

Transcriptional and structural analyses of *Amsacta moorei* entomopoxvirus protein kinase gene (AMV197, *pk*)

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Abstract The *Amsacta moorei* entomopoxvirus (AMEV) genome has 279 open reading frames (ORFs) among which is the AMV197, composed of 900 nt and potentially encoding a protein of 299 amino acids. Sequence-derived amino acid analysis suggested it to be a serine/threonine protein kinase (PK) having conserved PK and serine/threonine PK domains. For transcriptional analysis of the AMV197 *pk* gene, Ld652 cells were infected with AMEV and mRNA was isolated at different times thereafter. RT-PCR analysis indicated that the transcription of the AMV197 *pk* gene started at 4 h post infection (h p.i.) and continued to be expressed through 24 h p.i. Infection of Ld cells in the presence of Ara-C (inhibits DNA replication), followed by RT-PCR showed that AMV197 *pk* is transcribed as an early gene. Transcription was initiated at 54 nt upstream of the translation start site. The vaccinia virus early promoter element G was also found at the correct position (−21) in the AMV197 *pk* gene. Rapid amplification of the 3' ends of the AMV197 *pk* transcript showed that there are two polyadenylation start points. They are located at 22 and 32 nucleotides downstream of translation stop site. Also, the translational stop site and poly (A) signal of AMV197 *pk* are overlapped. The termination signal TTTTGT sequence of vaccinia virus early genes was found just upstream of the 3' end of AMV197 *pk* gene. Conserved amino acid subdomains of the AMV197 PK were found by sequence comparisons with PK's from other organisms. Analysis of the protein sequence of AMV197 *pk* gene reveals close identity with PK genes of other organisms.

Keywords *Amsacta moorei* entomopoxvirus (AMEV) · Protein kinase · Transcriptional analysis

Introduction

Poxviruses are a family of large DNA genome viruses pathogenic for many species of mammals, birds and insects. Their genomes are dsDNA molecules with hairpin termini (Moss 2001). The International Committee on the Taxonomy of Viruses has divided *Poxviridae* family into two subfamilies: the *Chordopoxvirinae* (poxviruses of vertebrates) and the *Entomopoxvirinae* (insect poxviruses). The subfamily *Entomopoxvirinae* is a related but distinct member of the *Poxviridae* family. These viruses share many biological features of the poxviruses of chordates, but instead infect the larvae of a number of insect families. Entomopoxviruses (EPVs) have been isolated from several insect orders including Coleoptera, Lepidoptera, Orthoptera, and Diptera (Arif 1995). EPVs have structural similarities to orthopoxviruses and a number of vertebrate poxvirus gene homologues have been found in entomopoxviruses (Bawden et al. 2000). Because of this homology, it is reasonable to expect similarities in gene regulation at both groups.

Amsacta moorei entomopoxvirus (AMEV), genus betaentomopoxvirus, has been reported to infect agriculturally important pests, such as *Estigmene acrea* and *Lymantria dispar* included lepidopteran insects. A characteristic pathology is associated with each of the four orders of insects infected. The course of infection in lepidopteran larvae is relatively rapid, ranging from 1 to 3 weeks. Symptoms of the disease vary among hosts. For example, *Estigmene acrea* larvae infected with AMEV show little signs until late in the infection, when motility

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and coordination are adversely affected. EPV-infected *Elasmopalpus lignosellus* larvae change color, from brown-striped to red, with hemolymph becoming whitish-blue, possibly because of the accumulation of spheroid (a single major structural polypeptide) occlusion bodies (Roberts and Granados 1968).

The 232-kb AMEV genome has been completely sequenced and contains 292 open reading frames (ORFs). Among these ORFs, AMV153 and AMV197 suggested it to be a serine/threonine protein kinase (PK) having conserved PK and serine/threonine PK domains (Bawden et al. 2000).

Serine/threonine protein kinases are phospho-transferases that transfer the phosphate groups onto serine and/or threonine residues of protein substrates. Improper functioning of these enzymes is often manifested in several malignancies since modification of proteins by phosphorylation at a limited number of amino acid residues is identified as a general method of controlling activities of protein synthesis, cell division and modulation of metabolic enzymes (Hunter 1987) in eukaryotes and prokaryotes. Phosphorylation of numerous cellular and viral proteins is also observed in virally infected cells, which suggests that protein kinases may have a role in regulating a wide variety of viral infections (Leader and Katan 1988; Kann et al. 1999).

The PK of some vertebrate viruses is known to be packaged within the virion particle and shown to phosphorylate a variety of proteins of viral and non-viral origin (Lin et al. 1992; Rempel and Traktman 1992; Lin and Broyles 1994). Viruses often utilize many of the regulatory mechanisms as cells, and this is particularly evident for the poxviruses, such as vaccinia virus. DNA sequence analysis suggests that vaccinia virus may encode a protein kinase. The 34-kDa protein predicted from the sequence of the viral gene B1R bears a striking similarity to the catalytic domains of known protein kinases (Howard and Smith 1989; Traktman et al. 1989; Goebel et al. 1990). Most of the amino acids known to be conserved in the protein kinase family are located in the appropriate sites of the sequence predicted for the B1R protein. This protein kinase is expressed early in infections, is found in the viroosomes, and is also packaged into virions (Banham and Smith 1992). It appears to be an essential viral protein, and temperature-sensitive mutations that map to the B1R gene produce virus that cannot replicate its DNA at the restrictive temperature (Rempel et al. 1990; Rempel and Traktman 1992).

Amino acid alignment of AMV197 encoding a protein of 299 amino acids showed that this gene is an ortholog of B1R. However, there are no data on PKs of entomopoxviruses in literature. As part of our continuing work on functional analysis of AMEV genome, we report here on structural and transcriptional analyses of AMV197 *pk* gene as part of basic studies to understand its function in the context of the infection process.

Our results show that the AMV197 *pk* gene is an early gene whose transcripts are detected at 4–24 h post infection (h p.i.). It has 54 nt region as 5' UTR and 32/22 nt regions as 3' UTRs. We also investigated the positions of the protein kinase subdomains in the PK sequence.

Materials and methods

Cell culture and virus *Lymantria dispar* (Ld652) cell line used in this study was obtained from Basil Arif and maintained in Excell-420 (SAFC Biosciences) and Grace's Insect serum-free medium (Gibco) supplemented with 10% heat-treated fetal bovine serum (Gibco). Penicillin (50 IU) and streptomycin (50 µg/ml) were added to cell line growth media to prevent microbial contamination. AMEV was kindly supplied by Basil Arif. Replication of AMEV in the cell line has been described previously (Goodwin et al. 1990). For virus production, T75 flasks containing Ld652 cells at a density of 9×10^6 cells/ml were infected with wild type AMEV at a low m.o.i (0.5 p.f.u./cell) The virus was harvested when the cytopathic effect was complete at 4-day post infection (h p.i.). Subsequently, the supernatant was centrifuged at $1,000 \times g$ for 5 min to remove intact cells and cellular debris. The resulting supernatant contained the extra-cellular virus was stored at 4°C. Virus titer was determined by end point dilution assay (EPDA) (Darling et al. 1998).

Messenger RNA (mRNA) isolation Ld652 cells were seeded at a density of 1×10^7 /T75 flask and infected at an m.o.i of 2 with AMEV. At 2 h p.i., the medium was removed and replaced with fresh medium. Cells were harvested at various times after infection (0, 1, 2, 4, 7, 12, and 24 h) and pelleted at $300 \times g$ for 5 min. The pellet was washed with phosphate-buffered saline ($1 \times$ PBS). Messenger RNA (mRNA) was isolated with the PolyATtract System 1000 kit (Promega) following the manufacturer's instructions and quantified at 260 nm. An aliquot of 10 µg mRNA was treated with 200 U of RNase free DNase I at 37°C for 30 min to remove any residual DNA and then extracted with phenol-chloroform and quantified after precipitation.

Time course analysis of AMV197 *pk* specific transcripts D-Nase I treated mRNA preparations were screened for the presence of AMV197 *pk* gene-specific transcripts by RT-PCR. Briefly, 2 µg of mRNA and 75 µM reverse oligo (dT) anchor primer (Roche) were denatured at 70°C for 5 min and then placed on ice. First-strand cDNA was synthesized by the addition of 2.5 µl M-MuLV 10X buffer, 2 µl of 10 mM dNTPs, 100 U M-MuLV reverse transcriptase (BioLabs). DEPC-treated water was added to make a final volume of 25 µl. The reaction proceeded at 37°C for 1 h,

followed by heating at 70°C for 15 min. PCR was performed in a 50 ml volume containing 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 0.1% Tween 20, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 0.2 mM primers PKSP4 and PKR (Table 1), 2.5 U of *Taq* DNA polymerase, and 1 µl of completed RT-PCR reaction mixture. Amplification consisted of 1 cycle of denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final cycle of elongation at 72°C for 7 min. PCR amplification was also done on mRNA isolated from non-infected cells to confirm that there was no cellular contamination.

The temporal class analysis of the AMV197 *pk* transcript Ld652 cells were infected with AMEV at a multiplicity of infection of 2. In order to inhibit DNA synthesis, cells were continuously exposed to cytosine arabinoside (Ara-C) (100 µg/ml; Sigma) starting at 1 h prior to virus infection. The inhibitor was maintained at this level throughout the infection. At 0 and 16 h p.i., cells were harvested, and mRNAs were isolated from virus-infected cells in the presence and absence of Ara-C by using PolyATtract System 1000 kit (Promega) according to the manufacturer's protocol. The isolated mRNAs were DNase treated, quantified at 260 nm, subjected to RT-PCR and subsequently to PCR as above. RNA samples from mock infected cells and 0 h p.i. were processed as control.

Determination of the 5' UTR of AMV197 *pk* transcript The 5' untranslated region of the AMV197 *pk* mRNA was obtained by rapid amplification of the cDNA ends (Frohman et al. 1988) using a 5' RACE kit (Roche) following the procedure supplied by the manufacturer with a set of three specific primers. First strand cDNA was synthesized from 2 µg mRNA isolated at 16 h p.i. using gene-specific primer (PKSP1R; see Table 1). The first-strand cDNA was then isolated and tailed with dA. This was followed by two consecutive nested PCRs with specific primer (see Table 1). PKSP2R primer was used for the first PCR with an oligo

dT anchor primer (Roche). PKSP3R primer was used for the second PCR in combination with a PCR anchor primer (Roche). The amplified fragments were cloned into pGEMT-Easy (Promega) and 11 clones were analyzed by automated sequencing.

Determination of the 3' UTR of AMV197 *pk* transcript To determine the 3' terminus of the AMV197 *pk* transcript, 3' RACE was performed. The first strand cDNA was synthesized from mRNA using oligo (dT) anchor primer and M-MuLV reverse transcriptase. Then, the first strand cDNA was amplified by PCR using a gene specific primer PKSP4 and a PCR anchor primer corresponding to the oligo (dT) anchor primer (Table 1). The first amplification product was then used as a template for the second PCR amplification with the second gene specific primer PKSP5R and PCR anchor primer. The PCR product was isolated from gel, cloned in pGEM-T (Promega) and 13 clones were sequenced.

Amino acid sequence comparison The predicted amino acid sequence of AMEV was examined by using Expasy-Prosit program and compared to those of other PK family members (2 viral PKs, 2 PKs from vertebrates and a PK from *S. cerevisiae*) with the aid of Clustal W program.

Results

Time course transcriptional analysis of AMV197 *pk* gene To determine whether the putative AMV197 gene was expressed during viral infection, mRNA was extracted from AMEV infected Ld652 cells at 0, 1, 2, 4, 7, 12, and 24 h p.i. and from mock infected cells. The samples were analyzed by RT-PCR. A single band with an expected size of 521 bp as *pk* transcript was first detected at 4 h p.i.,

Table 1 Primers used for 5' RACE (PKSP1R, PKSP2R, and PKSP3R), 3' RACE (PKSP4, PKSP5) and RT-PCR (PKSP4, PKR) experiments

Primer name	Sequences
PKSP1	5'-CAT AAT AAT CTA TTA CTA TAT ATC-3'
PKSP2	5'-CCA TTA TGA TAT AGT AAT GGT ATT CC-3'
PKSP3	5'-GCA TTT CTA CAA ACA ACG G-3'
PKSP4	5'-CCG TTG TTT GTA GAA ATG C-3'
PKSP5	5'-CAA CAC TTG TTT AAC TAA TTC ACC-3'
PKR	5'-GGC AAA CTT CCA CTA TAC C-3'

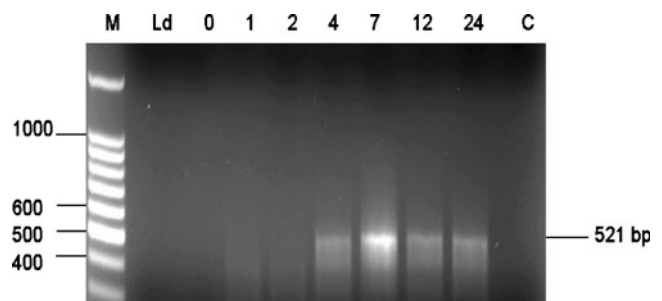


Fig. 1 Transcriptional pattern of AMV197 *pk* gene detected by RT-PCR analysis. *M* represents a 100-bp ladder DNA marker (Promega). *Ld* indicates DNA amplified from the RNA from mock infected Ld652 cells. Numbers show time points hours (h) post-infection for each RNA samples extracted from infected cells. *C* shows negative control for the PCR. The amplicons were electrophoresed in a 1.2% agarose gel, and their sizes (in base pairs) are indicated at the right

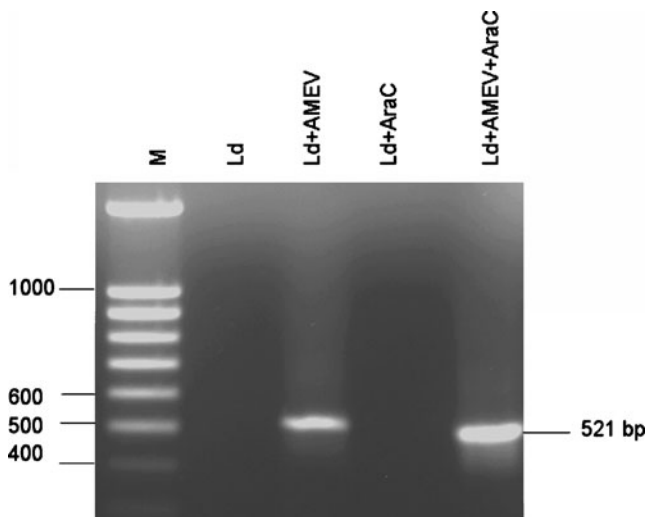


Fig. 2 Temporal class of AMV197 *pk* gene determined by RT-PCR analysis in the presence of DNA synthesis inhibitor. *M* represents a 100-bp ladder DNA marker (Promega; lane 1). mRNA's extracted from Ld652 cells in the absence (lane 2) or presence (lane 4) of Ara-C and from AMEV-infected Ld652 cells in the absence (lane 3) or presence (lane 5) of Ara-C were subjected to RT-PCR analysis using primers specific for the AMV197 *pk* gene. The amplicons were electrophoresed in a 1.2 % agarose gel, and their sizes (in base pairs) are indicated at the right

reached to maximum level at 7 h p.i. and started to decrease after this point (Fig. 1).

The transcriptional class analysis of the AMV197 *pk* gene In order to determine the expression class, we employed an RT-PCR approach to detect the transcriptional products of AMV197. To that aim, Ld652 cells were

infected with AMEV in the presence or absence of Ara-C, an inhibitor of DNA replication. mRNA was extracted from cells at 16 h p.i. and analyzed for the presence of AMV197 transcript by RT-PCR (Fig. 2). AMV197 transcript was observed at this time point (Fig. 2, lane 3) and, apparently, it was not affected by the presence of inhibitor of DNA (Fig. 2, lane 5). Because viral DNA replication is not required for the expression of the AMV197 gene, it belongs by definition to an early gene class. This result is in agreement with other viral *pk* gene's transcription classes (Liu et al. 2001; Gershburg et al. 2007).

Transcription initiation site of AMV197 *pk* gene The transcription start site for AMV197 gene was identified by 5' RACE analysis using mRNA extracted from infected cells at 16 h p.i. 5' RACE was performed using three specific primers (PKSP1R, PKSP2R, and PKSP3R) (Table 1). Amplified 5' ends of cDNA fragments were cloned in pGEMT-Easy vector and 11 clones were sequenced. The 9 clones obtained for AMV197 showed that the transcription initiation site (+1) is at the adenine (A) located 54 nt upstream of the translational start site (Fig. 3).

Transcription termination site of AMV197 *pk* gene To determine the 3' terminus site of the AMV197 gene, a 3' RACE was performed on mRNA. The first strand cDNA was synthesized using the oligo(dT)-anchor primer and M-MuLV reverse transcriptase. The amplification of the 3' RACE PCR products were cloned and 13 clones were sequenced. The polyadenylation starts at 22 nt downstream of the translational stop point in 8 clones and 32 nt

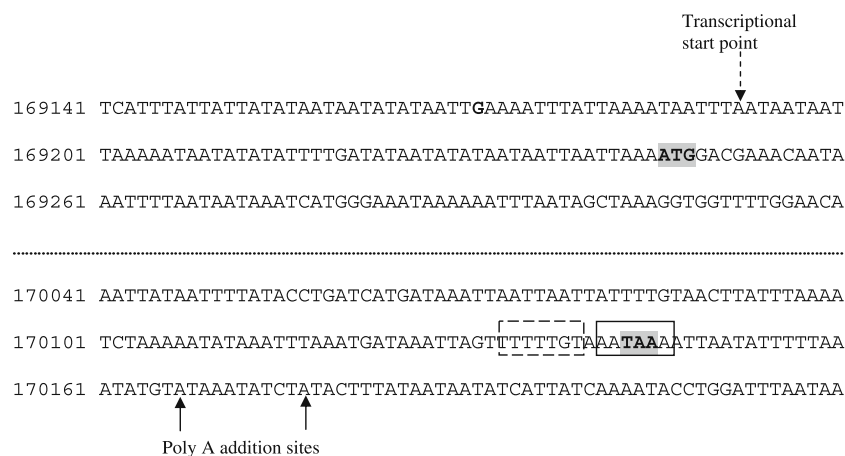


Fig. 3 The locations of transcription initiation and termination sites of AMV197 *pk* gene. The translation start and stop codons are shaded gray. The dashed arrow indicates the 5' termini (transcriptional start points) of AMV197 *pk* gene revealed by sequencing of 5' RACE clones. The bold *G* at -21 shows the universal *G* residue that is found

at the promoter region of vaccinia virus early genes. The poly (A) addition sites that are obtained by sequencing of 3'RACE clones are indicated by the straight arrows. The box indicates poly (A) signal which is overlapped with the translation stop codon and the dashed box indicates transcription termination signal

AMEV-PK	-----	
VACV-B1-RPK	-----	
MSEV-PK	-----	
PIM-1	-----MLLSKINSLAHL	12
MOS	MAHSTPCSQTS LAVPNHFSLVSHVTVPSEGVMPSPLSLCRYLPRELSPSVDSRSCSIPLV	60
CDC28	-----	
AMEV-PK	-----MDETI--NFNNK-SWEIK-----NLIAGKGGFGTVYKLCCKNDNN	36
VACV-B1-RPK	-----MNFQGLVL--TDNCKNQWVVG-----PLIGKGGFGSIYTTNDNN---	37
MSEV-PK	-----MIL--KSVRGNIQVVS-----ECLVNEYIINIHLLEDDN--	33
PIM-1	RARPCNDLHATK LAPGKE--KEPLESQYQVG-----PLLGSGGFGSVYSGIRVADN-	61
MOS	APRKAGKLF LGTTPPRAP--GLPRRLAWF SIDWEQVCLMHR LGS GGFSGVYKATYHGVP-	117
CDC28	-----MSGELANYKRLEK-----VGBGTYGVVYKALDLRPG-	31
I		
AMEV-PK	NCYAIKIEPSDNGPLFV-EMHFYKINK-NEIKNFIDAKNLSYLGIPLLYH-NGIIKKN	93
VACV-B1-RPK	--YVVKIEPKANGSLFT-EQAFYTRVLKPSVIEEWKSHNIKHVGLITCKA-FGLYKSI	93
MSEV-PK	--KIKIASIEDGPLFC-EINFIYRYCK-----EEMVKD VNILPYVD-SGIDKYK-	79
PIM-1	LPVAIKHVEKDRISDWG-ELPNGTRVPMEVLLKVKVSSDFSGVIRLLDWF E-RPDSFVLI	119
MOS	--VAIKQVNKCTEDLRASQRSFWAELNIAGLRHDNI VRRVVAAS TRTPEDSNLSGTI IMEF	175
CDC28	--QGQRVVALKKIRLESEDEGVPSTAI REISLLKELKDDNI VRLYDIVHSD-AHKLYLVF	88
II III		
AMEV-PK	IEYRYIVIDYYEFNLNDILKK-YIKLPIITIKITIQILYILEYLHKKKYTHNDIKKNNI	152
VACV-B1-RPK	VEYRFLVINRLGADLDAVIRANNRPLKRSVMLIGIEILNTIQMFHEQGYSHGDIKASNI	153
MSEV-PK	-KLRFLVLPKLDYNLKQISIN-----NIYNLSKDIIFALKYIHSKEYIHSDIKDENI	130
PIM-1	LERPEPVQDLDFDIT ERGALQ-----EDLARGFFWQVLEAVRHCHNCV LHRDIKDENI	173
MOS	GGNVTLHQVIYDATRSPEPLSCRQQLSLGKCLKYSLDVVNGLLFLHSQSLHLDLK PANI	235
CDC28	EFLDLDLKRYMEGIPKQDPLG-----ADIVK KFMQLCKGIAYCHSHRILHRDLK PQNI	142
IV VI		
AMEV-PK	MFNSS-LTKVYLIDYGLIYNMNSNQEYN--IKCSN---DGTLEYLP-LITHLFG LKTYMG	205
VACV-B1-RPK	VLDQIDKNKLYLV DYG LVS KFMS GEHVFP FIRNPNKMDNGTLEFTP-IDSHKGYVVSRRG	212
MSEV-PK	LYDKN-NNKFYLTDFGNITKHTK-----FITNCKLVNNGTYKYIG-IDMHNG-ISTYKS	182
PIM-1	LIDL S-RGEIKLIDFGSGALLKDT-----VYTFDGT RVYSPPEWIRYHRYHGRSA	223
MOS	LISEQ--DVCKISDFGCSQKLQDLRGRQ---ASPPHIGGTYTHQA-PEILKGEIATPKA	288
CDC28	LINKD--GNLKLGD FGLARAFGVP-----LRAYTHEIVTLWYRAPEVLLGGKQYSTGV	193
VII VIII		
AMEV-PK	DIESLMYNIIEWYSGSLPWI KYKKNVILK KLDFFNTCLTNSPIEICKLYNIK NAPS IY	265
VACV-B1-RPK	DLET LGYCMIRWLG GILPWTKISETKNCALVSATKQKYVNNTATLLMTSLQYAPRELLQY	272
MSEV-PK	DLESFGYLLYDILYG-LPWNNKLDKDI INIKKDFIDNVI ICN-NKLSKYFLYLS-----	234
PIM-1	AVWSLGI LLYDMVCGDI PFEHDEE I IKGQVFRQTVSSECQHLIKWCLSLRPSDRPSFEE	283
MOS	DIYSFGITLWQMTTREV PYSGEPEYVQYAVVAYNLRPSLAGAVFTASLTGKALQN-IIQS	347
CDC28	DTWSIGCIFAEMCNRP I FSGDSEIDQIFKIFRVLGTPNEAIWPDIVYLPDFKPSFPQWR	253
IX		
AMEV-PK	NYNFI PDHDKL INYFVTYLKS KNINLNDKLVFCK-----	299
VACV-B1-RPK	----ITMVNSLTYFEEPNYDEFRIHLMQGVVY-----	300
MSEV-PK	----LIENDKNIEY-VTL LK TLE-----	252
PIM-1	IRNHPWMQGDLLPQAASEIHLHLSLSPGSSK-----	313
MOS	CWEARGLQRPSAELLQRDLKAFRGT LG-----	374
CDC28	RKDL SQVVP SLDFR GIDLLDKLLAYDPINRISARRAAIHPYFOES	298

Fig. 4 Comparison of the amino acid sequence of AMV197 PK with other PK's of other organisms. The sites of the conserved catalytic subdomains are numbered with Roman numbers and shaded. AMEV-PK, *Amsacta moorei* entomopoxvirus protein kinase (Accession no: NC002520); VACV-B1-RPK, Vaccinia virus protein kinase (Accession no: YP233065); MSEV-PK, *Melanoplus sanguinipes* entomo-

poxvirus protein kinase (Accession no: AF063866); PIM-1, *Mus musculus* protein kinase (Accession no: AAA39930); MOS, serine/threonine-protein kinase-transforming protein (*mos*) of Mouse (Accession no: P00538); CDC28, cell division control protein 28 of *S. cerevisiae* (Accession no: P00546)

downstream of the translational stop point in 5 clones of sequenced 13 clones as shown in Fig. 3.

Amino acid sequence alignment of AMV197 pk gene When the deduced amino acid sequence of AMV197 *pk* gene was analyzed in Expasy-Prosites, it was observed that the location of protein kinase catalytic domain is between residues of 12 and 299.

The amino acid sequence of AMV197 PK was also compared with other PK family members (with especially

B1R). Major subdomains conserved in various PKs were also readily identified on the AMV197, but a few subdomains were difficult to be ascertained (Fig. 4).

At the N-terminal extremity of the catalytic domain, there is a region (subdomain I) containing a glycine (G)-rich stretch of residues (21GFGTVY26) and nearby there is the lysine residue (42 K) of subdomain II (39–42). The region from subdomain I to II has been shown to be involved in ATP binding (Hanks and Hunter 1995). In the PROSITE database,

this ATP binding region is one of only two regions that have been selected to build a protein kinase signature. The sequence (aa 21–42) in this region matches (although not exactly) the protein kinase signature consensus pattern I: [LIV]-G- $\{P\}$ -G- $\{P\}$ -[FYWMGSTNH]-[SGA]- $\{PW\}$ -[LIV-CAT]- $\{PD\}$ -x-[GSTACLIVMFY]-x-(5,18)-[LIVMFYWC-STAR]-[AIVP]-[LIVM FAGCKR]-K, where K is the residue that binds ATP (square brackets are used to explain the acceptable alternative amino acids for a given position and the curly brackets explain the amino acids that are not acceptable at a given position). The sequences of 54E and 98YIV100 show homology to subdomains III and IV, respectively. Subdomain V is not clearly discernable.

Subdomain VI is highly conserved among protein kinases and is used to build the protein kinase signature consensus pattern II. The sequences 142YTHNDIKKN-NIMF154 matches well with the protein kinase signature consensus pattern II, [LIVMFYC]-x-[HY]-x-D-[LIVMFY]-K-x-(2)-N-[LIVMFYCT] (3), where the aspartic acid residue (146D) is probably the residue important for the catalytic activity of the enzyme (Knighton et al. 1991; Liu et al. 2001). The residue at position 148 is K, indicating that it is a serine/threonine specific protein kinase. Subdomain VII is found in a highly conserved region, 163LIDYG167.

Subdomain VIII has a highly conserved triplet, A-P-E, in most protein kinases. This region is thought to lie close to the catalytic site and to flank a common autophosphorylation receptor site (Selten et al. 1986; Hunter 1987). No identical sequence is found within the AMV197 PK sequence. Another sequence, T-L-E, in the appropriate region has been found and thought to be subdomain VIII in accordance with the vaccinia 34-kDa protein kinase subdomain VIII (Traktman et al. 1989). In this study, the possible change of the APE motif to TLE is clearly explained. It is found that this motif within published kinase sequences reveals some heterogeneity and demonstrates that the final glutamic acid is the only invariant residue (Selten et al. 1986). Replacement of alanine with other neutral residues (glycine, leucine and proline) has been observed. Alanine (neutral), leucine, and isoleucine (nonpolar) have been found as substitutes for the central proline. Subdomain IX is thought to be placed at the sequences 206DIESLM211, but may also include the 212YNIIEWYSG220 sequence. Subdomains X and XI are not so clearly defined in AMV197 PK but amino acid residues 221 to 225 may potentially constitute subdomain X.

Discussion

Although poxviruses are known to incorporate enzymes such as DNA-dependent RNA polymerase (Baroudy and Moss 1980; Shchelkunov et al. 1993), poly (A) polymerase (Moss et al. 1975), mRNA guanyltransferase and mRNA

methyltransferase (Martin et al. 1975; Barbosa and Moss 1978) into virions, retention of a protein kinases had not previously been shown. The studies on poxvirus protein kinases were initially conducted on vaccinia virus B1R and F10L protein kinases. They were followed by investigations on other poxvirus PKs; Fowlpox virus (FWPV-PK, Poxviridae; Afonso et al. 2000) Myxoma virus (MYXV-PK, Poxviridae; Cameron et al. 1999), *Molluscum contagiosum* virus subtype 1 (MOCV1-PK, Poxviridae; Senkevich et al. 1996), and Variola major virus (VARV-PK, Poxviridae; Massung et al. 1993). However, to date, data on the *Amsacta moorei* Entomopoxvirus (AMEV) protein kinase (*pk*) gene have not been reported.

In order to study the mechanisms controlling AMEV gene regulation, we have, as a first step, initiated transcriptional and structural analyses of the *pk* gene.

Based on the temporal expression, the AMV197 *pk* gene was classified as an early gene, just as B1R belongs to vaccinia virus. The transcripts were detected early after infection (by 4 h p.i. and maximized at 7 h p.i.) a time relegated primarily to the synthesis of non-structural proteins, including enzymes involved in replicating the genome and modifying DNA, RNA, and proteins (<http://www.virustaxonomyonline.com/virtax/lpext.dll?f=templates&fn=main-h.htm>).

The transcriptional start and stop sites were determined by 5' and 3' RACE analysis. 5' RACE showed that AMV197 *pk* gene has a short 5' UTR of 54 nt. Also, the promoter region has a universal G residue at -21 flanked by variable and A-T-rich sequence (Fig. 4). This sequence matches well with the vaccinia virus early promoter element identified by Davison and Moss (1989).

The 3' UTR results revealed the transcripts with heterogeneous 3' ends were derived from cleavage sites starting 22 nt and 32 nt downstream of the translation stop site, which overlaps the polyadenylation signal AAUAAA. RNA polyadenylation plays an important role in the control of viral and eukaryotic gene expression and involves cleavage of the nascent transcript and addition of a poly (A) tail with 150–200 adenylate residues (Gilmartin 2005). One cleavage site for the AMV197 transcripts was mapped to nt 170167. The other cleavage site was mapped downstream of the first one, with only ten nucleotides separating them at nt 170177 (Fig. 4). The heterogeneity of the cleavage site usage in AMV197 transcripts may simply reflect selection flexibility, which is common in mammals (Pauws et al. 2001).

We examined the AMV197 gene sequence for the presence of termination signal (TTTTTGT) normally found just upstream of the 3' ends of early poxvirus mRNAs (Yuen and Moss 1987). However, no termination signal was found at that region. Further analysis of the AMV197 *pk* gene sequence showed that the termination consensus sequence is located in the coding sequence of AMV197

(Fig. 4) as in the case of vaccinia virus early gene and flanked by the polyadenylation signal.

Protein kinase catalytic domains range from 250 to 300 amino acid residues, corresponding to 30 kDa. Fairly precise boundaries for the catalytic domains have been defined through an analysis of conserved sequences as well as by assays of truncated enzymes (Hanks et al. 1988). The location of the catalytic domain within the protein is not fixed but, in most single subunit enzymes, it lies near the carboxyl terminus, the amino terminus being devoted to a regulatory role. In protein kinases having a multiple subunit structure, subunit polypeptides consisting almost entirely of a catalytic domain are common. The AMEV catalytic domain also covers almost the entire sequence of the PK gene, starting from the 12th amino acid. Also, the amino terminus of AMV197 PK catalytic domain lies as close as ten residues from the first conserved glycine, as indicated for some catalytic domain polypeptides by Hanks et al. (1988).

A large number of *pk*-related genes have been sequenced and analyzed for their amino acid sequence patterns. Hanks et al. (1988) have reported the identification of 11 conserved regions in the catalytic domains of PKs on the basis of an alignment of 65 different members of the PK family. In AMV197 PK, we clearly identified the subdomains I, II, VI, VII, VIII and predicted subdomains III, IV, IX and X. However, the subdomains V and XI were difficult to locate.

In this paper, we showed that AMV197 gene belongs to early viral gene class by RT-PCR analysis. The vaccinia early promoter region and the transcription termination signal confirm this result. Thus, this gene is transcribed by enzymes derived and packaged within the virion core (Banham and Smith 1992). Early gene expression is independent of viral replication and depends only on previous assembly of a competent virus particle and a subsequent partial uncoating of the infecting virus. When the predicted amino acid sequence was compared with those of other PKs, major subdomains which are conserved in various PKs could be readily identified, but a few subdomains were difficult exactly assign. The identified subdomains were homologous to counterparts of PKs of other organisms. However, these subdomain sequences presented here are only preliminary data. In order to confirm the validity of these results, genetic studies will have to follow.

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