Comparison of the thymidine kinase genes from three entomopoxviruses

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The entomopoxviruses (insect poxviruses) of eastern spruce budworm (Choristoneura fumiferana), two year cycle spruce budworm (C. biennis) and the Indian red army worm (Amsacta moorei) are being studied in our laboratory for their potential as biological insecticides and expression vectors. These viruses characteristically replicate in the cytoplasm of insect cells and produce occlusion bodies that serve to protect the virion from the environment. By analogy to mammalian poxviruses, they should also contain a viral thymidine kinase (TK) that functions in viral DNA synthesis. The replication of the A. moorei entomopoxvirus was inhibited by bromodeoxyuridine whereas the baculovirus of Autographa californica was insensitive to this drug. This result was a biochemical indication that entomopoxviruses contained a kinase that phosphorylated this nucleoside analogue and thus viral DNA synthesis was inhibited. TK genes from the three different insect poxviruses were identified, cloned and sequenced. The sequences of the TK genes of the entomopoxviruses were closely related and exhibited 63.2% identity and 9.9% similarity at the protein level. However, there was only 36.7% identity and 13.6% similarity when these enzymes were compared to their mammalian poxvirus counterpart in vaccinia virus. Finally, one entomopoxvirus TK gene was expressed in Escherichia coli mutants lacking the enzyme. These bacteria were converted to a phenotype that could incorporate radioactive thymidine into their chromosomal DNA. The results presented in this paper provide impetus for the design of a recombinant entomopoxvirus expression system in which foreign genes could be introduced into the viral TK locus under selective pressure from bromodeoxyuridine.

The thymidine kinase (TK) gene is a valuable target site into which foreign genes may be introduced. For example, investigators have inserted genes into vaccinia virus and herpesviruses at this locus through a process of homologous recombination (Mackett et al., 1982; Panicali & Paoletti, 1982; Roizman & Jenkins, 1985). Recombinant viruses lacking a functional TK may be selected with media containing bromodeoxyuridine (BrdU) in conjunction with cell lines that are also deficient in TK.

Many laboratories, including our own, have been using baculoviruses to produce recombinant proteins in large quantities (Luckow & Summers, 1988; Miller, 1988; Vialard et al., 1990). Recently, our laboratory has concentrated on the study of insect poxviruses (entomopoxviruses) with hopes of employing them as alternative insect expression vectors (Yuen et al., 1990; Banville et al., 1992). Our group has been studying three different entomopoxviruses. One virus (CfEPV) infects the eastern spruce budworm (Choristoneura fumiferana), another (CbEPV) multiplies in the two year cycle spruce budworm (Choristoneura biennis) and the third (AmEPV) infects either the Indian red army worm (Amsacta moorei) or the saltmarsh caterpillar (Estigmene acrea) (Arif, 1984; Arif & Kurstak, 1991). Unlike baculoviruses, entomopoxviruses multiply and form occlusion bodies in the cytoplasm, rather than the nucleus of their respective host cells (Goodwin et al., 1990; Granados & Roberts, 1970). We recently sequenced the major occlusion body proteins of two different entomopoxviruses (Banville et al., 1992; Yuen et al., 1990) and identified their strong late promoters (Pearson et al., 1991). Another group has also sequenced the gene for the occlusion body protein of A. moorei entomopoxvirus (AmEPV; Hall & Moyer, 1991). We are considering the use of the promoters from these genes in the construction

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of entomopoxvirus-based expression vectors designed to introduce foreign genes into the TK locus of these insect viruses. The TK genes of a large number of different poxviruses have been reported (Binns et al., 1988; Boyle et al., 1987; Esposito & Knight, 1984; Feller et al., 1991; Gershon & Black, 1989; Gruidl et al., 1992; Martin Hernandez & Tabares, 1991; Schnitzlein & Tripathy, 1991; Upton & McFadden, 1986; Weir & Moss, 1983). A high degree of similarity can be observed at the N termini and nucleotide-binding and nucleoside-binding sites (Black & Hruby, 1990; Martin Hernandez & Tabores, 1991). These similarities can be used to construct oligonucleotide probes for the identification of similar genes in other viruses.

To begin with, we showed that AmEPV possessed a TK\(^+\) phenotype and that the virus was sensitive to inhibition by BrdU. This nucleoside analogue is known to interfere with DNA synthesis through the formation of bromouridylic acid by the catalytic activity of TK in the nucleotide salvage pathway. The phosphorylated analogue is incorporated into DNA and causes chain termination and mismatch pairing. In the presence of BrdU, \textit{de novo} synthesis of thymidylate from folate and uridylic acid is favoured since this pathway is independent of TK. TK\(^-\) viruses are not affected by BrdU when grown in cells that also lack a functional TK gene, whereas TK\(^+\) viruses cannot replicate in the presence of BrdU. In Fig. 1 we clearly demonstrated that AmEPV was unable to grow in the presence of BrdU (25 \&g/ml) in a TK\(^-\) gypsy moth cell line (IPLB-LD-652 TK\(^-\)). This cell line was selected in our laboratory by continuously passaging IPLB-LD-652 cells (Goodwin et al., 1990) in the presence of increasing concentrations (10 to 100 \&g/ml) of BrdU (Kit et al., 1966). The absence of virus production was demonstrated with plaque assays and confirmed by the lack of c.p.e. on infected cells (Fig. 1a, c). On the other hand, cells infected with AmEPV in the absence of BrdU formed cytoplasmic occlusion bodies, became detached, and produced high virus titres (Fig. 1a, b). In contrast to entomopoxviruses, there is no evidence that baculoviruses synthesize TK, except one report in the literature which suggests that a baculovirus of \textit{Trichoplusia ni} might possess a TK gene (Kit, 1985). Since we also generated an Sf9 TK\(^-\) cell line capable of supporting the baculovirus of \textit{Autographa californica} (AcNPV), we decided to perform an experiment analogous to the one described above using this baculovirus. It was surprising to find that BrdU (100 \&g/ml) had no effect upon the replication of AcNPV, suggesting that it does not have a functional TK gene. Thus, AcNPV served as an appropriate negative control for the effects of BrdU on DNA virus replication in insect cells.

Since entomopoxviruses possessed a functional TK, we decided to clone and identify its gene from the DNA of our three viruses. Genomic DNA from CbEPV, CfEPV and AmEPV was prepared as previously described (Banville et al., 1992) and digested with various restriction endonucleases (Accl, Asel, BamHI, DraI, EcoRI, EcoRV, HindIII, Ndel, PvuI and XmnI). Restriction fragments containing the TK gene were identified by Southern blot hybridization using probes prepared from a polymerase chain reaction (PCR) fragment specific for each TK gene. These PCR probes
Fig. 2. PCR analysis and Southern blot hybridization of the TK genes from three entomopoxviruses. (a) PCR products obtained by amplification of a TK gene fragment using TK1 and PK2’ degenerate oligonucleotide primers and genomic DNA of AmEPV (lane 1), CfEPV (lane 2) and CbEPV (lane 3). The lane marked L contains a 1 kb ladder (BRL). In (b) and (c), plasmid and EPV DNAs were digested with EcoRI and electrophoresed on a 0.8% agarose gel. The blot was probed with the TK consensus oligonucleotide DK39. (b) Agarose gel stained with ethidium bromide. (c) Autoradiogram of the Southern blot. Lanes 1, pKK223-3 + AmTK; lanes 2, pUC18 + AmTK; lanes 3, pUC18 + CfTK; lanes 4, pUC18 + CbTK; lanes 5, AmEPV; lanes 6, CfEPV; lanes 7, CbEPV; lane L as in (a).

were constructed using consensus oligonucleotide primers TK1 (GGNCCCCATGTTYTCNGG) and PK2’ (AARAAAYTGNCCYTCRTC) where N represents any base, Y represents C or T, and R represents A or G residues. These sequences were derived from the most conserved regions of poxvirus TK genes (Boyle et al., 1987; Martin Hernandez & Tabares, 1991; Upton & McFadden, 1986). PCR products, shown in Fig. 2(a), were radioactively labelled and hybridized to restriction fragments ranging in size from 1 to 13 kb (data not shown). EcoRI digestion of the three different genomes produced the smallest TK-containing fragments: 1.5 kb for AmEPV, 1.15 kb for CbEPV and 1.18 kb for CfEPV. Each of these fragments was cloned into pUC18 and sequenced. A Southern blot (Fig. 2b, c), probed with a degenerate oligonucleotide [GCTAATAAT(G/A)(G/A)AAAAAA(G/A)TTATTGT(T/A)GCTGGATTATA] DK39 derived from the TK consensus sequence, identified the EcoRI fragments from the genomic DNA which contained the TK gene. The blot also demonstrated that these inserts were present in the pUC18 recombinant plasmids.

The cloned EcoRI fragments containing TK genes from the three different entomopoxviruses were sequenced by the dideoxynucleotide chain termination method. The degenerate oligonucleotides, TK1 and PK2’, were used for the first round of sequencing reactions for all three TK genes. Specific primers, derived from these results, were synthesized in order to complete the DNA sequencing of both strands. Analysis of the DNA data indicated that open reading frames (ORFs) from the three viruses encoded putative TK proteins of slightly different lengths: 186 amino acids (M, 21406) for CbEPV, 185 amino acids (M, 21279) for CfEPV and 182 amino acids (M, 21221) for AmEPV. While we were preparing this manuscript, another group also sequenced the TK gene of AmEPV (Gruidl et al., 1992) and their data are in agreement with ours. We have compared the TK gene sequences from the three different entomopoxviruses and these results are shown in Fig. 3. Although close similarity is evident within the ORFs of the three TK genes, the flanking sequences are quite different. There is 98.4% identity at the DNA level between the ORFs of CbEPV and CfEPV, 77.1% identity between those of CbEPV and AmEPV, and 76.1% between CfEPV and AmEPV. Comparison of the three genes revealed 74.3% identity and 15.6% similarity.

Early and late genes of poxviruses are transcribed by a viral RNA polymerase. Early promoters of vaccinia virus, including the TK gene promoter, appear to be composed of three stretches of nucleotides: a critical
region, a spacer and an initiation sequence (Davison & Moss, 1989). The critical region has a general consensus sequence [AATAAAATGGTTTTTA(A/T)] which lies between 13 and 28 nucleotides upstream of the mRNA transcription start site. This region appears to be analogous to the TATA box of other eukaryotic promoters. The 11 nucleotide spacer and the 7 nucleotide transcription initiation region are more variable in composition and are situated downstream of the critical region. Similar transcriptional signals may be present in the upstream promoter regions of the three TK genes sequenced. For example, the sequence AATATT-GAAAAAATA, found in the potential critical region of the TK gene promoters of CbEPV and CFEV, bears some resemblance to the analogous region of the vaccinia virus TK promoter, which has the sequence ATAAAGTGAACAAATA. The potential critical region of the AmEPV TK promoter, TTTTTTGGAAAAAATA, is less similar. Finally, a consensus sequence for a poxvirus termination signal (TTTTTNT) for transcription of early genes (Yuen & Moss, 1987) is found downstream from the translational stop codons of all three entomopoxvirus TK genes. Thus, it appears that some transcriptional signals present in the early genes of other poxviruses are found in the TK genes of the entomopoxviruses presented here.

The protein sequences of entomopoxvirus and vaccinia virus TKs were aligned and compared in Fig. 4. There was 98.4% identity and 1.1% similarity between the TKs of CbEPV and CFEV, 62.2% identity and 9.9% similarity between CbEPV/CFEV and AmEPV proteins, 29.9% identity and 15.8% similarity between CbEPV and vaccinia virus enzymes. Overall, the four TK proteins exhibited 36.7% identity and 13.6% similarity.

TK plays a central role in the nucleotide salvage pathway by catalysing the production of dTMP from thymidine and ATP. Previous investigators (Black & Hruby, 1990) have demonstrated that mammalian and viral TK proteins contain seven highly conserved domains: DINKAFKSK, DNKNPFKSI, YNDATFTMK, RICYNENNTIN, TFARKPFNNIV, DNPNNFIKNI and NNFALEATKLCDVE.

Fig. 3. Comparison of the TK gene sequences of CbEPV (Cbtk), CFEV (Cftk) and AmEPV (Amtk). Asterisks (*) indicate perfectly conserved residues across all four sequences; plus signs (+) specify residues where conservative substitutions occurred.

Fig. 4. Amino acid sequence comparison of CbEPV, CFEV, AmEPV and vaccinia virus TK proteins predicted from DNA sequence data. The sequences were aligned manually. There is 36.7% identity and 13.6% similarity between the four proteins. Asterisks (*) indicate perfectly conserved residues across all four sequences; plus signs (+) specify residues where conservative substitutions occurred.
domains. The same regions are evident in the entomopoxvirus proteins and the high degree of conservation is especially true in the five regions in the first three-quarters of the protein. The function of domain I only (near the N terminus) is known and it appears to play a role in ATP binding prior to catalysis. This region is highly conserved among the entomopoxviruses. Functional TK forms a tetrameric complex during catalysis (Black & Hruby, 1990). However, the sites for nucleoside binding and the protein regions involved in tetramer formation have yet to be elucidated.

There is no doubt that the genes we cloned coded for functional TK molecules. The AmEPV TK gene when introduced into TK- vaccinia virus allows the virus to grow in the presence of methotrexate on human 143 TK- cells (Gruidl et al., 1992). In our laboratory, E. coli strains deficient in TK (KY895 and JF1642) (Hiraga et al., 1967; Liu & Manning, 1986) were transformed with a bacterial expression plasmid containing the TK gene of AmEPV, pKKAmTK (Fig. 2b, c). Bacterial colonies were cultivated on nitrocellulose filters which were placed on top of a TK selection medium containing [Me-14C]thymidine. As expected, TK+ cells incorporated radioactive thymidine, whereas TK- cells did not (data not shown). However, negative controls of KY895 and JF1642 bacteria, containing only the expression plasmid (pKK223-3) without the TK gene, did not incorporate radioactive thymidine. These experiments indicated more directly that the TK gene of AmEPV was active.

In summary, we cloned, sequenced and compared the TK genes of three entomopoxviruses. The TK proteins encoded by these genes were closely related and the N termini of the entomopoxvirus proteins were nearly identical for the first 27 residues. In addition, entomopoxvirus TKs shared about 51-3% protein similarity with vertebrate poxvirus TKs such as vaccinia (Weir & Moss, 1983), fowlpox (Binns et al., 1988), swinepox (Feller et al., 1991; Schnitzlein & Tripathy, 1991), African swine fever (Martin Hernandez & Tabares, 1991) and variola (Esposito & Knight, 1984) viruses. Slightly less similarity was observed with cellular TK proteins of humans (Bradshaw & Denninger, 1984) and mice (Lin et al., 1985). The seven conserved domains previously reported in poxvirus TK proteins were apparent (Black & Hruby, 1990). Domain I is known to bind ATP for phosphorylation, whereas the roles of the other conserved regions in thymidine binding and subunit interaction are not yet known.

We previously sequenced the major occlusion body genes from AmEPV (Banville et al., 1992) and CbEPV (Yuen et al., 1991) and found them to be substantially different. However previous work in our laboratory indicated that another CbEPV gene product, nucleoside triphosphate phosphohydrolase I (NPHase I), shared significant homology with the NPHase I of vaccinia virus (Yuen et al., 1991). Similar results (Hall & Moyer, 1991) indicated that the NPHase I of CbEPV and vaccinia virus also bore striking homology to the analogous gene product of AmEPV. The close relatedness of the TK genes found in entomopoxviruses with those of other poxviruses further justifies the classification of entomopoxviruses within this family.

TK from AmEPV normally functions at 25 to 28 °C, but we demonstrated thymidine incorporation at 37 °C by TK- bacteria expressing the viral protein. Another laboratory also found the same gene product to be active at 37 °C in mammalian cells infected with a recombinant vaccinia virus (Gruidl et al., 1992). In addition, BrdU clearly inhibited the replication of wild-type AmEPV in a TK- cell line. These results indicated that the TK of AmEPV was functional and suggest that the enzyme could be used in conjunction with BrdU to select recombinant virus deficient in TK. We are currently constructing expression vectors consisting of the flanking regions of the TK gene and strong promoters designed to drive the expression of foreign genes. Our intent is to interrupt the TK gene of wild-type entomopoxvirus and insert a foreign gene at this site by a process of homologous recombination through selective pressure from BrdU. In addition, entomopoxviruses have an extremely restricted host range and most viruses will only infect a particular species of insect larvae (Arif & Kurstak, 1991). Consequently, recombinant entomopoxviruses, generated in the laboratory, may have potential as biological insecticides as well as alternative insect virus expression vectors.

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