The *pnk/pnl* gene (ORF 86) of *Autographa californica* nucleopolyhedrovirus is a non-essential, immediate early gene

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*Autographa californica* nucleopolyhedrovirus (AcMNPV) ORF 86, located within the HindIII C fragment, potentially encodes a protein which shares sequence similarity with two T4 bacteriophage gene products, RNA ligase and polynucleotide kinase. This AcMNPV gene has been designated *pnk/pnl* but has yet to be assigned a function in virus replication. It has been classified as an immediate early virus gene, since the promoter was active in uninfected insect cells and mRNA transcripts were detectable from 4 to 48 h post-infection and in the presence of cycloheximide or aphidicolin in virus-infected cells. The extremities of the transcript have been mapped by primer extension and 3' RACE–PCR to positions 18 from the translational start codon and 15 downstream of the stop codon. The function of *pnk/pnl* was investigated by producing a recombinant virus (Acdel86lacZ) with the coding region replaced with that of lacZ. This virus replicated normally in *Spodoptera frugiperda* (Sf 21) cells, indicating that *pnk/pnl* is not essential for propagation in these cells. Virus protein production in Acdel86lacZ-infected Sf 21 cells also appeared to be unaffected, with normal synthesis of the IE-1, GP64, VP39 and polyhedrin proteins. Shut-down of host protein synthesis was not abolished in recombinant infection. When other baculovirus genomes were examined for the presence of *pnk/pnl* by restriction enzyme digestion and PCR, a deletion was found in AcMNPV 1.2, *Galleria mellonella* NPV (GmNPV) and *Bombyx mori* NPV (BmNPV), suggesting that in many isolates this gene has either never been acquired or has been lost during genome evolution. This is one of the first baculovirus immediate early genes that appears to be non-essential for virus survival.

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**Introduction**

The complete sequence of *Autographa californica* nucleopolyhedrovirus (AcMNPV) clone C6 was determined by Ayres *et al.* (1994). Computer-aided analysis of the AcMNPV genome identified 154 nonoverlapping, methionine-initiated potential open reading frames (ORFs) of at least 150 nucleotides (nt). Many of the products of these genes have yet to be assigned a function. Comparing baculovirus sequences with previously characterized genes from other organisms provides a valuable indication of the potential role of a predicted protein product. Subsequent deletion of the gene from the AcMNPV genome may confirm the presumed function. In addition to directed mutagenesis of virus sequences to elucidate gene function, natural deletions in baculovirus genomes can also provide useful information about virus replication. In this study we have combined the two approaches outlined above to characterize a predicted baculovirus gene product with similarity to two bacteriophage T4 proteins, an RNA ligase and a polynucleotide kinase, encoded by genes 63 and pseT respectively. Interestingly, these prokaryotic gene products are involved in the control of the host response to virus infection (Snyder & Kaufmann, 1994). The AcMNPV gene was originally designated ORF 86 or *pnk/pnl*, even though enzyme activity had yet to be associated with the putative gene product (Ayres *et al.*, 1994). We have continued to use the latter name for this gene in this report. In an attempt to identify the function of *pnk/pnl*, the coding region was removed from the AcMNPV genome and the resulting recombinant virus was compared to wild-type virus to determine if the absence of *pnk/pnl* affected virus growth in Sf
21 cells. In parallel studies, experiments were performed to analyse the activity of the promoter and to map the transcripts. Finally, we have examined the occurrence of this gene in a different strain of AcMNPV, clone 1.2 (Croizier & Quiot, 1981), in a closely related *Galleria mellonella* nucleopolyhedrovirus (*GmMNPV*) isolate (Croizier & Quiot, 1981) and in *Bombyx mori* nucleopolyhedrovirus (*BmNPV*) clone 7 (Croizier et al., 1994).

**Methods**

### Cells and viruses.

Two *AcMNPV* strains were used in this study: 1.2 (Croizier & Quiot, 1981) and C6 (Possee, 1986). A *GmMNPV* clone (Croizier & Quiot, 1981) was also used. These strains and the viral recombinant baculovirus *Acdel86lacZ* were propagated and titrated by plaque assay in *Spodoptera frugiperda* IPLB-Sf 21 cells (Vaughn et al., 1977), cultured in TC 100 medium supplemented with 5 or 10% (v/v) foetal bovine serum (Gibco-BRL) as described previously (King & Possee, 1992).

To inhibit *AcMNPV* DNA replication, virus-infected Sf 21 cells were treated with aphidicolin (5 µg/ml; Sigma) 1 h after virus inoculation. For inhibition of protein synthesis, cycloheximide (100 µg/ml; Sigma) was added to the cells 30 min before infection.

### Construction of *pnk*/*pnl*-deficient recombinant baculovirus.

The transfer vector pAcADD1 was generated by the insertion into pUC18 of two sequences flanking the *pnk/pnl* coding sequence. A single BamHI restriction enzyme site replaced the coding region.

DNA fragments corresponding to the sequences upstream and downstream of *pnk/pnl* were generated by PCR. The plasmid pATHindIII C, containing the HindIII C restriction fragment of *AcMNPV* clone C6 within pAT153, was used as a template. The oligonucleotide primers were: HC 2800 (5' AGATTTGTG 3'); position 74213 relatively to C6 positive strand); HCR 5088 (5' GCATGTCATGCATGTCATGTCTTATAGC 3'; position 72135 relatively to C6 positive strand); HCR 5088 (5' GGTACAAGTTGCCGTACATAACAATACACATCG 3'; position 76501, complementary strand); HC 1 (5' GGATAGGCCCACCGTCAAGTTCTG 3'; position 71415, positive strand) and HCR 718 (5' GCTTGGATCCATGCTTATATATG 3'; position 72131, negative strand). The product generated using HC 2800 and HCR 5088 was digested with BamHI and EcoRI, and inserted into a pUC18 cloning vector.

The product generated using HC 2800 and HCR 5088 was digested with BamHI and EcoRI, and inserted into a pUC18 cloning vector.

To construct the transfer vector pAcADD2, the *Escherichia coli lacZ* gene was excised from a modified pCH110 plasmid (Possee & Howard, 1987) and inserted into the BamHI site of pAcADD1 in the correct orientation for expression: lacZ inserted in the opposite orientation in pAcADD1 produced pAcADD3.

A baculovirus recombinant deficient in *pnk/pnl* was obtained by co-transfection of *Sf* 21 cells with C6 DNA (500 ng) and pAcADD2 (10 µg) using DOTAP reagent (Boehringer Mannheim). Recombinant baculovirus *Acdel68lacZ* was plaque purified as described in King & Possee (1992), by selecting plaques staining blue in the presence of X-Gal.

### Southern blot analysis.

Southern blot analysis of *Acdel68lacZ* was performed as described (King & Possee, 1992) with the following modification: DNA probes were labelled with digoxigenin (Boehringer Mannheim) and hybridization was detected by chemoluminescence (Tropix).

### Virus growth curve.

*Sf* 21 cells (6 x 10⁵) were infected with AcMNPV C6 or *Acdel68lacZ* at an m.o.i. of 5 p.f.u. per cell in 25 cm² flasks. Following inoculation, cells were washed twice and recovered with 5 ml of medium culture. Progeny virus was harvested until 96 h post-infection (p.i.). The samples were titrated in a plaque assay (King & Possee, 1992) and the mean values of three separate experiments used to plot a replication curve.

### Transient expression experiments.

*Sf* 21 cells (2.5 x 10⁵) in 25 cm² flasks were transfected with pAcADD2 or pAcDD3 (10 µg) alone or co-transfected with AcMNPV C6 DNA (500 ng). Plasmid pAcIE1-lacZ (gift from C. Persillon), which contains the lacZ gene under the control of AcMNPV ie-1 promoter, was used as a positive control. At 24, 48 and 72 h post-transfection (p.t.), cells were fixed with 2% formaldehyde–0.2% glutaraldehyde in PBS solution and β-galactosidase production was monitored in the presence X-Gal [0.1% X-Gal; 4 mM KFe(CN)₆; 4 mM K₃Fe(CN)₆; 2 mM MgCl₂] for 12 h at 28 °C in a dark room.

### SDS–PAGE of infected cell proteins and immunoblotting.

*Sf* 21 cells (2.5 x 10⁵) were infected with wild-type (wt) or recombinant *Acdel68lacZ* virus at an m.o.i. of 20 and incubated at 28 °C. Protein labelling was performed as described by King & Possee (1992) using 50 µCi Pro-mix ³²P cell labelling mix (Amersham). After labelling for 2 h, cells were harvested, pelletted and washed once with 1 ml PBS. The cellular pellets were then lysed in dissociation mix (50 mM Tris–HCl, pH 6.9; 25% glycerol; 10% SDS; 25% β-mercaptoethanol; 0.25% bromophenol blue). Samples were heated in a boiling water bath for 5 min, then loaded in 7.5 or 10% SDS–polyacrylamide gels and proteins separated using denaturing conditions (Laemmli, 1970). Proteins were then semi-dry blotted on a nitrocellulose membrane (Trans-blot transfer medium, Bio-Rad). The membrane was incubated overnight in Tris–NaCl buffer (TBS: 137 mM NaCl; 20 mM Tris–HCl, pH 7.5; 0.1% SDS–Tween 20; 0.05% N-dodecyl sodium sulphate) followed by incubation in a blocking buffer (5% dried milk and 0.1% Tween 20, GP64-EFP monoclonal antiserum AcV₆₁, IE1 and VP39 polyclonal antiserum were obtained previously (Blissard & Roehrmann, 1989; Obresnik et al., 1994; Thiem & Miller, 1989). A rabbit polyclonal antiserum against polyhedrin protein was obtained by injection of polyhedrin extracted from gel, using standard techniques (Ausubel et al., 1994); an immuno-adsorption against *AcMNPV* poly (lacking polyhedrin gene) infected *Galleria mellonella* larvae antigens was carried out just before used. A monoclonal antibody directed against β-galactosidase (Promega) was also used. Western blots were incubated with appropriate dilutions of polyclonal or monoclonal antibodies.

Immunoreactive proteins were detected using the appropriate secondary antibody linked to the peroxidase (Diagnosics Pasteur) followed by incubation with a chemoluminescent substrate (Amersham), following the manufacturer's instructions.

### RNA isolation and primer extension analysis.

Total RNA was isolated as described by Ausubel et al. (1994) from 2 x 10⁶ *AcMNPV* C6 infected (m.o.i. 20) and mock infected cells, at different times p.t. Briefly, cells were lysed with GIT buffer (4 M guanidium isothiocyanate; 20 mM sodium acetate, pH 5.2; 0.1 mM dithiothreitol; 0.5% N-lauroylsarcosine). The lysate solution was then layered onto a cushion of 5.7 M CsCl and centrifuged for 18 h in a Beckman Ti-50 rotor at 35 000 r.p.m. at 20 °C. The pellet was resuspended in 5 mM EDTA, 0.5% N-lauroylsarcosine, 5% β-mercaptoethanol at 4 °C, extracted with acid phenol–chloroform and RNA was precipitated with ethanol in the presence of 0.5 vol. 7.5 M ammonium acetate. RNA was resuspended in DEPC-treated water and quantified by absorbance at 260 nm. For primer extension, 40 µg of total RNA were annealed at 55 °C to the 30 base primer ORF86PE3 labelled with [γ-³²P]ATP. This primer is complementary to the *pnk/pnl* mRNA between +82 to +112 relative to the translation initiation site (Fig. 1). Extension was done with AMV RT polymerase at 42 °C for 1 h as described in the manufacturer’s protocol (Promega). Half of the sample was loaded on the gel. To measure accurately the size and to locate the
**Fig. 1.** Virus nucleotide sequences and predicted open reading frames of ORF 86 (pnn/pnl) in AcMNPV C6 and 1.2. The major deletion in the 1.2 sequence overlaps the two putative domains of pnn/pnl. Nucleotide differences are denoted by lower-case letters. Locations of two consensus TATA boxes, a canonical early consensus initiation motif CAGT, the related CATT sequence that is used as a transcription start, and the polyadenylation signal just downstream of the TAA stop codon are denoted in bold. The positions of the primers used are indicated by arrows. Transcription initiation as determined by primer extension is indicated by an arrow just upstream of the ATG start codon (position \(\theta\)), and the position of the poly(A) chain site attachment is indicated by asterisks downstream of the TAA stop codon (position \(\theta\)). Putative RNA ligase and polynucleotide kinase domains are indicated by open and filled boxes respectively. Other motifs (NTP binding site, tyrosine kinase phosphorylation site) are underlined. The putative start codon of ORF 87 and stop codon of ORF 85 are also indicated just before and after the pnn/pnl region.
Fig. 2. Sequence comparison of the predicted ORF 86 product (PNK/PNL) with T4 RNA ligase (LIGT4) and T4 polynucleotide kinase (PNKT4) respectively (A, B). Amino acid identity is indicated by a vertical line and similarity by a colon. Gaps introduced into the sequences are represented by dashes. The ATP/GTP binding site conserved in both PNK/PNL and PNKT4 (B) and the GTP binding site conserved in both PNK/PNL and LIGT4 (A) are boxed.

start of transcription, plasmid pATHindIIIIC DNA was sequenced using the same primer. Extension and DNA sequencing reactions were analysed in an 8% polyacrylamide gel.

Results and Discussion

Similarity of PNK/PNL to T4 bacteriophage RNA ligase and polynucleotide kinase

A brief description of pnk/pnl was previously reported by Ayres et al. (1994). It is located between nt 17231 and 24213, within the HindIII C fragment of AcMNPV, on the opposite strand of DNA to that which contains the polyhedrin gene (4520 to 5255). It has a coding potential of 694 amino acids (Fig. 1). A GRAIL analysis (Uberbacher & Mural, 1991) indicated that this putative ORF has a high coding probability (Ayres et al., 1994). The coding region is preceded by two TATA motifs between positions 548-542 and 597-591 relative to the ATG and a CAGT motif (33-36) which is characteristic of an early transcription start site (Blissard & Rohrmann, 1990; Pullen & Friesen, 1995) (Fig. 1). Typical polyadenylation motifs are found within 200 nt downstream of the TAA translation stop site (Fig. 1).

Appropriate searches of protein databases revealed significant similarities of the pnk/pnl predicted protein sequence with two gene products of bacteriophage T4. The amino-terminal portion (residues 1 to 128) is strongly related to the T4 gene 63 that encodes a protein which has an RNA ligase activity (Silber et al., 1972; Snopek et al., 1977) (31% identity, 72% similarity, z-score = 316-2, E = 3 x 10^-11). The car-
boxy-terminal portion of the predicted protein (residues 393 to 694) is related to the T4 gene pseT that encodes a polynucleotide kinase (Midgley & Murray, 1985) (26% identity, 66% similarity, z-score = 411.7, E = 1.8 x 10^{-16}) (Fig. 2).

Further analysis of these protein sequences revealed that a conserved motif (consensus KXDG) essential for GTP binding and characteristic for catalysis in nucleotidyl transfer (Cong & Shuman, 1993) is present just before the ligase domain (residues 103 to 106) (Figs 1 and 2); a similar motif is found in the T4 RNA ligase (Fig. 2; K99-EDG). A conserved motif for NTP binding (consensus GXXXXGKS; residues 401 to 408) was identified in the polynucleotide kinase domain of Pnk/Pnl (Fig. 1). Two potential tyrosine kinase phosphorylation sites (consensus R/KX[2,3]D/E[2,3]Y) were identified at positions 196 and 624 (Fig. 1). The former motif is also conserved in T4 RNA ligase (residues 182 to 189) (Fig. 2).

The two T4 genes are not needed for virus replication in common E. coli hosts, but are indispensable in the clinical isolate CT196 (Depew & Cozzarelli, 1974; Sirotkin et al, 1978; Runnels et al, 1982). This isolate has an insertion containing the ppr locus. Both proteins are involved in the repair of tRNA^{305} (Amitsur et al, 1987), which is selectively cut by the E. coli ppr gene product. The ppr locus encodes two physically associated restriction activities (Amitsur et al, 1992; Morad et al, 1993), the EcoPrl (Tyndal et al, 1994) and the anticodon nuclease activity (ACNase; Abdul-Jabbar & Snyder, 1984), which is inhibited under normal conditions. Upon infection, the product of T4 gene Stp inactivates the EcoPrl activity, but activates the ACNase activity (Kaufmann et al, 1986; Penner et al, 1995). The T4 polynucleotide kinase and the T4 RNA ligase are thus involved in counteracting the attempt by the host to block the incoming infection.

Other organisms, like wheat (Pick & Hurwitz, 1986; Pick et al, 1986) and yeast (Xu et al, 1990a,b), have a single protein with both RNA ligase and polynucleotide kinase activities. In yeast, this protein is involved in the processing of tRNAs. Mutants in this function are non-viable (Phizicky et al, 1992).

Although no analysis of the activities of the putative AcMNPV pnk/pnl product has yet been done, all the sites that have been proven to be necessary for the proposed functions are conserved, including the lysine-containing domains that catalyse GTP binding (K103-IDG). Overexpression of pnk/pnl would allow a test for the activities to be done and generation of antibodies to map its cellular localization.

Various roles may be suggested for pnk/pnl based on what is known about the T4 system: a role in the activation of virus late promoters similar to that proposed by Sirotkin et al (1978) for T4 or a role in cell–virus interaction [such as the shut-off of cellular protein production in favour of the virus-encoded polypeptides or the inhibition of host defence mechanisms, as occurs in T4 (Penner et al, 1995)]

**Deletion of pnk/pnl from the AcMNPV C6 genome**

To investigate the role of pnk/pnl in virus infection, the complete coding region was replaced with that of lacZ to derive a recombinant baculovirus (AcDel86lacZ). The heterologous sequences introduced into the AcMNPV genome remained under the control of the pnk/pnl promoter. The virus was produced by co-transfecting insect cells with infectious AcMNPV C6 DNA and pAcDD2 and subsequent selection of plaques which stained blue in the presence of X-Gal. Several independent recombinant viruses were isolated by three rounds of plaque purification and amplified to working stocks. The virus on the left.

![Fig. 3. Western blot analyses of steady-state levels of selected early and late proteins in wt- and AcDel86lacZ-infected Sf 21 cells from 3 to 18 h p.i. or 24 to 72 h p.i. The early proteins analysed were IE-1 and GP64. Late proteins were VP39 and polyhedrin. The protein bands are indicated by arrows. The numbers above each line indicate time p.i. (h). The protein analysed is indicated at the top and the virus on the left.](Image)
similar growth curve, with a lag phase between 0 and 15 h p.i., followed by a burst of infectious virus released into the cell culture medium. After 40 h p.i. the virus titres remained stable until 96 h p.i. (5 to 7.5 x 10^6 p.f.u./ml, that is 5 to 7.5 p.f.u. per cell). No differences were observed in the budded virus (BV) production kinetics nor in the final BV titres between parental and recombinant viruses in cell culture. In addition, injection of G. mellonella larvae with both viruses did not reveal any difference, either in the doses or time of mortality (data not shown).

**Effect of the deletion on protein expression**

Although infectious virus production appeared to be unaffected by the absence of a functional pnk/pnl, we examined protein synthesis in Sf 21 cells infected with AcDel86lacZ or AcMNPV. Time-course analyses of parental and recombinant virus were performed and levels of selected proteins were analysed by Western blotting. Fig. 3 shows the expression of two early genes, GP64 and IE1, and two late genes, VP39 and polyhedrin, in wt- and recombinant-infected Sf 21 cells. No consistent differences were observed either in the time or level of expression of those proteins between AcMNPV C6- and AcDel86lacZ-infected cells.

Pulse-labelling cells infected with AcMNPV C6 or AcDel86lacZ with a mixture of [35S]methionine/cysteine at various intervals between 6 and 48 h p.i. was also carried out (Fig. 4). Consistent with Western blot experiments, the synthesis of virus proteins in parental- and recombinant-infected cells was not modified. There was production of polyhedrin protein in a similar fashion from 18 until 48 h p.i., with an identical increase in the expression at the late time-points. Host protein synthesis was identically reduced in AcMNPV- and AcDel86lacZ-infected cells. Those results suggest that pnk/pnl is not directly involved, at least in an essential way, in the activation of late promoters or host protein synthesis shut-down. It is conceivable that there is redundancy in the mechanisms controlling host protein synthesis shut-down. In this situation, the presence of a functional pnk/pnl in most situations could be dispensable.

**Activity of the pnk/pnl promoter in uninfected Sf 21 cells**

The presence of a CAGT early gene promoter motif upstream of pnk/pnl (Fig. 1) suggested that it might be an early gene capable of transcription in the absence of other virus proteins. Insect cells were transfected with pAcDD2 or pAcDD3, which contain the β-galactosidase coding sequence in either orientation under the control of the putative pnk/pnl promoter. A positive control was provided by cells transfected with pAcIE1-lacZ, which contains the β-galactosidase coding region under the control of the AcMNPV ie-1 (immediate early) gene promoter. The cells were harvested at 24, 48 and 72 h p.t. and monitored for β-galactosidase production using X-Gal staining. LacZ expression was detectable in cells transfected with pAcDD2 and pAcIE1-lacZ, but not pAcDD3, where the lacZ sequences were inserted antisense to the gene promoter. β-Galactosidase was thus produced in the absence of other virus gene products, indicating that pnk/pnl has an active promoter of the immediate early type.

When Sf 21 cells were co-transfected with pAcDD2 and AcMNPV DNA a higher expression of β-galactosidase was observed (Table 1). This may indicate that viral factors transactivate this promoter.

**Transcriptional mapping and temporal regulation of pnk/pnl**

Primer extension analysis was used to analyse the presence of pnk/pnl transcripts throughout virus infection and to
promoter region, the main transcript starts in the sequence promoters are efficiently transcribed by host RNA polymerase. sequencing ladder generated with the same primer from pAT153 surrounding the start point are indicated on the right.

Determination of the 5\textsuperscript{pnk} of both aphidicolin and cycloheximide (Fig. 5). The specific high stability of the RNA synthesized earlier in infection.

Using \textit{lacZ} is added to the medium. In addition, the activity of the promoter was monitored this variability in the \textit{pnl} promoter, both clones C6 and 1.2 are identical in the coding sequence of \textit{AcMNPV} C6-infected S\textit{f} 21 cells at 4, 8, 12, 12 with aphidicolin (12a), or with cycloheximide (12c), 18, 24, 48 h p.i. and mock infected (Mi) S\textit{f} 21 cells. RNA (40 \mu g) from each time-point was incubated with an end-labelled 30 base primer complementary to the \textit{pnk}/\textit{pnl} nucleotide sequence at positions +82 to +112 from the ATG (Fig. 1). Half of the reaction products were loaded on a 8% sequencing gel, in parallel with a sequencing ladder generated with the same primer from pAT153HindIII C. Molecular mass markers (lane M; lengths in nucleotides) and sequences surrounding the start point are indicated on the right.

determine the mRNA start site (Fig. 5). A single major cDNA product was detected between 4 and 48 h p.i. with some minor start sites from the neighbouring nucleotides. Peak levels of \textit{pnk}/\textit{pnl} mRNA accumulation appeared to be between 8 and 12 h p.i. The major start site of transcription maps 18 nt upstream of the ATG translation initiation codon, on the underlined A of the sequence GTATGC\textsuperscript{ATT} (Figs 1 and 5).

To confirm the nature of this promoter, this experiment was done in the presence of aphidicolin and cycloheximide, inhibitors of DNA replication and protein synthesis, respectively. Only immediate early promoters are transcribed when aphidicolin is added to the culture medium. Moreover, late and very late promoters are not transcribed when aphidicolin is added to the medium. \textit{pnk}/\textit{pnl} was expressed in the presence of both aphidicolin and cycloheximide (Fig. 5). The specific \textit{pnk}/\textit{pnl} RNAs present at later times of infection may reflect a high stability of the RNA synthesized earlier in infection.

In addition, the activity of the promoter was monitored using \textit{lacZ} as a reporter gene. By Western blot analysis with an antibody specific for \beta-galactosidase, a 116 kDa protein was shown to be present in low quantities in \textit{Acde86lacZ}-infected cells from 6 up to 96 h p.i. (data not shown), consistent with its expression under the \textit{pnk}/\textit{pnl} promoter. \textit{Acde86lacZ}-infected cells did not appear to accumulate significant quantities of \beta-galactosidase, suggesting a low activity of the promoter.

A basal baculovirus immediate early promoter is constituted by a \textit{TATA} box followed by an initiator, generally a C\textit{AGT} box (Blissard & Rohrmann, 1990; Pullen & Friesen, 1995); the two boxes are separated by 23 to 27 nt in the early genes \textit{ie-0}, \textit{ie-1}, \textit{ie-2} and \textit{pe-38} (Lu & Miller, 1995). Those promoters are efficiently transcribed by host RNA polymerase. Although the C\textit{AGT} early motif was present in the \textit{pnk}/\textit{pnl} promoter region, the main transcript starts in the sequence GTATGC\textsuperscript{ATT}. This indicates that the initiator box of \textit{pnk}/\textit{pnl} is not the C\textit{AGT} sequence located only 7 nt from the second TATA box and 56 from the first, but is the related C\textit{ATT} sequence 29 nt downstream the second TATA. This spacing corresponds to the usual distance between the initiator and the \textit{TATA} box for genes transcribed by the RNA pol II (Roeder, 1991). The insect initiator consensus deduced by Cherbas & Cherbas (1993) is (A/C/T)/CA(G/T)T and the \textit{pnk}/\textit{pnl} initiator fits into this consensus. Other baculovirus early genes, such as \textit{LdMNPV} early gene \textit{vPK} (Bischoff & Slavicek, 1994), \textit{LdMNPV} early gene G22 (Bischoff & Slavicek, 1995) or \textit{AcMNPV} \textit{lef-4} gene (D. Durantel and others, unpublished results) have their transcription initiator site within a similar sequence (TC\textsuperscript{ATT}).

To map the 3\textsuperscript{end} of the \textit{pnk}/\textit{pnl} transcripts, 3\textsuperscript{RACE} was performed on mRNA isolated from \textit{AcMNPV}-infected cells at 8 h p.i. One \textit{pnk}/\textit{pnl}-specific cDNA with a size between 135 and 140 nt was isolated and sequenced (data not shown). The last nucleotide homologous to the genomic sequence was located in a series of six A beginning 15 nt downstream of the stop codon (Fig. 1). A typical polyadenylation site, AATAAA, was located 9 nt upstream of the determined 3\textsuperscript{end}, at the level of the stop codon. Taken together, the results from primer extension and 3\textsuperscript{end} mapping predict a transcript of 2115 bp [excluding the poly(A) tail].


Polymorphism of the \textit{pnk}/\textit{pnl} region in other baculoviruses

A certain degree of variability in the genome of \textit{AcMNPV} has been observed. Two of twelve clones analysed by Lee & Miller (1978), coming from a natural population, had a deletion in the \textit{HindIII} C region, where \textit{pnk}/\textit{pnl} is located, but the precise mapping of this deletion has not been done. Restriction enzyme analysis of genomic DNA from several baculovirus isolates (\textit{AcMNPV} C6, 1.2, \textit{GmMNPV} and \textit{BmNPV}) confirmed this variability in the \textit{HindIII} C fragment (data not shown). \textit{AcMNPV} 1.2, \textit{BmNPV} and \textit{GmMNPV} have a deletion in this region. By PCR analysis with primers HC 716 and HCR 2800, which amplify the complete \textit{pnk}/\textit{pnl} coding sequence, it was possible to determine that there is an internal deletion of about 1.3 kbp in both \textit{AcMNPV} 1.2 and \textit{GmMNPV}. No amplification was obtained with \textit{BmNPV} DNA (data not shown). To characterize further the deletion within \textit{AcMNPV} 1.2, this region of the virus genome was sequenced and compared with the homologous region in \textit{AcMNPV} C6 (Fig. 1). Alignment of the DNA sequences showed that there is a major deletion of 1349 bp between positions +163 to +1513 relative to C6 \textit{pnk}/\textit{pnl} ATG and various other minor deletions and point mutations, particularly in the promoter sequence. We have not checked if the promoter is still active. In this case, the polypeptide encoded would have a size of 37 amino acids due to the presence of an in-frame stop codon. Upstream of the \textit{pnk}/\textit{pnl} promoter, both clones C6 and 1.2 are identical in the region sequenced. The variability observed is restricted to the coding sequence of \textit{pnk}/\textit{pnl} and control sequences.
The sequence of BmNPV T3 has recently been released (accession no. L33180). When these data were analysed, it became evident that AcMNPV ORFs 84, 85 and 86 were absent from the BmNPV genome. This explains the lack of DNA amplification since the primers used are in the region that is not present in BmNPV. Interestingly, in the pnk/pnl flanking regions of the AcMNPV C6 genome, ORF 87 (1 kbp) and ORFs 71 to 83 (9 kbp), there is a high degree of sequence identity between the two viruses (92\% and 90.4\% respectively). The divergence of the sequences between AcMNPV C6 and BmNPV T3 starts around the putative translation start of pnk/pnl (nt 74217 on the C6 genome).

In the OpMNPV sequence (Ahrens et al., 1997; accession no. U75930), this gene does not exist. In the flanking region (AcMNPV ORFs 85 and 87, OpMNPV ORFs 87 and 88), the identity between both viruses is lower than average (23-9 and 39-5, respectively compared to 56\% average identity at the amino acid level).

Immediate early genes are usually involved in basic functions, and their deletion modifies the course of infection. From this point of view pnk/pnl is an exception, because it can be deleted without affecting virus survival or late gene expression. If this gene is dispensable, why is it maintained in AcMNPV C6? It is possible that the gene is needed in some circumstances, for example in different hosts, and its deletion may impair isolates like 1.2. Accordingly, pnk/pnl may be a host-range gene, but we have not yet found the conditions in which it is required. Insertion of the gene in viruses that do not normally contain it may help in the search for its function.

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