ld652Y cells maximum levels are obtained at 8–12 hr postinfection (p.i.) prior to DNA replication. By 48 hr p.i. OPEP-2 is shut off and is undetectable. To aid in elucidating the function of this OpMNPV-specific gene an *opep-2* deletion mutant was generated and was compared to wild-type virus to determine if its absence affects viral growth in Ld652Y tissue culture cells.

INTRODUCTION

Baculoviruses are large double-stranded DNA viruses pathogenic for a variety of insect species. As such, they have the potential to be used for biological pest control in forestry and agriculture in both naturally occuring and engineered forms (Wood and Granados, 1991). The baculovirus *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) is a naturally occuring pathogen of the Douglas fir tussock moth and is used as a control agent during outbreaks of this economically important forestry pest (Cunningham, 1988; Shepherd *et al.,* 1984).

The OpMNPV genome is approximately 130 kb and has been shown to be essentially colinear with the archetype baculovirus *Autographa californica* MNPV (AcMNPV) which has been shown to code for approximately 150 ORFs of 50 nucleotides or longer (Ayres *et al.,* 1994; Leisy *et al.,* 1984). A great many of the AcMNPV genes that have been studied to date have homologs in OpMNPV. Exceptions to this, for example, are the *p94* and *p35* genes found between *p26* and *pp34* in AcMNPV but which are not present in OpMNPV. The *p35* gene inhibits apoptosis and is essential for virus replication in some cell types but can be functionally replaced by the OpMNPV *iap* gene (Crook *et al.,* 1993). These results suggest that baculoviruses have been obtaining unique genes that will allow them to survive in specific environments. Baculovirus genome sizes have been reported to range from 88 to 160 kb (Rohrmann, 1992). This suggests that genes identified by the sequencing of the AcMNPV genome do not represent the entire repertoire of baculoviral genes.

We have been investigating the function of immediate early genes in the baculovirus OpMNPV. As part of this investigation the genomic region between *ie1* and *ie2* was sequenced and was found to contain a >2000-bp insert relative to the same region in the archetype virus AcMNPV. Three ORFs were identified in this region that we have called *p8.9* (Wu *et al.,* 1993b), *opep-2* (*O. pseudotsugata* MNPV early protein-2), and *opep-3.* The predicted proteins from these ORFs do not have similarity to any known baculovirus or nonbaculovirus proteins. Of significant interest is that upstream of each ORF is an early gene transcriptional start site motif (TATA-N_{23-27}-CAGT). Most other genes described to date that contain the early gene motif have been shown to play significant roles in the infection cycle of baculoviruses. These genes include the transcription factors *ie0, ie1, ie2, p34 (pe38),* the budded virus (BV)-specific protein *EFP*(*gp64*), and the phosphoprotein *pp31* (*p39*) (Blissard and Rohrmann, 1989; Lu and Miller, 1995). The structural similarity to these other early genes suggested that these unique OpMNPV genes may play an important role in virus replication. In this article we report on the molecular characterization of one of these genes, *opep-2.* In addition, we

The nucleotide sequence data reported in this article have been deposited with the Genbank Database under Accession No. U72650.

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have generated an *opep-2* deletion virus to study the role of OPEP-2 in the viral infection cycle and the effects of its absence on other viral proteins.

METHODS

Cells and virus

Lymantria dispar (Ld652Y) cells and *Spodoptera frugiperda* (Sf9) cells were maintained in TC100 medium as described (Summers and Smith, 1987). OpMNPV and the deletion virus, vΔopep-2, were propagated in Ld652Y cells as previously described (Quant-Russell *et al.,* 1987).

DNA sequencing and plasmid constructs

Plasmid clones of *opep-2* were constructed from a 1.9-kb *Pst*I restriction fragment from the cosmid Op47 (Leisy *et al.,* 1984). DNA sequencing was carried out using Sequenase Version 2.0 (Amersham) as described (Toneguzzo *et al.,* 1988). DNA and predicted protein sequences were analyzed and compared to the Genbank, EMBL, PIR, and SWISSPROT databases using the UWGCG and DNA Strider sequence analysis packages (Devereux *et al.,* 1984; Marck, 1988).

A bacterial fusion protein was produced from plasmids that fused the OPEP-2 ORF to glutathione *S*-transferase (GST) in the bacterial expression vector pGEX-3X using standard techniques (Smith and Johnson, 1988). The resulting fusion protein consisted of GST fused to amino acids 11–236 of OPEP-2.

A 1.8-kb *Ava*II fragment containing the *opep-2* ORF was cloned downstream of the T7 RNA polymerase promoter in pBS+ (pOp6-7Ava1.8A). This plasmid was linearized with *Hin*dIII and used to generate run-off transcripts for use in *in vitro* translation.

The opep-2–β-galactosidase fusion construct for deletion of *opep-2* from the OpMNPV genome was made by inserting the *lacZ* gene from the pMC1871 plasmid (Shapira *et al.,* 1983) in frame with the *opep-2* gene at the *Eco*RI and *Sca*I sites. The chloramphenicol acetyltransferase (CAT) reporter constructs were made by inserting the CAT gene into the *Eco*RI and *Sca*I sites followed by digestion with *Pst*I–*Xma*III and *Pst*I–*Psp*5II to make Xma-opep2CAT and Psp-opep2CAT, respectively.

RNA isolation and Northern blots

Total RNA from OpMNPV-infected Ld652Y cells for Northern blots and 5′ transcript mapping was prepared as described (Chirgwin *et al.,* 1979; Turpen and Griffith, 1986). Total cellular RNA for cDNA synthesis was extracted from infected cells (mock and 8 and 48 hr p.i.) with Trizol (BRL) according to the manufacturer's instructions. For Northern blotting, total RNAs (5 μg per lane) were separated by electrophoresis in 1.25% agarose gels containing 6% formaldehyde, 1× MOPS buffer [20 m*M* MOPS (3-[*N*-morpholino]propanesulfonic acid, pH 8.0), 5 m*M* sodium acetate, 1 m*M* EDTA] (Thomas, 1983). Separated RNAs were transferred to Nytran nylon membrane (Schleicher & Schuell) by capillary blotting. Hybridization was carried out at 60° in 6× SSC (1× is 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0), 10× Denhardt's solution (1× is 0.02% polyvinylpyrolidone, 0.02% BSA, 0.02% Ficoll 400), 0.1% SDS, 100 μg/ml denatured DNA from salmon sperm, 100 μg/ml yeast RNA. Single-stranded RNA probes complementary to *opep-2* mRNA were synthesized using T7 RNA polymerase. After hybridization, blots were washed twice for 15 min in 2× SSC, 0.1% SDS at 60° and twice for 15 min in 0.1× SSC, 0.1% SDS at 75° and exposed to Kodak XAR film with an intensifying screen.

cDNA synthesis

First-strand cDNA was synthesized from total RNA using primer XBEdT [5′ CTCGAGGGATCCGAATTC(T_{17}) 3′] which incorporates *Xba*I, *Bam*HI, and *Eco*RI sites immediately 5′ to the poly(T) tail. Briefly, 5 μg total RNA and 10 pmol XBEdT in 20 μl H_2O were heated to 65° for 3 min to denature, then quenched on ice. To the denatured RNA–primer mix 4 μl of 5× reverse transcription buffer (1× is 50 m*M* Tris–Cl, pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$), 33 units RNAguard (Pharmacia), 10 m*M* dithiothreitol, 0.5 m*M* each dATP, dTTP, dCTP, dGTP, and 200 units of Superscript RNase H^- reverse transcriptase (BRL) prewarmed to 37° was added and incubated at 42° for 60 min. Reverse transcriptase was inactivated at 65° for 10 min and 30 μl of 0.1× TE (1× is 10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA, pH 8.0) was added — this cDNA stock was used in the amplification reaction.

Second-strand cDNA ends were synthesized by combining 2 μl of cDNA stock with 50 pmol of each primer XBE (5′ CTCGAGGGATCCGAATTC 3′) and p25-1 (5′GTCATAACCACAACGGATGC 3′), in 20 m*M* Tris–HCl, pH 8.4, 50 m*M* KCl, 2 m*M* $MgCl_2$, in a final reaction volume of 50 μl. The reaction mix was heated at 94° for 5 min followed by addition of 2.5 units *Taq* DNA polymerase (BRL), annealing at 42° for 5 min, and extension at 72° for 40 min. cDNA ends were then amplified for 40 cycles using the following conditions: 94° for 40 sec, 45° for 30 sec, 72° for 1 min. The final extension was at 72° for 15 min. The resulting amplified products were digested with *Eco*RI and *Hin*cII, gel purified, and cloned into pBS+ (Stratagene).

S1 nuclease protection and primer extension

5′ S1 nuclease protection analysis was performed as described (Theilmann and Stewart, 1991) using 10 μg of total RNA. DNAs were labeled with ^{32}P[dATP] and T4 polynucleotide kinase (Sambrook *et al.,* 1989). S1 nuclease-protected fragments were analyzed on denaturing sequencing gels [8% polyacrylamide, 4.6 *M* urea, 1× TBE (100 m*M* Tris-borate [pH 8.3], 20 m*M* EDTA)] using M13 sequencing ladders as size markers.

Primer extension reactions using 10 μg total RNA were performed as previously described (Chisholm and Henner, 1988). A 17-mer oligonucleotide (5′ GTCTCCACCATTAACTG 3′) complementary to the sequence of the *opep-2* transcript, 149 bp downstream from the start of the predicted ORF, was used as a primer for the extension and to obtain a sequencing ladder from pOpPst1.9B. Primer extension products and sequencing ladders were analyzed on 6% denaturing polyacrylamide gels as described above.

Transfections and CAT assays

Transfections were performed using 1 μg of plasmid DNA and 10 μl lipofectin (Gibco) using the manufacturer's recommended protocol. CAT assays were performed as described by Neumann *et al.* (1987)

Western blot analysis

Time course analysis of OpMNPV-infected Ld652Y cells were performed as previously described, using an m.o.i. of 10–20 (Blissard and Rohrmann, 1989). At each time point, cells were pelleted, washed once with PBS (10.1 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, 2.7 m*M* KCl, 136 m*M* NaCl, pH 7.2), resuspended in PBS, and lysed with an equal volume of 2× protein sample buffer [1 × PSB; 125 m*M* Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromphenol blue (Laemmli, 1970)]. Proteins were separated using denaturing gel electrophoresis (SDS–PAGE; Laemmli, 1970) and transferred to Immobilon membranes (Millipore) using standard techniques (Sambrook *et al.,* 1989). Rabbit polyclonal antiserum was raised against the GST OPEP-2 fusion protein using standard techniques (Harlow and Lane, 1988) and used for immunoprecipitation and to probe Western blots. Polyclonal antisera to ODVP-6E and monoclonal antisera to IE1 and P34 have been described previously (Theilmann *et al.,* 1996; Theilmann and Stewart, 1993; Wu *et al.,* 1993a). GP64-EFP monoclonal antisera AcV_5 have also been described previously (Blissard and Rohrmann, 1989). Monoclonal antisera against capsid and polyhedrin proteins (Pearson *et al.,* 1988; Quant *et al.,* 1984) were a gift from George Rohrmann. Monoclonal antisera against OPEP-3 was also used (D. A. Theilmann, manuscript in preparation). Western blots were incubated with appropriate dilutions of polyclonal or monoclonal antisera. Immunoreactive proteins were detected using rabbit anti-mouse or goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (Jackson Laboratories) followed by a chemiluminescent substrate (Amersham), following the manufacturer's instructions.

In vitro translation

pOp6-7Ava1.8A was linearized with *Hin*dIII and run-off transcripts were synthesized with T7 RNA polymerase. Transcripts were phenol extracted, ethanol precipitated, and resuspended in 10 μl H_2O. Transcribed RNA (0.9 μl) was translated in 3.5 μl nuclease-treated rabbit reticulocyte lysate (Promega), 0.1 μl methionine-free amino acid mix (1 m*M*), 3.3 units RNAguard (Pharmacia), 4 μCi [^{35}S]methionine (final volume 5 μl) for 2 hr at 30°. One microliter was removed for loading as the total translation reaction. Immunoprecipitations were performed by combining 4 μl of *in vitro*-translated proteins with 300 μl IP buffer (250 m*M* NaCl, 50 m*M* HEPES, pH 7.0, 5 m*M* EDTA, 1 m*M* PMSF, 0.5 m*M* dithiothreitol, and 0.1% NP-40), 5 μl protein G–Sepharose (Pharmacia), and 0.5 μl undiluted polyclonal antisera. Samples were rocked for 1 hr at 4°, pelleted 1 min, washed twice with IP buffer, and resuspended in 10 μl 1× protein sample buffer.

Cell fractionation

Nuclear and cytoplasmic fractions of OpMNPV-infected Ld652Y were prepared as previously described (Jarvis *et al.,* 1991). Briefly, 2×10^6 cells were harvested with sterile rubber policemen and pelleted in 15-ml conical tubes at 3000 rpm for 5 min. Cell pellets were washed once with 1 ml PBS. Washed pellets were resuspended in 100 μl NP-40 lysis buffer (10 m*M* Tris–HCl, pH 7.9, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.5% NP-40). Samples were chilled 5 min on ice and nuclei pelleted at 1000 *g* for 5 min. The supernatant was removed and 100 μl 2× PSB added. The nuclear pellet was resuspended in 100 μl NP-40 lysis buffer and 100 μl 2× PSB added. DNA in each sample was sheared with a 25-gauge needle. For the total cell protein sample, 100 μl 2× PSB was added to whole cells resuspended in 100 μl NP-40 buffer, and DNA sheared with a 25-gauge needle.

Isolation of occlusion-derived virus and budded virus

Budded virus (BV) was purified using the methods of Summers and Smith (1987). Occlusion-derived virus (ODV) were isolated from OpMNPV polyhedral inclusion bodies (PIBs) obtained from infected *O. pseudotsugata* larvae (2 mg). PIBs were resuspended in 200 μl H_2O and heated at 70° for 20 min, then pelleted at 12,000 *g* for 5 min at 4°. The pellet was resuspended in 180 μl H_2O, 20 μl dilute alkaline saline (DAS; 1 *M* Na_2CO_3, 50 m*M* NaCl), and solubilized at 65° for 5 min and quenched on ice for 2 min. DAS-insoluble material was pelleted at 12,000 *g* for 10 min at 4° and the soluble fraction removed to a fresh tube. The pellet was resuspended in 60 μl 10 m*M* Tris–HCl, pH 7.5. An equal volume of 2× PSB was added to both the pellet and the supernatant fractions.

Construction of *opep-2* deletion virus

The *opep-2* deletion viruses were constructed by co-transfection of opep-2β-gal and purified OpMNPV viral DNA into Ld652Y cells by $CaPO_4$ precipitation (Summers and Smith, 1987). Cells were incubated at 27°, and the culture supernatant was harvested after visible pathological effects were evident. β-Galactosidase-expressing vi-

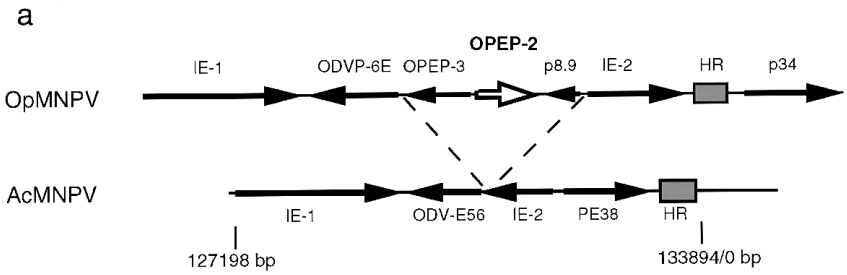

FIG. 1. (a) Comparison of the OpMNPV and AcMNPV *ie1–ie2* gene regions. Arrows indicate the orientation and location of the ORFs. The name of the ORF is given above the arrow. IE-1, IE-2, and P34 (IE-1, IE-2, and PE38 are the corresponding homologs in AcMNPV) are transcriptional transactivators. OPEP-2, OPEP-3, and P8.9 do not have corresponding homologues in AcMNPV. HRs are nonhomologous enhancer elements of OpMNPV and AcMNPV (Cochran and Faulkner, 1983; Theilmann and Stewart, 1992b). The base-pair coordinates based on the AcMNPV sequence are indicated (Ayres *et al.,* 1994) and dashed lines highlight the approximate insertion point of the OpMNPV unique region. The OpMNPV and AcMNPV maps are drawn to the same scale. (b) Nucleotide and amino acid sequence of *opep-2.* The consensus early transcription initiation motif, CACAGT, is overlined in bold, the transcription initiation site is indicated by a bent arrow. GATA motifs in both orientations are underlined as well as the CACGTG motif. The TATA box 5′ of *opep-2* ORF is boxed; polyadenylation signals (AATAAA) 3′ to the ORF are also boxed; letter designations correspond to Fig. 3b. The 3′ termination sites are indicated by asterisks above the nucleotide. Direct repeats in the promoter region are double underlined. Relevant restriction sites are indicated.

ruses were isolated by plaque assay. Positives were confirmed through three rounds of plaque purification. Restriction digest, PCR, and Western blot analyses were used to confirm the deletion of the *opep-2* ORF from the viral genome. Two isolates of the deletion virus, vΔopep-2 10 and 11 were used to generate BV stocks for use in subsequent studies. The PCR reactions were performed using 10 ng of viral DNA, 10 pmol each of primers opep2d (5′ GAATTCTAACACACGGTTTTGACCAA 3′) and opep2e (5′ TATTGTGTTTGACACTGC 3′) in 25 μl PCR buffer (10 m*M* Tris–HCl, pH 8.4, 0.05% Tween 20, 0.05% NP-40, 1.5 m*M* $MgCl_2$, 0.25 m*M* dNTPs, 3.75 units *Taq* DNA polymerase). The reaction was hot started at 95° for 2 min, followed by 30 cycles at 95° for 1 min, 50° for 1 min, 72° for 1 min. The final extension was 72° for 2 min.

RESULTS

Identification of *opep-2*

The *opep-2* ORF was identified in the genomic region between the *ie1* and the *ie2* genes (Theilmann and Stewart, 1991, 1992a). Within this region, some rearrangements in gene order and orientation were found, when compared to AcMNPV (Fig. 1a). The *ie2* gene of OpMNPV and its homologue in AcMNPV are in opposite orientations (Carson *et al.,* 1991; Theilmann and Stewart, 1992a). Another early gene, *p34* (*pe38* in AcMNPV) (Krappa and Knebel-Morsdorf, 1991; Wu *et al.,* 1993a), is transcribed in the same orientation in both viruses, but is downstream of the enhancer region of OpMNPV, whereas *pe38* is upstream of an *hr* enhancer region (Ayres *et al.,* 1994; Theilmann and Stewart, 1992b). The *ie1* gene and the downstream late gene *odvp-6e* and its AcMNPV homologue *odv-e56* have similar arrangements in both OpMNPV and AcMNPV (Braunagel *et al.,* 1996; Theilmann *et al.,* 1996). In the center of this region in OpMNPV are three ORFs not present in AcMNPV or BmNPV. *p8.9* was the first unique gene found in this region (Wu *et al.,* 1993b). *opep-2* is immediately upstream of *p8.9* and is transcribed off the opposite strand. It too is unique to OpMNPV, as AcMNPV and *Bombyx mori* (Bm) NPV do not have homologous sequences in their genomes (Ayres *et al.,* 1994; Maeda, 1994). The third gene, *opep-3,* is also unique to OpMNPV and will be described elsewhere (D. A. Theilmann, manuscript in preparation).

The *opep-2* ORF was found to be 708 nt in length and coded for a predicted protein of 236 amino acids with a predicted molecular weight of 25.4 (Fig. 1b). Analysis of the predicted amino acid sequence did not identify any recognizable motifs. In addition, no significant homologies to known proteins have been identified by comparison with protein and nucleic acid databases (Genbank, EMBL, SWISSPROT, PIR). One noticeable feature of the predicted protein sequence was the high valine and alanine content, constituting 27.4% of the total amino acids.

An early gene transcription initiation motif (TATAA-N_{27}-CAGT) was found 17 bases upstream of the start methionine of the *opep-2* ORF (Fig. 1b). This early gene motif is conserved in several baculovirus early gene promoters. Upstream of the TATA box a number of possible regulatory motifs were found, which included four GATA motifs, one CACGTG, and two 13-bp direct repeats. Six polyadenylation signal sequences (AATAAA) were found downstream of the TAA stop codon of the *opep-2* ORF (Fig. 1b).

Transcriptional mapping and temporal expression of *opep-2*

Temporal expression of *opep-2* was analyzed by Northern blot of total RNAs extracted from OpMNPV-infected Ld652Y cells at various times postinfection and hybridized to a strand-specific RNA probe homologous to the entire *opep-2* ORF (Fig. 2a). Three *opep-2* tran-

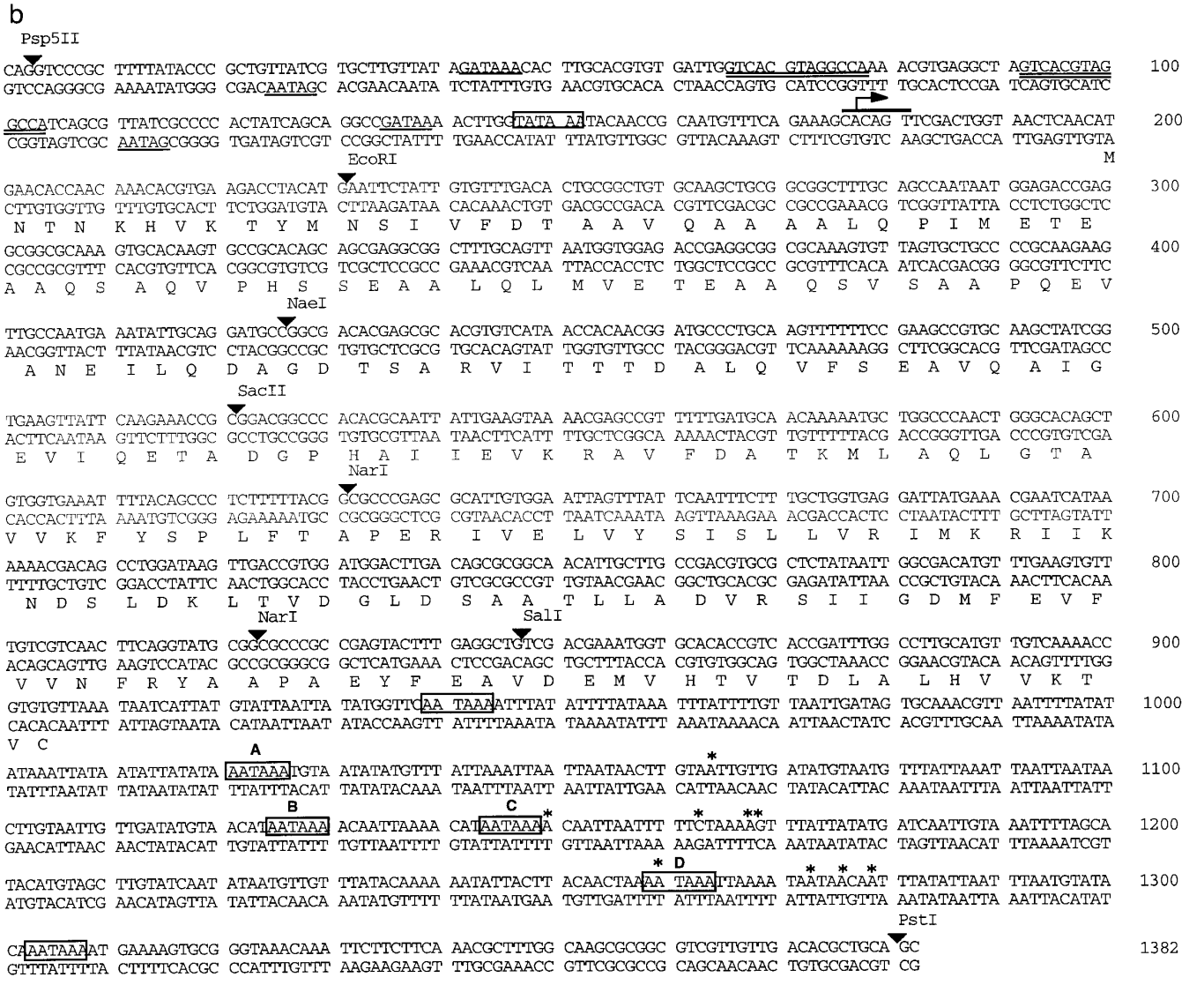

FIG. 1 — *Continued*

scripts of 1.1, 0.98, and 0.88 kb were detected from 1 to 36 hr p.i. (Fig. 2b). At late times (18–120 hr p.i.), two larger transcripts of 6.8 and 1.6 kb were detected. A smaller 616-base strand-specific RNA probe (Fig. 2a) was also used to probe a similar Northern blot and identical results were obtained (data not shown). The detection of three major early transcripts homologous to *opep-2* 1 hr p.i. was unusual as the only OpMNPV early gene that is expressed as multiple transcripts immediately upon infection is *ie1,* which is spliced. It was therefore possible that *opep-2* was spliced or there were multiple transcript initiation or termination sites.

To determine which of these was correct, *opep-2* transcripts were mapped by 5′ and 3′ S1 nuclease protection analysis, primer extension and 3′ RACE. 5′ S1 nuclease protection analysis indicated a single 248-nt protected fragment at 2, 12, and 48 hr p.i. (Fig. 3a), corresponding to transcription initiation at the CACAGT motif 17 bases from the 5′ end of the *opep-2* ORF (Fig. 1b). An additional 254-nt protected fragment was consistently detected at 48 hr p.i.

Precise mapping of the 5′ end of the *opep-2* transcript was also determined by primer extension, using a 17-nt oligonucleotide, complementary to the sequence of the *opep-2* transcript 146 bases downstream of the start methionine of the *opep-2* ORF. A single extension product of 185 nt was also observed at 8 hr p.i. (Fig. 3b), mapping the transcription initiation site of *opep-2* to the first A of the CACAGT of the early gene motif (Fig. 1b). A 185-nt extension product was detected at 48 hr, as well as several additional extension products of varying sizes (Fig. 3b).

3′ S1 nuclease analysis was attempted using several different probes, none of which were successful in ob-

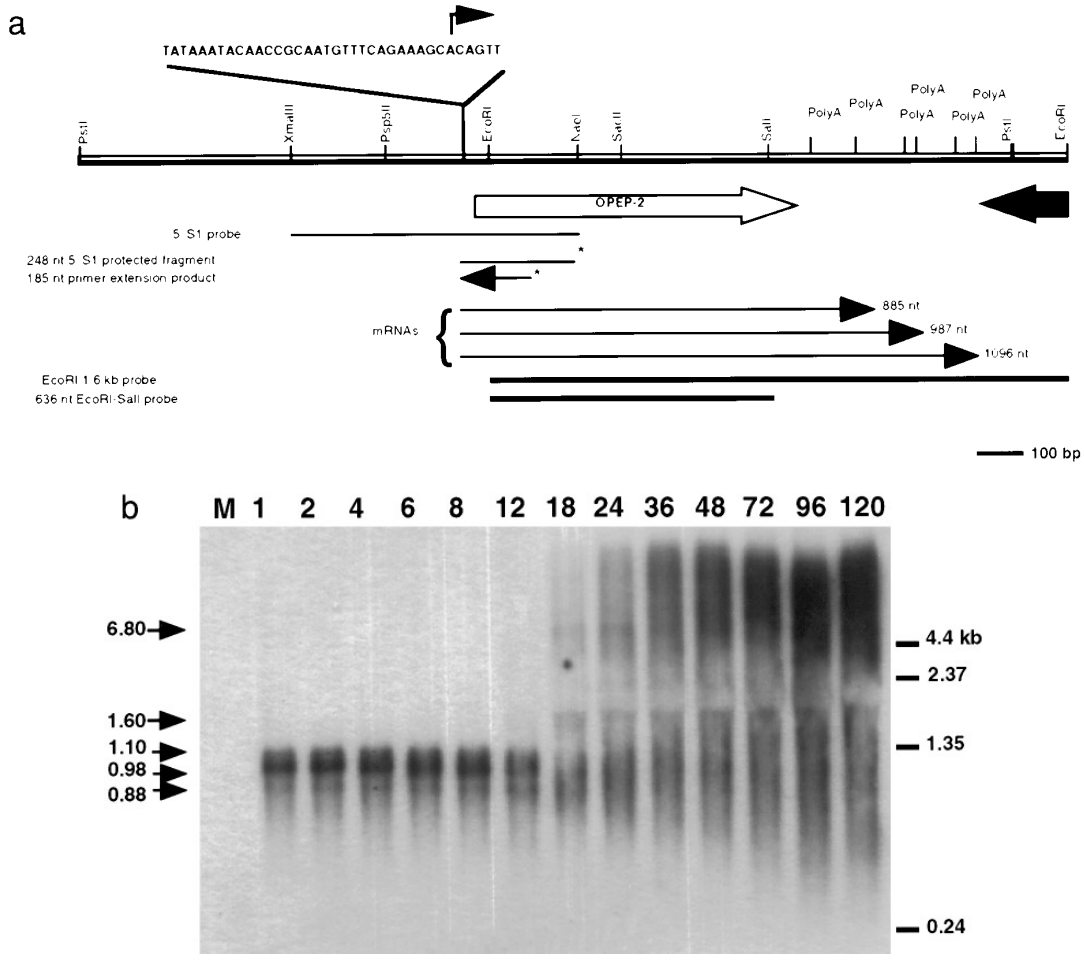

FIG. 2. (a) Schematic diagram of *opep-2* gene region. The 5′ S1 nuclease probe and protected fragment and primer extension product are indicated below the ORF. Locations of *opep-2* mRNAs as determined by 5′ and 3′ mapping are illustrated by arrows. Complementary RNA probes used for Northern blots are indicated by thick black lines. (b) Northern blot analysis of expression of *opep-2* in OpMNPV-infected Ld652Y cells. Total RNA (5 μg) from 1 to 120 hr p.i. was probed with a strand-specific RNA probe homologous to the *opep-2* ORF (*Eco*RI 1.6 kb). Approximate sizes of three major early transcripts detected are indicated on the left, size markers are given on the right. M, mock-infected cells; lane numbers correspond to hours postinfection.

taining a protected fragment (data not shown). A possible reason for this was the greater than 80% A+T content of the region downstream of the OPEP-2 ORF. Therefore, to accurately map the 3′ end, 3′ RACE was performed on total RNA isolated from infected cells at 8 and 48 hr p.i. Nine *opep-2*-specific cDNAs were isolated and sequenced. The results shown in Fig. 3c indicated there were three clusters of termination sites, downstream from polyadenylation signals at 885, 987, and 1096 nt (Figs. 1c and 3c), corresponding to the sizes of the three transcripts of 887, 998, and 1103 nt. These transcripts correspond in size to the three early transcripts detected by Northern blot. Based on our transcriptional mapping data, it therefore appears that the three *opep-2* transcripts arose due to multiple termination signals.

The very early expression of *opep-2* transcripts and initiation from an early gene motif (Fig. 3b) suggested that the promoter would be functional in uninfected cells and would not require viral factors for activation. To test this we constructed two *opep-2* CAT reporter constructs, Xma-opep2CAT and Psp-opep2CAT, which contained 388 and 176 bp of 5′ promoter sequences, respectively. Each construct was transfected into Ld652Y cells and assayed for CAT activity. The results shown in Fig. 3d show that both constructs were actively expressed and gave approximately equivalent levels of CAT. This result shows that the *opep-2* promoter is active in uninfected Ld652Y cells, similar to the *ie1, ie2,* and *p34* promoters. In addition, 176 bp of 5′ sequence appear to be sufficient for full activity in the absence of viral factors.

Temporal expression of OPEP-2

To further analyze the expression of OPEP-2, a polyclonal antiserum was generated against an OPEP-2 fusion protein. The steady-state levels of OPEP-2 were then analyzed from 2 to 120 hr p.i. by Western blot. A single 32-kDa band was detected by 2 hr p.i. and declined to

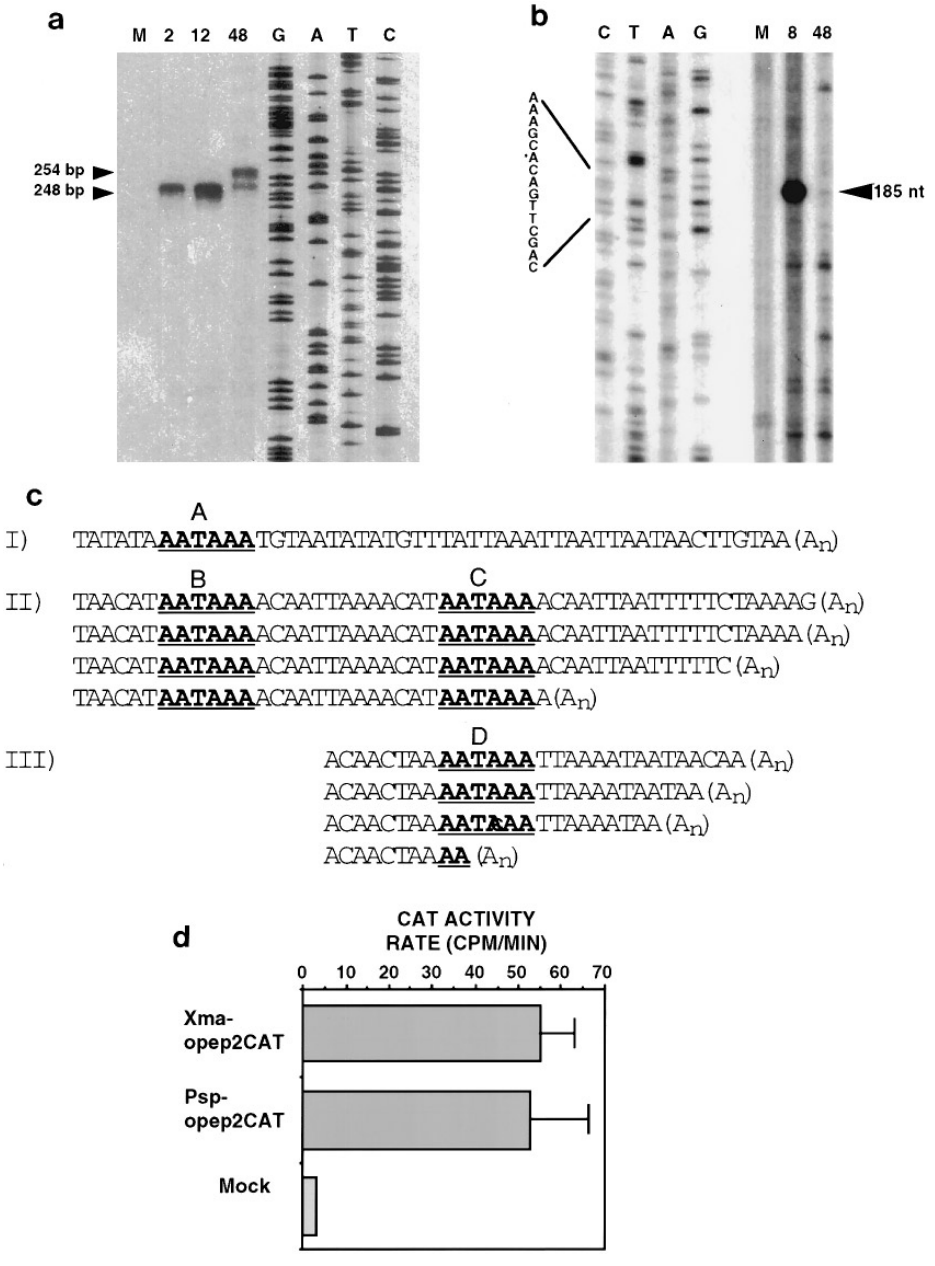

FIG. 3. Transcriptional mapping of *opep-2* and transfection analysis of the *opep-2* promoter.(a) 5′ S1 nuclease protection analysis of *opep-2.* Total RNAs from mock (M) and 2, 12, and 48 hr p.i. were hybridized with 5′-labeled DNA, indicated in Fig. 2a. A single protected fragment of 248 nt is indicated on the left. Sequencing ladder for sizes is M13 DNA, primed with universal forward primer. (b) Primer extension product of *opep-2.* Total RNAs from mock (M) and 8 and 48 hr p.i. were incubated with primer XW2. A single 185-nt product is indicated on the right. Size and sequence location was determined from a sequencing ladder made with the same primer. Sequence and initiation site are schematically shown on the left. (c) Sequence of 3′ RACE cDNAs. The polyadenylation signals are indicated by the letter designations A–D which correspond to Fig. 1b. (d) CAT activity of *opep-2* CAT constructs transfected into Ld652Y cells. Xma-opep2CAT and Psp-opep2CAT contain 388 and 176 bp of 5′ promoter sequences relative to the transcriptional start site of *opep-2.* The results shown are the average CAT expression from two separate transfections. Transfections were repeated more than five times.

barely detectable by 48 hr p.i. (Fig. 4a). The steady-state levels of OPEP-2 peaked at 12 hr p.i., prior to DNA replication, which starts at approximately 18 hr p.i. (Bradford *et al.,* 1990). The 32-kDa size observed in the Western blot was unexpected since the predicted size of OPEP-2 based on the amino acid sequence was 25.4 kDa.

This large difference between the predicted and the observed size suggested that the protein may be post-translationally modified or the aberrant migration was an inherent property of the protein. In addition, it was possible that OPEP-2 antisera was not detecting the correct viral protein. To investigate this further, transcripts of the *opep-2* ORF were translated *in vitro* using a rabbit reticulocyte lysate system. Three proteins 25, 29, and 32

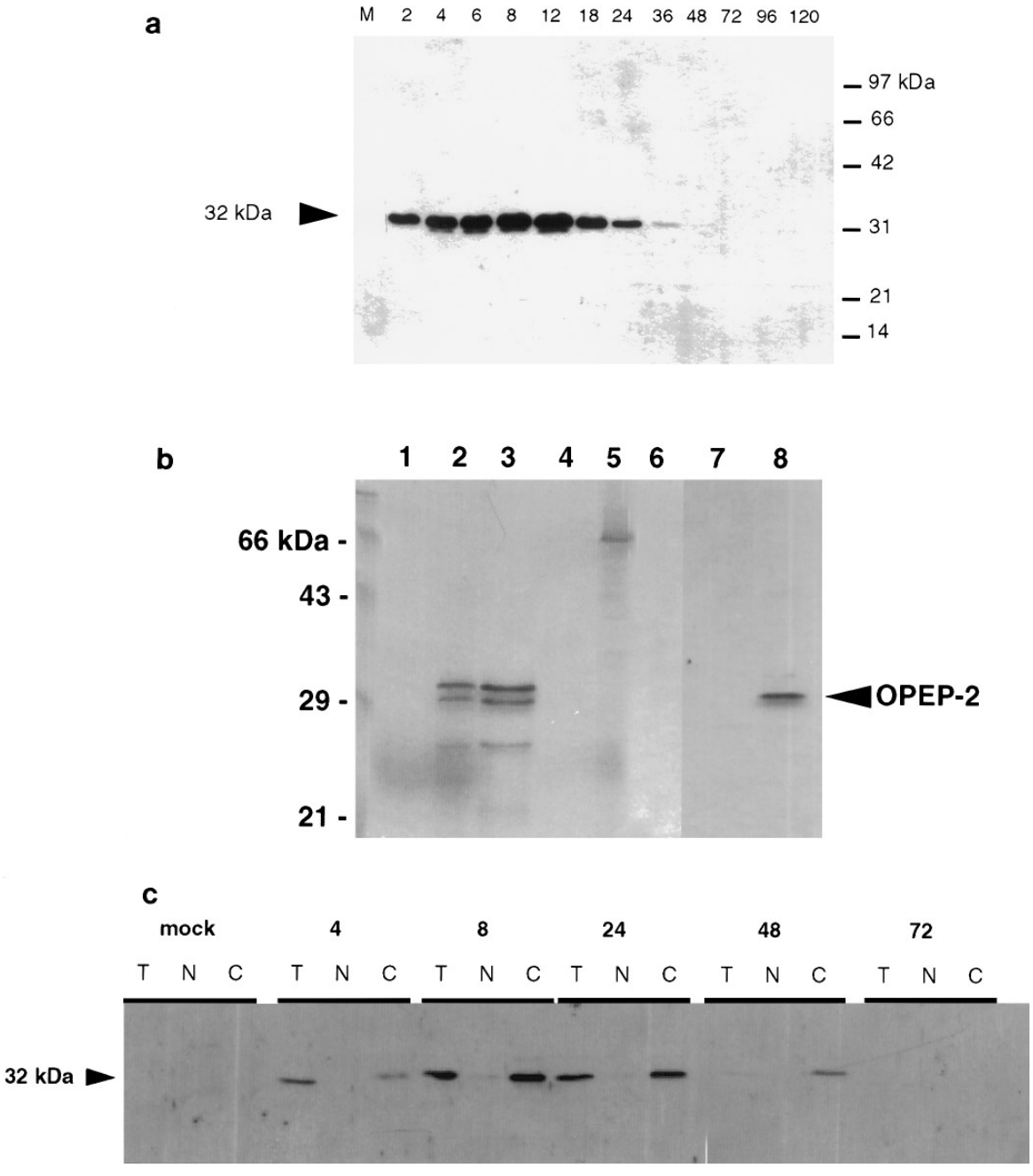

FIG. 4. (a) Western blot analysis of OPEP-2 in OpMNPV-infected Ld652Y cells from 2 to 120 hr p.i. OPEP-2-specific proteins were identified using polyclonal antisera and detected with HRP-labeled secondary antibody and a chemiluminescent substrate; M, mock-infected cells; lane numbers correspond to hours p.i. Size standards are indicated on the right, the single 32-kDa immunoreactive protein is indicated on left. (b) *In vitro* translation of cloned *opep-2* ORF using rabbit reticulocyte lysate. Lane 1, RNA-free negative control; lane 2, unprecipitated *opep-2* translation products; lane 3, *opep-2* translation products immunoprecipitated with OPEP-2-specific polyclonal antisera; lane 4, immunoprecipitated by nonspecific polyclonal antisera; lane 5, translation products of luciferase RNA-positive control; lane 6, as in lane 5 but immunoprecipitated with OPEP-2 polyclonal antiserum; lanes 7 and 8, Western blot analysis of mock (lane 7) and OpMNPV-infected Ld652Y cells at 8 hr p.i. (lane 8). The 32-kDa-migrating OPEP-2 is indicated by an arrow on the right. Size markers are indicated on the left. (c) Cellular localization of OPEP-2 by Western blot analysis of total, nuclear, and cytoplasmic proteins from OpMNPV-infected Ld652Y cells at 4, 8, 24, 48, and 72 hr p.i. M, mock-infected cells; T, total; N, nuclear; C, cytoplasmic fractions.

kDa in size were specifically immunoprecipitated by the OPEP-2 polyclonal antiserum (Fig. 4b). Western blot analysis of infected cell extracts from the same gel indicated an OPEP-2-specific band migrating at the same position as the 32 kDa *in vitro*-translated product. The smallest *in vitro* translation product migrated at 25 kDa, the predicted size of OPEP-2. Rabbit reticulocyte lysate systems have been shown to have kinase activity (Joshi *et al.*,

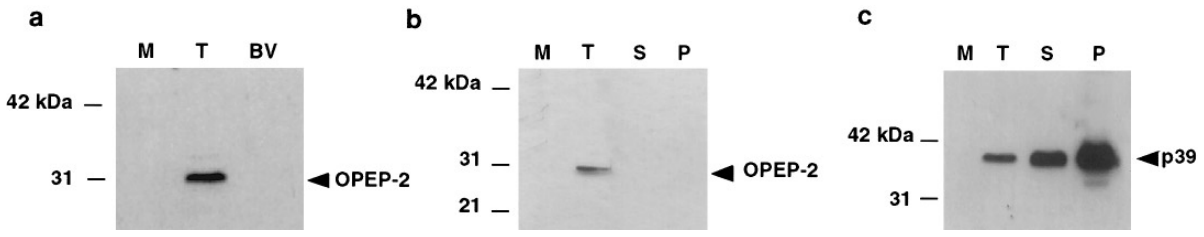

FIG. 5. Western blot analysis of purified BV and ODV. (a) Purified budded virus analyzed by Western blotting and probed with OPEP-2 polyclonal antiserum. M, mock-infected cells; T, total protein 8 hr p.i. (b)Alkaline soluble (S) and insoluble (P) fractions of ODV were analyzed by Western blot and probed with OPEP-2 or (c) with P39-capsid polyclonal antisera.

1995) . Therefore, the largest two *in vitro* translation products may have migrated as higher molecular weight proteins due to varying levels of phosphorylation by kinases present in the rabbit reticulocyte lysate system. The migration of OPEP-2 from infected cells as a 32-kDa protein may be due to the amino acid composition of the protein and/or posttranslational modifications such as phosphorylation.

Cellular localization of OPEP-2

To determine cellular localization of OPEP-2, OpMNPV-infected Ld652Y cells at various times postinfection were fractionated using a detergent-based protocol into nuclear and cytoplasmic fractions and analyzed by Western blot. OPEP-2 was detected in the total cell and cytoplasmic fractions up to 48 hr p.i. (Fig. 4c).

To determine if OPEP-2 was a structural protein, both viral phenotypes were purified and analyzed by Western blot, probed with the OPEP-2-specific antisera. Figure 5a shows a Western blot of 5 μg of budded virus; no detectable OPEP-2 was observed. OPEP-2 was detected only in infected cell extracts (24 hr p.i. total protein). Analysis of ODV yielded similar results, as OPEP-2 was not detected in either ODV-P or ODV-S fractions (Fig. 5b). To confirm the presence of virus in the ODV samples, an identical blot was probed using P39-specific polyclonal antisera, and both the soluble and the insoluble fractions were positive for the 39-kDa capsid protein (Fig. 5c). These results indicate that OPEP-2 does not appear to be associated with either BV or ODV.

opep-2 deletion virus

To aid in determining the function of OPEP-2 in the viral infection cycle, we have constructed deletion viruses to see if its absence affected viral growth. Homologous recombination *in vivo* replaced the majority of the *opep-2* ORF with β-galactosidase in the OpMNPV genome, resulting in the isolation of two deletion viruses, vΔopep-2 10 and 11. Restriction digest, PCR, and Western blot analyses were performed to confirm deletion of the gene. Results of PCR analysis of purified viral DNAs using ORF-specific primers are shown in Fig. 6a. A single 728-bp fragment was amplified from the wild type (WT) DNA, and no amplified products were detectable in either isolate of the deletion viruses, indicating that the ORF was no longer present. To further confirm that *opep-2* expression had been eliminated, Western blot analysis of steady-state levels of OPEP-2 and β-galactosidase in WT- and vΔopep-2-infected Ld652Y cells was performed (Fig. 6b). OPEP-2 expression in WT OpMNPV-infected cells was detected from 6 hr p.i., and was undetectable by 48 hr p.i., as expected. In both vΔopep-2 10 and 11, OPEP-2 was not detected. Conversely, β-galactosidase was not detected in the WT infection, but was expressed in both of the deletion viruses from 6 hr p.i., and continued to the end of the time course at 72 hr p.i. The expression pattern of β-galactosidase under control of the OPEP-2 promoter differed from that of OPEP-2 in WT infections which declines to barely detectable levels by 48 hr p.i. It is believed that β-galactosidase persisted in the infected cell due to its stability, rather than the continued production of protein. These results confirmed that the *opep-2* ORF was deleted from the virus.

The isolation of vΔopep-2 10 and 11 indicated that OPEP-2 was not essential for growth of OpMNPV in cell culture. To determine if its absence affected aspects of OpMNPV infection, time course analyses of WT and vΔopep-2 10 and 11 were performed and steady-state levels of selected proteins were analyzed by Western blot. Figure 7a shows expression of four early genes, GP64-EFP, IE1, P34, and OPEP-3, in wild-type- and vΔopep-2-infected Ld652Y cells. GP64-EFP, a structural protein of BV, was first detected faintly at 6 hr p.i. and continues through to 72 hr p.i. in both WT and deletion viruses. A GP64-EFP 25-kDa-specific band is also detected at 48 hr p.i. in both WT and vΔopep-2-infected cells. Similarly no consistent changes in the expression of the viral transactivators IE1 and P34 were detected in WT- and vΔopep-2 10- and 11-infected cells. *opep-3* is another unique OpMNPV gene, adjacent to *opep-2* in the *ie1–ie2* region of the OpMNPV genome (D. A. Theilmann, manuscript in preparation). OPEP-3 was expressed starting at 6 hr p.i. and continued through to the end

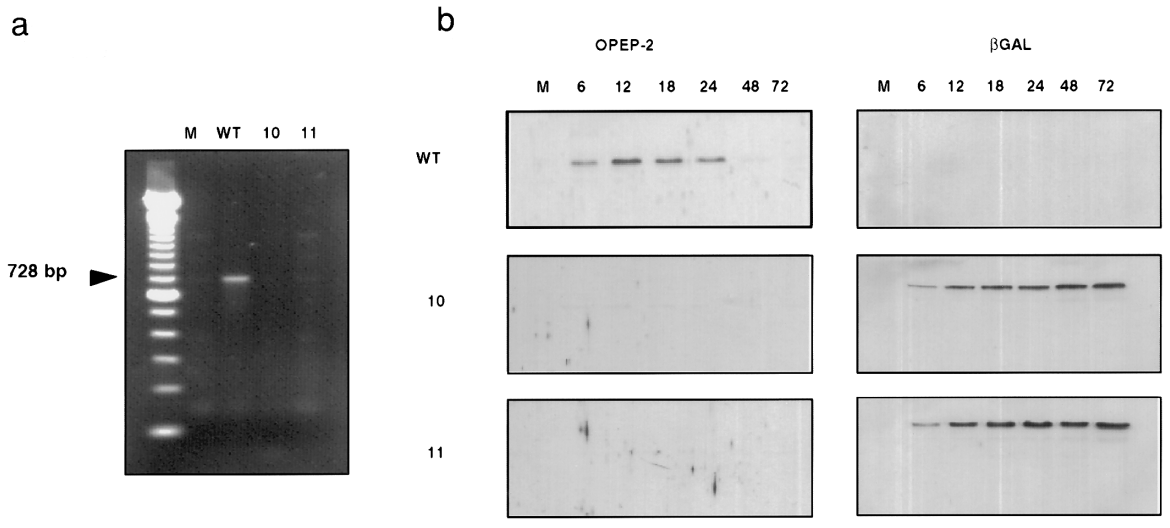

FIG. 6. Analysis of OPEP-2 deletion virus, vΔopep-2. (a) PCR analysis of WT and vΔopep-2 viral DNA. A single 728-bp fragment amplified from the WT DNA is indicated. M, mock; WT, WT OpMNPV; 10 and 11, vΔopep-2 isolates 10 and 11. (b) Western blot analysis of total cell proteins from WT- and vΔopep-2 10- and 11-infected Ld652Y cells from 6 to 72 hr p.i., probed with OPEP-2 polyclonal antisera or an anti-β-galactosidase monoclonal antibody.

of the time course. The expression of this gene was also unaffected by deletion of the *opep-2* ORF.

Figure 7b shows steady-state levels of three late genes, capsid, ODVP-6E, and polyhedrin in WT- and vΔopep-2-infected Ld652Y cells. Capsid protein was first detected at 48 hr p.i. in all three infections. ODVP-6E was detected weakly by 48 hr p.i. and was more prominent at 72 hr p.i. Polyhedrin was detected weakly at 24 hr p.i. and continued to increase up to 72 hr p.i. No differences were observed in expressions of these proteins in either the WT or the vΔopep-2 isolates 10 and 11.

DISCUSSION

In this paper, we report on the identification, mapping, and expression of a previously unidentified early gene, *opep-2,* of the baculovirus OpMNPV. There is no homologous gene in the complete sequence of either AcMNPV or BmNPV or any other known baculovirus sequence. *opep-2* is the second of three early genes found in the *ie1–ie2* intergenic region believed to be unique to OpMNPV. This region is one of only two large insertions in the OpMNPV genome relative to the AcMNPV genome (George Rohrmann, personal communication)

opep-2 is a unique early gene as it is transcribed as three transcripts immediately upon infection. The only early gene previously known to be expressed as multiple transcripts at similar times postinfection is *ie1* which is spliced. However, contrary to *ie1,* transcriptional mapping of *opep-2* indicated that the three mRNAs initiate at a single conserved baculovirus early gene transcriptional start site, CACAGT, but terminate using multiple polyadenylation signals at the 3′ end of the gene (Fig. 3c). The possible reason for this is that the region 5′ to the *opep-2* ORF is greater than 80% A+T and in such an environment the fidelity of the RNA polymerase may be affected. The *p8.9* gene, however, which also terminates in this A+T-rich region, utilizes only a single polyadenylation signal at early times p.i. (Wu *et al.,* 1993b)

The *opep-2* early gene initiation site, CACAGT, is similar to other baculovirus early genes including the OpMNPV genes *ie1, ie2, p34,* and *gp64-EFP,* all of which play important roles in OpMNPV replication (Blissard and Rohrmann, 1989; Theilmann and Stewart, 1991, 1992a; Wu *et al.,* 1993a). The *opep-2* CAT reporter constructs (Fig. 3d) showed that 176 bp 5′ to the CACAGT were sufficient for gene expression in uninfected Ld652Y cells. This region contains several conspicuous motifs—four GATA motifs (two in either orientation), a single CACGTG motif, and a 13-base direct repeat are present (Fig. 1b); a single copy of the direct repeat appears in the promoter of *EFP(gp64)* in AcMNPV and OpMNPV (Blissard *et al.,* 1989; Whitford *et al.,* 1989). The GATA and the CACGTG motifs have been previously shown to be host nuclear factor binding sites in lepidopteran cells (Kogan and Blissard, 1994; Krappa *et al.,* 1992) and may have roles in transcriptional regulation of *opep-2* or adjacent genes. This small promoter should provide an excellent model for dissecting the role of these elements in baculovirus early gene expression.

Western blots of total infected cell proteins probed with an OPEP-2-specific polyclonal antibody detected a single protein migrating at 32 kDa (Fig. 4a) which is significantly larger than the predicted size of 25 kDa. Post-translational modifications are a likely cause for the difference between predicted and observed sizes. *In vitro* translation of the cloned *opep-2* gene in rabbit reticulo-

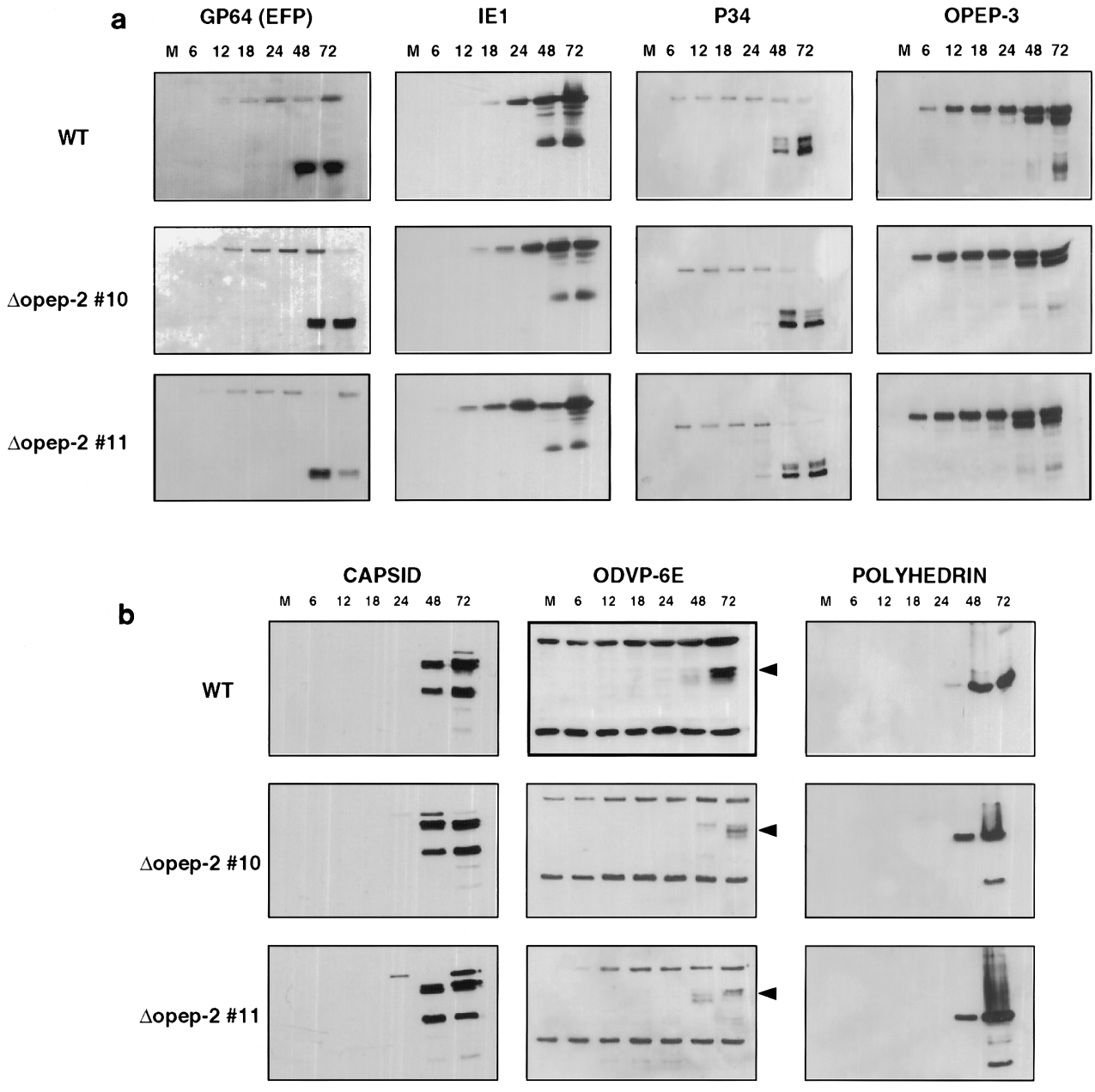

FIG. 7. Western blot analyses of steady-state levels of selected early (a) and late (b) proteins in WT- and vΔopep-2 10- and 11-infected Ld652Y cells from 6 to 72 hr p.i. The early proteins analyzed were GP64-EFP, IE1, P34, and OPEP-3 . Late proteins were capsid, ODVP-6E, and polyhedrin. The ODVP-6E-specific bands are indicated by the arrowheads. The numbers above each line indicate hr p.i. The protein analyzed is indicated on top and the virus on the left.

cyte lysate and immunoprecipitation with the OPEP-2-specific polyclonal antisera indicated three major bands, 25, 29, and 32 kDa (Fig. 4b). The 25-kDa band corresponds to the predicted size of OPEP-2, based on its amino acid sequence, and the largest band, 32 kDa, comigrates with the OPEP-2 protein from infected cells. Rabbit reticulocyte lysate systems have kinase activities (Joshi *et al.,* 1995), and therefore the 25- and 29-kDa proteins may be incompletely phosphorylated OPEP-2. There are 37 tyrosine, serine, and threonine amino acids of the 236 in OPEP-2, providing many potential sites for phosphorylation. Further studies will be required to determine if OPEP-2 is an OpMNPV phosphoprotein.

Analysis of nuclear and cytoplasmic protein fractions and total protein of infected cells indicated that OPEP-2 is predominantly a cytoplasmic protein (Fig. 4c). OPEP-2 was not detected in association with either the purified extracellular virus or the purified and fractionated occlusion-derived virus (Fig. 5) and is, therefore, not believed to have a structural role.

To aid in the elucidation of the possible role for OPEP-2 in the viral infection cycle, *opep-2* was de-

leted from the genome by homologous recombination *in vivo.* Viable deletion mutants were obtained which indicated that *opep-2* does not play an essential role in OpMNPV infection of Ld652Y cells in culture. Possible effects of the absence of OPEP-2 on expression of other selected early and late proteins was investigated by Western blot analysis of total proteins from both WT- and vΔopep-2-infected Ld652Y cells. No consistent differences in expression of GP64-EFP, IE1, P34, OPEP-3, capsid, ODVP-6E, or polyhedrin from WT- or vΔopep-2-infected Ld652Y cells were observed (Fig. 7).

It is surprising, considering that *opep-2* is expressed immediately upon infection and is tightly regulated, that its deletion did not affect viral growth. It is possible that the function of OPEP-2 is very tissue or host specific and is not required in Ld652Y cells. For example, the apoptosis-inhibiting gene of AcMNPV, *p35,* is required in Sf21 cells for viable virus production but is not required in TN368 cells (Clem and Miller, 1993). To identify the function of OPEP-2 it may be necessary to obtain additional OpMNPV-permissive cell cultures, or it could be required *in vivo.* We are currently actively pursuing *in vivo* bioassays to address this question.

In conclusion, this article describes the identification, expression, and characterization of a unique OpMNPV early protein, OPEP-2, that expands the repertoire of baculovirus genes not represented by the genomic sequences of AcMNPV and BmNPV. Deletion of the gene from the genome of OpMNPV results in viable virus, and has no effect on expression of selected proteins in the infection cycle and is not associated with either BV or ODV. It is expressed from 2 to 48 hr p.i., with peak levels occurring before DNA replication. The promoter structure of *opep-2* contains a baculovirus early gene motif and several recognizable promoter regulatory elements suggesting that *opep-2* is a true baculovirus early gene. The evolutionary conservation of these elements, along with its tight temporal regulation, suggests that *opep-2* does serve a role in the infection cycle of the virus and may have evolved to be utilized in a specific niche which has not yet been identified.

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