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

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\textbf{\it 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 \textit{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 \textit{pnk/pnl} was investigated by producing a recombinant virus (Acdel86lacZ) with the coding region replaced with that of \textit{lacZ}. This virus replicated normally in \textit{Spodoptera frugiperda} (Sf 21) cells, indicating that \textit{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 expression of \textit{pnk/pnl} by restriction enzyme digestion and PCR, a deletion was found in AcMNPV 1.2, \textit{Galleria mellonella} NPV (GmNPV) and \textit{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.

\textbf{Introduction}

The complete sequence of \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 (Crozier & Quiot, 1981), in a closely related Galleria mellonella nucleopolyhedrovirus (GmMNPV) isolate (Crozier & Quiot, 1981) and in Bombyx mori nucleopolyhedrovirus (BmNPV) clone 7 (Crozier et al., 1994).

**Methods**

- **Cells and viruses.** Two AcMNPV strains were used in this study: 1.2 (Crozier & Quiot, 1981) and C6 (Possee, 1986). A GmMNPV clone (Crozier & Quiot, 1981) was also used. These strains and the viral recombinant baculovirus A6el86acZ 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 previously described (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) was added to the cells 30 min before infection.

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

  The transfer vector pACDD1 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: WC 2800 (5′ ACGTGATCCATTGTGCTTATTAGC 3′; position 74213 relatively to C6 positive strand); HCR 5088 (5′ GGATACCGGCTGCAATAACAATAATCGC 3′; position 76501, complementary strand); HC 1 (5′ GGATACCGGCTGCAATAACAATAATCGC 3′; position 72131, negative strand). The product generated using WC 2800 and HCR 5088 was digested with BamHI and EcoRI, and inserted into a pUC18 cloning vector. The resulting construct was then digested with BamHI and HindIII1 to insert the previously digested PCR product obtained with primers HC 1 and HCR 718 to derive pACDD1.

  To construct the transfer vector pACDD2, the Escherichia coli lacZ gene was excised from a modified pCH110 plasmid (Possee & Howard, 1987) and inserted into the BamHI site of pACDD1 in the correct orientation for expression: lacZ inserted in the opposite orientation in pACDD1 produced pACDD3.

  A baculovirus recombinant deficient in pnk/pnl was obtained by co-transfection of SF 21 cells with C6 DNA (500 ng) and pACDD2 (10 µg) using DOTAP reagent (Boehringer Mannheim). Recombinant baculovirus A6el86acZ 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 A6el86acZ 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 A6el86acZ 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 10⁶) in 25 cm² flasks were transfected with pACDD2 or pACDD3 (10 µg) alone or co-transfected with AcMNPV C6 DNA (500 ng). Plasmid pAcEI-lacZ (gift from C. Persill)on, 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 K₃Fe(CN)₆; 4 mM K₅Fe(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 10⁶) were infected with wild-type (wt) or recombinant A6el86acZ 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-labelled cell labelling mix (Amersham). After labelling for 2 h, cells were harvested, pelleted and washed once with 1 ml PBS. The cellular pellets were then lysed in suspension 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 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) containing 10% dried milk and 0.1% Tween 20. GP64-EPF monoclonal antiserum AcV₅, IE1 and VP39 polyclonal antiserum have been described previously (Blissard & Rohrmann, 1989; Ohresser et al., 1994; Thiém & Müller, 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 (Diagnostics 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.i. 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 ORF68PE3 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 (pnk/pnl) in AcMNPV C6 and 1.2. The major deletion in the 1.2 sequence overlaps the two putative domains of pnk/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 -18), and the position of the poly(A) chain site attachment is indicated by asterisks downstream of the TAA stop codon (position +15). 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 pnk/pnl region.
start of transcription, plasmid pATHindIII C DNA was sequenced using the same primer. Extension and DNA sequencing reactions were analysed in an 8% polyacrylamide gel.

Determination of the messenger 3' end. One-hundred ng of mRNA extracted at 8 h p.i. was hybridized with the primer oligo(dT)-XhoI ([N]CTCGAGGATCTpM) at 45 °C. First strand cDNA synthesis was done with AMV RT at 45 °C for 60 min. The resulting mixture was PCR-amplified by adding a second primer, HCR 806 (Fig. 1) according to the TITAN RT–PCR System instructions (Boehringer Mannheim).

The product obtained was subjected to a second amplification with the same primers for 35 cycles. The specific fragment of 140 bp was gel purified, cloned into pUC19, and sequenced with universal and reverse primers.

Sequence analysis. The length polymorphism of the pnk/pnl region was analysed by PCR. The PCR reaction was carried out in a Thermocycler thermo cycler (Eurogentec) with primers HC 716 (5' GTCGAGATCT TAAAATTCCAAGTAATCCCTGC 3') and HCR 2800 (5' CGCTGGATCC ATGTTGCACGTTTCGCGTC 3') using pATHindIII C as a template. Those primers allow amplification of the complete pnk/pnl coding sequence; the start and the stop codons are underlined.

The pnk/pnl region in AcMNPV isolate 1.2 was sequenced in an Applied Biosystems model 373A sequencer by the dye terminator method (ABI PRISM Dye terminator cycle sequencing, Perkin Elmer). For manual sequencing, the Taq dye deoxy terminator cycle sequencing kit (Pharmacia) was used.

Computer analysis. Comparison of pnk/pnl with sequences present in databases (EMBL, SWISS-PROT and PIR) was done with the programs FASTA (Pearson & Lipman, 1988), BLAST (Altschul et al., 1990) and BLITZ (Smith & Waterman, 1981). The GCG package (Devereux et al., 1984), version 8.1, was employed to search for motifs. 1.2 (accession number X99292), C6 (accession number L22858), BmNPV (accession number L33180) and OpMNPV (accession number U75930) sequences were aligned using MACAW (Schuler et al., 1991).

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 72131 and 74213, 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 48 to 42 and 97 to 91 relative to the ATG and a CAGT motif (33 to 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 141 to 292) 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 = 3·6·10−11). The car-

![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.](image-url)
boxy-terminal portion of the predicted protein (residues 393 to 694) is related to the T4 gene *psdT* 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[X2,3]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, 1982). This isolate has an insertion containing the *ppr* locus. Both proteins are involved in the repair of tRNA*{Ac}* (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 *Ecopr* (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 *Ecopr* 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. 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.

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The stability of the plaque-purified virus stocks was tested by five passages in Sf 21 cells; no differences in restriction enzyme patterns or production of β-galactosidase were noted (data not shown) indicating that *pnk/pnl* is not essential for the virus replication in Sf 21 cells.

One-step growth curves were determined for *AcMNPV* C6 and *Acdel86lacZ*. Recombinant and wt viruses produced a
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 Sf21 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 Sf21 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 identical between 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 Sf21 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

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**Table 1. Percentage of cells expressing β-galactosidase upon transfection with the different vectors (10 µg) in the presence (500 ng) or absence of viral DNA**

<table>
<thead>
<tr>
<th>Vector</th>
<th>− Virus DNA</th>
<th>+ Virus DNA</th>
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<tbody>
<tr>
<td>pAcDD2</td>
<td>5% (8/165)</td>
<td>37% (196/525)</td>
</tr>
<tr>
<td>pAcDD3</td>
<td>0% (0/187)</td>
<td>0% (0/267)</td>
</tr>
<tr>
<td>pAcIE1-lacZ</td>
<td>2% (3/154)</td>
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**Fig. 4.** Protein synthesis in cells infected with AcMNPV C6 or Acdel86lacZ. Polyacrylamide-SDS gel (10%) of proteins labelled with [35S]methionine/cysteine at 6, 12, 18, 24 and 48 h p.i. in cells infected with AcMNPV C6 (lanes 1, 3, 5, 7 and 9, respectively) or Acdel86lacZ (lanes 2, 4, 6, 8 and 10, respectively). Sf21 cells (2.5 x 10^6) were infected at an m.o.i. of 10 p.f.u. per cell and pulse-labelled for 2 h after each time-point with 50 µCi of a mixture of [35S]methionine/cysteine. The polyhedrin protein is indicated on the right by an arrow and molecular mass markers are on the left.
promoter region, the main transcript starts in the sequence.

Although the CAGT early motif was present in the promoters are efficiently transcribed by host RNA polymerase. 

surrounding the start point are indicated on the right.

Fig. 5. Determination of the 5’ end of pnk/pnl transcripts and kinetics of transcription. Primer extension analysis of total RNA isolated from AcMNPV C6-infected Sf 21 cells at 4, 8, 12, 12 with aphidicolin (12a), or with cycloheximide (12c), 18, 24, 48 h.p.i. and mock infected (Mi) Sf 21 cells. RNA (40 µg) from each time-point was incubated with an end-labelled 30 base primer complementary to the pnk/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 pAT153 HindIII. 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 pnk/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 GTATGCATT (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 cycloheximide is added to the culture medium. Moreover, late and very late promoters are not transcribed when aphidicolin is added to the medium. pnk/pnl was expressed in the presence of both aphidicolin and cycloheximide (Fig. 5). The specific pnk/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 lacZ as a reporter gene. By Western blot analysis with an antibody specific for β-galactosidase, a 116 kDa protein was shown to be present in low quantities in Acel86lacZ-infected cells from 6 up to 96 h.p.i. (data not shown), consistent with its expression under the pnk/pnl promoter. Acel86lacZ-infected cells did not appear to accumulate significant quantities of β-galactosidase, suggesting a low activity of the promoter.

A basal baculovirus immediate promoter is constituted by a TATA box followed by an initiator, generally a CAGT box (Blissard & Rohrmann, 1990; Pullen & Friesen, 1995); the two boxes are separated by 23 to 27 nt in the early genes ie-0, ie-1, ie-2 and pe-38 (Lu & Miller, 1995). Those promoters are efficiently transcribed by host RNA polymerase. Although the CAGT early motif was present in the pnk/pnl promoter region, the main transcript starts in the sequence GTATGCATT. This indicates that the initiator box of pnk/pnl is not the CAGT sequence located only 7 nt from the second TATA box and 56 from the first, but is the related CATT sequence 29 nt downstream the second TATA. This spacing corresponds to the usual distance between the initiator and the 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)TT and the pnk/pnl initiator fits into this consensus. Other baculovirus early genes, such as LaDNPV early gene vPK (Bischoff & Slavicek, 1994), LaDNPV early gene G22 (Bischoff & Slavicek, 1995) or AcMNPV lef-4 gene (D. Duranteau and others, unpublished results) have their transcriptional promoter site within a similar sequence (TCTTT).

To map the 3’ end of the pnk/pnl transcripts, 3’ RACE was performed on mRNA isolated from AcMNPV-infected cells at 8 h.p.i. One pnk/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’ end, at the level of the stop codon. Taken together, the results from primer extension and 3’ end mapping predict a transcript of 2115 bp [excluding the poly(A) tail].

Polymorphism of the pnk/pnl region in other baculoviruses

A certain degree of variability in the genome of AcMNPV has been observed. Twelve of twelve clones analysed by Lee & Miller (1978), coming from a natural population, had a deletion in the HindIII C region, where pnk/pnl is located, but the precise mapping of this deletion has not been done. Restriction enzyme analysis of genomic DNA from several baculovirus isolates (AcMNPV C6, 1.2, GmMNPV and BmNPV) confirmed this variability in the HindIII C fragment (data not shown). AcMNPV 1.2, BmNPV and GmMNPV have a deletion in this region. By PCR analysis with primers HC 716 and HCR 2800, which amplify the complete pnk/pnl coding sequence, it was possible to determine that there is an internal deletion of about 1·3 kbp in both AcMNPV 1.2 and GmMNPV. No amplification was obtained with BmNPV DNA (data not shown). To characterize further the deletion within AcMNPV 1.2, this region of the virus genome was sequenced and compared with the homologous region in 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 pnk/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 pnk/pnl promoter, both clones C6 and 1.2 are identical in the region sequenced. The variability observed is restricted to the coding sequence of pnk/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 (92-1 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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