The *gp64* locus of *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus contains a 3′ repair exonuclease homologue and lacks *v-cath* and *ChiA* genes

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*Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus (*AgMNPV*) is one of the most successful biological insecticides. In this study, we cloned and sequenced a 12.5 kbp *BamHI*-D restriction endonuclease fragment of the *AgMNPV* isolate 2D genome that includes the *gp64* gene. We compared this highly conserved region with that of other baculoviruses. *AgMNPV* contained two genes, *p22.2* and *v-trex*, in common with *Choristoneura fumiferana* MNPV (CMNPV) that were not present in other baculoviruses. The *v-trex* gene has homology to a eukaryotic 3′ repair exonuclease and appears to have been acquired from an invertebrate host. The *v-trex* gene product has the potential to be involved in virus recombination or UV-light tolerance. Multigene phylogenetic analysis suggested that *AgMNPV* is most closely related to *Orgyia pseudotsugata* MNPV (OpMNPV). *AgMNPV* differed from other group I NPVs in that *ChiA* and *v-cath* gene homologues were missing from the region downstream of the *gp64* gene. Proteinase assays and genetic probes suggest the *v-cath* gene is absent from *AgMNPV*.

**INTRODUCTION**

Baculoviruses are exclusive pathogens of arthropods, mostly from lepidoptera. Baculoviruses are a diverse family of viruses with large double-stranded DNA genomes that range from 80 to 180 kbp in size. The earliest studies of baculovirus molecular biology focused on *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*). These studies led to the development of baculovirus expression vectors that are widely used to produce foreign proteins.

Baculoviruses are widely recognized as effective biological insecticides. The insecticidal properties of *AcMNPV* have been improved by adding foreign genes and/or by inactivating accessory viral genes (for review see Inceoglu et al., 2001). The knowledge from these studies is currently being applied to baculovirus species with specificity for economically important insect pests. Of these, *AgMNPV* has been most extensively and successfully applied for biological control of *Anticarsia gemmatalis*, especially in Brazil (Moscardi, 1999). *AgMNPV* exhibits a high degree of genetic heterogeneity (Croizier & Ribeiro, 1992). *AgMNPV* isolate 2D (*AgMNPV-2D*) is a prototype as its genomic profile, based on restriction endonuclease (REN) analysis, represents 40% of field isolates (Maruniak et al., 1999).

The *AgMNPV*-2D genome was previously mapped by restriction endonucleases (Johnson & Maruniak, 1989; Maruniak et al., 1999). In the current study, we sequenced the 12.5 kbp *BamHI*-D REN fragment of *AgMNPV*-2D. A number of *AgMNPV* sequences have been published including homologous repeat (*hr*) regions (Croizier & Ribeiro, 1992; Garcia-Maruniak et al., 1996), the polyhedrin gene (Zanotto et al., 1992) and the egt gene (Rodrigues et al., 2001). A region of *AgMNPV*-2D common to this study was also published (Razuck et al., 2002). That region contains homologues of the *AcMNPV* genes *p94*, *p26*, *p10* and the 3′ end of the *p74* gene. The 5′ end of an ORF homologous to the *Choristoneura fumiferana* (*Cf*) MNPV gene *p22.2* (ORF 126) is also present. Most recently, the *AgMNPV* *gp64* gene sequence was published (Pilloff et al., 2003). Although the sequence was derived from the SF isolate of *AgMNPV*, it is identical to the corresponding sequence in our study. This study focuses on the genes downstream of *gp64* that, to our knowledge, have not been previously described.

Comparative studies suggest that there are two evolutionarily divergent groups of NPVs; group I and group II (Bulach et al., 1999; Herniou et al., 2001; Zanotto et al., 1993). Single
gene comparisons of AgMNPV suggest that AgMNPV belongs to the group I NPVs that include AcMNPV, CjMNPV, Bombyx mori NPV (BmNPV), Orzya pseudotsugata MNPV (OpMNPV), Epiphyas postvittana NPV (EppoNPV), Choristoneura fumiferana defective MNPV (CjDEF), Antheraea pernyi NPV (AnpNeNPV), Hyphantria cunea NPV (HycuNPV), Anagraphe falcifera MNPV (AnfaMNPV), Rachophius ou NPV (RavoNPV) and Perina nuda NPV (PenuNPV).

To further define the evolutionary relationship of AgMNPV among other baculoviruses, we compared the AgMNPV BamHI-D REN fragment with the corresponding regions of three other group I NPVs: AcMNPV, BmNPV, OpMNPV, EppoNPV, HycuNPV and CjMNPV.

In this study, we focus on two features of this AgMNPV-2D BamHI-D REN fragment: (a) the presence of a baculovirus gene with a third repair exconuclease motif; and (b) the absence of ChiA and v-cath homologues downstream of gp64. We also report on a replication assay analysis of two hr-like repeat elements that were discovered during this study.

METHODS

Cells lines and viruses. Spodoptera frugiperda (SF-9) cells, Lymantria dispar (L6252Y) cells and Trichoplusia ni (Tn5B1-4) cells were used to propagate wild-type (w) AcMNPV (strain E2), OpMNPV and AgMNPV-2D viruses, respectively. Cells were cultured at 27 °C in TMN-FH medium (Hink, 1970) containing 10 % (v/v) fetal bovine serum.

Southern blot hybridizations. To locate the AgMNPV gp64 gene, a plasmid (pAcEcoRI-H) containing the EcoRI-H fragment from the AcMNPV-2D genome was digested with BamHI, which generates three fragments each containing portions of the gp64 ORF. The fragments were labelled with [32P]dCTP using random 9-mers (Rediprime kit, Amersham), and used to probe a plasmid library of AgMNPV-2D HindIII fragments by Southern blot hybridization. For Southern blot hybridizations, membranes containing RNAs of cloned AgMNPV-2D DNA fragments were incubated in prehybridization buffer (6 × SSC, 0.5 % SDS, 5 × Denhardt’s solution, 100 μg denatured salmon sperm DNA ml⁻¹) for 2 h at 60 °C, and then hybridized with the [32P]-labelled AgMNPV probe DNA overnight in prehybridization buffer + 0.01 M EDTA. The membrane was then washed for 15 min at 60 °C in 50 ml 2 × SSC, 0.1 % SDS, and for another 15 min in 0.1 × SSC, 0.1 % SDS, and exposed to X-ray film for 72 h.

PCR protocol. Vent polymerase (New England Biolabs) was used for PCR screening for a v-cath gene homologue in AgMNPV-2D. 100 ng of viral template DNA was combined with 250 μM dNTPs, 2 U Vent polymerase, 1 μM of each primer, 1× Thermopol buffer (New England Biolabs) in a final volume of 100 μl. PCR mixtures were topped with 30 μl mineral oil. v-cath primers used were: 5'-GGCTTGTGCGGCGCGTGCTGGC-3' and 5'-ACCTTAAATAACCCCTCCTCTCCCAAA-3'. 30 PCR cycles were run: 95 °C 1 min, 40 °C 1 min, and 72 °C 1 min. Parallel PCR reactions were run with OpMNPV and AcMNPV template DNA to ensure the specificity of v-cath primers. PCR products were also sequenced to ensure that the v-cath ORF region was being amplified. PCR reactions with primers to the AgMNPV gp64 gene (5'-GGCGGTGGCGTGCGCCTGGGTA-3' and 5'-GACCAACGGCGGTGGCTTCTTTT-3') were also included in parallel amplifications to ensure AgMNPV DNA template quality.

Cloning, sequencing and nucleotide sequence analysis. The 12.5 kbp BamHI-D REN fragment of AgMNPV-2D was cloned into the BamHI site of plasmid, PBS(-) (Stratagene), resulting in the construct pBS-Ag/BamHI-D. Sequencing was performed using rhodamine terminator cycle sequencing kits (Applied Biosystems) and an ABI Prism 377 genetic analyser sequencing machine. Template pBS-Ag/BamHI-D plasmid DNA was purified by CaCl2 gradient. Both strands were sequenced with a total mean redundancy of approximately 2–4. The entire 12.5 kbp AgMNPV-2D region was conceptually translated and searched for homologous genes using BLASTX (http://www.ncbi.nlm.nih.gov). DNAStar MapDraw was used to locate remaining ORFs (greater than 50 amino acids). ORFs that overlapped identified homologues were excluded. AgMNPV genes were named using the same nomenclature as used for AcMNPV and where no gene names were previously assigned in AcMNPV, the AgMNPV gene (ORF) was named according to the AcMNPV ORF number with an Ag appended to signify the homologue (for example ORF119a).

Phylogenetic analysis. Protein homologue sequences were aligned with AgMNPV sequences using DNAStar MegaAlign and the CLUSTAL W method (Thompson et al., 1994) (gap penalty = 10, gap length penalty = 0-2, PAM 350 series protein weight matrix). For phylogenetic tree generation, alignments were saved as NEXUS files. NEXUS files were linked together and analysed by maximum parsimony using the program PAUP* 4.0 (Swofford, 1998). PAUP* 4.0 analysis employed a heuristic search for possible trees. Bootstrap values were calculated by performing 100 000 replicate heuristic tree searches with a parsimony optimality criterion. A bootstrap 50 % majority consensus tree was generated.

Assembly of the genomic segments of HycuNPV. The genomic regions flanking the HycuNPV gp64 gene were derived by assembling contigs with DNAStar SeqMan and overlapping GenBank sequence submissions. GenBank accessions and corresponding HycuNPV (Taxon 28288) ORFs are as follows: AF121457 (ChiA), AF120926 (v-cath) and AF190124 (gp64).

Origin of replication assay. AgMNPV repeat regions (R1 and R2), as well as AcMNPV hr5 were PCR-amplified and cloned by ligation into a pGEM-T plasmid (Promega). Amplified regions from AgMNPV corresponded to nt 808–1163 (366 bp, includes repeat 1) and nt 1002–1163 (162 bp, includes repeat 2) and nt 808–1163 (366 bp, includes repeats 1 and 2). Infection-dependent plasmid replication assays were performed as described earlier (Kool et al., 1993, 1994). SF-9 cells (2 × 10⁶) were transfected by calcium phosphate precipitation with 5 μg of a plasmid containing either R1, R2 or R1 and R2 or hr5 (positive control). At 24 h post-transfection, transfected cells were infected with AgMNPV or AcMNPV at an m.o.i. of 10 p.f.u. per cell and incubated at 27 °C. At 48–72 h post-infection, total DNA was extracted from cells (O’Reilly et al., 1992), and 13 μg of DNA was digested with DpnI (1.5 U μg⁻¹) at 37 °C for 12 h, followed by digestion with PstI (1.5 U μg⁻¹) for 12 h. DNA fragments were separated on agarose gels, transferred to Genescreen Plus membrane, and hybridized with a 32P-labelled pGEM-T plasmid DNA probe (9 × 10⁶ c.p.m. μg⁻¹) generated by random hexamer priming (Prime-It Rmt kit, Stratagene). Blots were prehybridized for 2 h at 65 °C in prehybridization solution (5 × SSC, 0.1 % laurylsarcosine, 0.02 % SDS, 10 % blocking reagent (Roche)). Hybridization was carried out for 14 h at 65 °C, in prehybridization solution plus probe (1 × 10⁶ c.p.m. ml⁻¹). Membranes were washed for 10 min in two changes of 2 × SSC/0.1 % SDS at room temperature, followed by two 15 min washes in 0.5 × SSC/0.1 % SDS at 65 °C. Membranes were exposed to phosphoscreens (Amersham) and screens were scanned on a Storm phosphorimager machine (Amersham).

V-CATH protease assays. Approximately 2 × 10⁶ Tn5B1-4 (High 5) cells were infected for 1 h at an m.o.i. of 2.5 p.f.u. per cell with AgMNPV, AcMNPV, or an AcMNPV recombinant virus containing an inactivated v-cath gene (AcMNPV-v-cath⁺⁻) (Slack et al., 1995). We also similarly infected L652Y cells with OpMNPV. At 75 h post-infection, cells were harvested for protease assays. Cells were pelleted...
RESULTS AND DISCUSSION

This study examines the organization and structure of genes in the gp64 locus of AgMNPV-2D. Southern blotting was used to first identify a gp64 gene containing a restriction fragment in the AgMNPV-2D genome. The AcMNPV gp64 gene was used to probe a HindIII digest of AgMNPV-2D DNA. A positively hybridizing 5-4 kbp HindIII-I AgMNPV-2D DNA fragment was identified. Based on a previously published AgMNPV-2D REN map (Johnson & Maruniak, 1989), the HindIII-I fragment was deduced to be nested inside a 12-5 kbp BamHI-D fragment. Consequently, the BamHI-D fragment was subcloned from the AgMNPV-2D genome to make the plasmid construct, pBS-Ag/BamHI-D. We sequenced the 12-5 kbp BamHI-D fragment directly and created new oligonucleotide sequencing primers as each consecutive 500 bp or so of sequence was generated.

Gene organization in the AgMNPV-2D BamHI-D REN fragment

The AgMNPV BamHI-D fragment was found to contain 17 baculovirus gene homologues including gp64. The arrangement of baculovirus gene homologues in the AgMNPV-2D BamHI-D fragment was compared to the corresponding

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**Fig. 1.** Gene organization of a 12-5 kbp region of AgMNPV-2D genome relative to group I NPVs. A 12-5 kbp BamHI-D REN fragment of AgMNPV-2D genome was sequenced in order to characterize the AgMNPV gp64 gene locus. This region was aligned with the corresponding DNA sequences from group I NPVs. The alignment was anchored at the gp64 gene and is schematically illustrated. The scale bar is relative to AgMNPV sequence. Represented sequences are shown in the following descending order; AgMNPV (Ag), CbMNPV (Cb), EppovNPV (Eppo), OpMNPV (Op), AcMNPV (Ac), RoauMNPV (Ro), BmNPV (Bm), HycuNPV, AnfaMNPV, PenuNPV, CDEF, AnpeNPV. For sequenced viral genomes, the relative sequence position is indicated in parentheses and the sizes of those regions are listed in a box in the lower right-hand corner. Gene sizes and orientation are represented as 'arrow-boxes'. Seventeen AgMNPV genes of interest are labelled at the top (just below the bp scale bar). Black-filled arrow-boxes represent 15 genes that are homologous between AgMNPV and AcMNPV. Dashed lines connect common homologues. The hatched arrow-box is a homologue shared between AgMNPV and CbMNPV. Open arrow-boxes on other viral regions are genes that are absent from the corresponding region of AgMNPV. Only the 3' ends of ORF119Ac and p74 are represented. They coincide with the portions of these genes that are included in the AgMNPV-2D BamHI-D REN fragment. A highly conserved 3 kbp ChiAl v-cath region, which is absent from AgMNPV, is represented at 1/9th scale in the main figure. That region is illustrated at full-scale in the inset box on the lower-middle section of the figure. Sequences and GenBank accessions were as follows; CbMNPV (NC_004778), EppovNPV (NC_003083), OpMNPV (NC_001875), AcMNPV (NC_001623), RoauMNPV (AY145471), BmNPV (L33180), CDEF (U72030), AnpeNPV (AB072731), PenuNPV (AF289055). For HycuNPV, regions flanking gp64 were assembled from contigs of GenBank submissions (see Methods).
known regions of the group I NPVs CjMNPV, EppeNPV, OpMNPV, BmNPV, RaouMNPV, AcMNPV, HycuNPV, AnfaMNPV, PenNPV, AnpeNPV and CjDEF (Fig. 1). AgMNPV-2D gene composition in the BamHI-D region most closely resembled CjMNPV as there were two common gene homologues that were not found in other NPVs. Those homologues were v-trex (CjMNPV ORF 113) and p22.2f (CjMNPV ORF 126). The predicted amino acid sequence identity between v-trex homologues was 68.4% and between p22.2f homologues was 61.6%. EppeNPV and OpMNPV were also more similar to AgMNPV than BmNPV, RaouMNPV and BmNPV. AgMNPV-2D lacked protein kinase-2 (pk-2), p35 and hr5. Curiously, AgMNPV-2D contained a remnant of the C terminus of the p94 gene similar to BmNPV. AgMNPV-2D differed from all other group I NPVs that have been analysed by lacking ChiA and v-cath genes downstream of gp64.

**Phylogenetic analysis**

To clarify the phylogenetic relationship of AgMNPV with other group I NPVs we performed multigene phylogenetic analysis of the proteins GP64, p24, gp16, PEP, ORF132Ac, AN, P26, P10, P74 (3' end), EGT and POLH, which are shared among AgMNPV, CjMNPV, OpMNPV, EppeNPV, RaouMNPV, BmNPV and AcMNPV (Fig. 2a). Multigene phylogenetic analysis was also done between group I and group II NPVs using the proteins PEP, AN, P10, P74, EGT and POLH (Fig. 2b). That analysis included the above-mentioned group I NPVs along with the group II NPVs: Lymantria dispar NPV (LdMNPV), Mamestra configurata NPV (MacoNPV) (A & B species), Spodoptera exigua MNPV (SeMNPV), Helicoverpa zea NPV (HzNPV), Heliothis zea SNPV (HzSNPV) and Spodoptera litura NPV (SpltNPV).

Our group I/group II phylogenetic analysis agrees well with others (Baluch et al., 1999; Herniou et al., 2001; Zanotto et al., 1993) showing a clear separation between group I and group II NPVs and concurring with the classification of AgMNPV as a group I NPV (Razuck et al., 2002; Rodrigues et al., 2001). Both phylogenetic analyses show AgMNPV to be most closely related to OpMNPV. In addition, AgMNPV was more closely related to CjMNPV and EppeMNPV than to AcMNPV, RaouMNPV and BmNPV. We had expected based on the presence of v-trex and p22.2f in both that CjMNPV would be the closest relative to AgMNPV. It is unlikely that AgMNPV and CjMNPV acquired v-trex and p22.2f independently. We must assume that v-trex and p22.2f were present in the other group I NPVs but as they diverged these genes were lost.

**Repeat regions**

Immediately downstream of ORF119Ac are found two short repeat sequences [Repeat 1 (R1) (102 bp: 891–992) and Repeat 2 (R2) (80 bp: 1005–1084)] with resemblance to baculovirus homologous repeats (hrs) (Fig. 3a). R1 and R2 each contain approximately 40 bp of semi-palindromic

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**Fig. 2.** Unrooted phylogeny of AgMNPV among other NPVs. Unrooted phylogenetic analysis of linked protein alignments was performed among several NPVs. Sequence sources and % identities are listed in Table 1. Branch lengths are proportional to the total number of amino acid changes between viruses. Bootstrap values representing support for the relationship have been placed near each node. The number of amino acid changes differing from AgMNPV is indicated in parentheses beside each virus name. The protein homologues used in phylogenetic analysis are listed above each tree. Only the C termini of P74 were included in the analysis to correspond with available AgMNPV sequence. (a) A phylogeny was constructed for the group I NPVs AgMNPV, AcMNPV, RaouMNPV, BmNPV, CjMNPV, EppeNPV and OpMNPV. Linked alignments produced a total of 3131 characters with one optimal tree being retained (Tree Score = 3026). (b) A phylogeny was also generated for the group I NPVs analysed in (a) and the group II NPVs HearSNPV, HzNPV, LdMNPV, MacoNPV-A, MacoNPV-B, SeMNPV and SpltNPV. Linked alignments produced a total of 2013 characters with one optimal tree being retained (Tree Score = 5036).
sequence. It is notable that region 897–1036 of our \textit{Ag}MNPV sequence is 77% identical to region 58–197 of \textit{Cf}DEF (accession U72030) (Fig. 3b). This homologous \textit{Cf}DEF region is found downstream of the \textit{Cf}DEF ChiA gene homologue (Fig. 1). Both R1 and R2 are also similar to previously published \textit{Ag}MNPV and \textit{Ac}MNPV repeat regions (Garcia-Maruniak \textit{et al}., 1996).

Among other functions, \textit{hrs} can serve as origins of DNA replication (Kool \textit{et al}., 1993). R1 and R2 regions were subcloned as PCR products separately or in tandem into pGEM-T (Promega) plasmids. R1/R2-containing plasmids were assayed in baculovirus infection-dependent DNA replication assays (Kool \textit{et al}., 1993). Assay conditions were confirmed by running parallel experiments with a similarly subcloned \textit{Ac}MNPV hr5 region. \textit{Ag}MNPV R1 and R2 regions did not support DNA replication in transient assays in the context of \textit{Ag}MNPV or \textit{Ac}MNPV viral infections (data not shown). This result does not fully exclude R1 and R2 as origins of replication. \textit{Ld}MNPV \textit{hrs}, for example, must be linked to AT-rich sequences before they can undergo replication (Pearson & Rohrmann, 1995), and the possibility exists that this region serves an enhancer function, analogous to \textit{Ac}MNPV (Guarino \textit{et al}., 1986) and \textit{Op}MNPV (Theilmann & Stewart, 1992) \textit{hrs}.

Table 1. Percent identities of \textit{Ag}MNPV protein sequences with other baculovirus homologues

Protein names are listed in the first column and baculovirus names are listed in the first row. The table corresponds to the data that were used to generate the phylogenies in Fig. 2. GenBank protein sequence accession numbers are listed in addition the percent identity of each sequence to the corresponding \textit{Ag}MNPV homologue. For the protein P74, we were able to use only the C-terminal region in comparisons. The amino acid residues used in the comparison are listed below the accession number in parentheses. \textit{Ag}MNPV sequences for the POLH (accession CA768441) and EGT proteins (accession AAK16408) were from other studies (Rodrigues \textit{et al}., 2001; Zanotto \textit{et al}., 1993).

![Fig. 3. AgMNPV repeat regions. Two repeat regions (R1 and R2) were identified to have some homology with hrs. (a) The R1 and R2 regions were located between ORF119\textsuperscript{Ac} and ORF120\textsuperscript{Ac}. (b) The DNA sequences of the R1 and R2 regions are shown aligned to a similar C/DEF sequence that is found downstream of the C/DEF ChiA gene. The alignment includes DNA residues 886–1090 of our AgMNPV sequence and DNA residues 46–252 of the C/DEF sequence (accession U72030). Common nucleotides are blocked in black. R1 and R2 regions are boxed.](http://vir.sgmjournals.org)
**v-trex gene**

During our analysis of ORF homologues we noticed that the AgMNPV homologue to CjMNPV ORF 113 also had homology to a type of group III exonucleases called Three prime Repair EXonucleases (TREXs) (Mazur & Perrino, 1999). For this reason we suggest the nomenclature, v-trex gene and V-TREX protein. TREX proteins are the most abundant 3′–5′ DNA exonucleases found in mammalian cells (Mazur & Perrino, 1999, 2001) and they are different from other type III exonucleases in that they function independently from the DNA polymerase complex (Mazur & Perrino, 1999, 2001). This characteristic would facilitate the acquisition of a foreign trex gene by baculoviruses. The v-trex gene could have been acquired from an invertebrate host. Insect proteins from *Anopheles gambiae* PEST and *Drosophila melanogaster* are 39-0% and 33-3% identical to AgMNPV V-TREX (Fig. 4a). Neither of the insect proteins has been characterized but they both contain common exonuclease motifs; Exo I, Exo II and Exo III. These motifs form the active sites of type III exonucleases (Bernad *et al*., 1989; Koonin & Deutscher, 1993). The Exo III motif of V-TREX contains the consensus sequence ‘HXAXXD’ (Fig. 4b), which is a hallmark of TREX type exonucleases (Mazur & Perrino, 1999).

TREX proteins may be involved in DNA mismatch repair, DNA UV-damage repair and in DNA recombination (Mazur & Perrino, 1999), and any of these activities would be augmentative to AgMNPV’s survival in the environment. V-TREX may even be contributing to the recombination-driven heterogeneity of AgMNPV isolates.

**ChiA and v-cath genes**

The largest single variation of this AgMNPV-2D BamHI-D region from other group I NPVs is the absence of a conserved 3 kb region downstream of gp64. Other group I NPVs, including *Eppo*NPV, *Op*NPV, *Ac*MNPV, *Raou*MNVP, BmNPV, CjMNPV, HycuNPV, CjDEF and

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**Fig. 4.** Comparison of AgMNPV V-TREX with other TREX homologues. Exonuclease motifs were identified in the V-TREX protein sequence. (a) The AgMNPV V-TREX protein is shown aligned to its protein homologue from CjMNPV (ORF113) and homologues from the insects *Anopheles gambiae* PEST (accession EAA11359) and *Drosophila melanogaster* (accession NP_608732). Consensus residues are blocked in black. Exonuclease motifs, Exo I, Exo II and Exo III, are found within the boxed regions. (b) The boxed regions in part (a) are aligned with exonuclease motifs of the TREX1 homologues of *Bos taurus* (*B*. *t*.; accession AF319575), *Homo sapiens* (*H*. *s*.; accession XM_003301) and *Mus musculus* (*M*. *m*.; accession NM_011637). Residues that are predicted to form part of the exonuclease active site are indicated by bolding and numbering above the alignment. All of these proteins contain the TREX consensus pattern HXAXXD in their Exo III motifs.
AnpeNPV, have the ChiA and v-cath genes downstream of gp64 (Fig. 1). The PeniNPV gp64 gene submission (accession AF289055) also contains the 3' end of a downstream v-cath gene. Thus, AgMNPV-2D lacking the v-cath and ChiA genes downstream of the gp64 gene is unusual. Among the group II NPVs LmMNPV, SeMNPV, SpltNPV, MacoNPV-A, MacoNPV-B, HzNPV and HearSNPV that lack gp64, the v-cath and ChiA genes are not linked to each other and do not have conserved genomic locations. The v-cath and ChiA genes are absent from the group II NPV Culex nigripalpus (Cutn)NPV (Afonso et al., 2001) and from the granuloviruses Plutella xylostella (Plx)GV (Hashimoto et al., 2000) and Phthorimaea operculella (Phop)GV (accession NC_004062). These facts begged the question of whether the ChiA and v-cath genes were absent from the AgMNPV-2D genome or were they simply relocated in the genome. In this study we made an effort to find evidence for an AgMNPV v-cath gene homologue.

Southern blotting was done to probe for an AgMNPV v-cath gene homologue. These experiments proved negative (data not shown). We also tried the approach of engineering PCR primers that could anneal to the conserved regions among v-cath gene homologues of the group I NPVs (Fig. 5a, b). We successfully used these primers to amplify v-cath gene fragments from OpMNPV and AcMNPV DNA but were unable to amplify any products from AgMNPV-2D DNA (Fig. 5c).

We also looked for V-CATH-specific proteinase activity in lysates from AgMNPV-infected T. ni cells using an azocasein-based proteinase assay. As positive and negative controls we assayed in parallel the lysates from T. ni cells that had been infected with either wt AcMNPV or AcMNPV+/-v-cath(-) (a virus containing a deletion of the v-cath gene) (Slack et al., 1995). The lysates from Ld652Y cells infected with OpMNPV were also included in proteinase assays as OpMNPV has a v-cath gene and is more closely related to AgMNPV than is AcMNPV. The basal proteinase activities of lysates from uninfected T. ni and Ld652Y cells were included to monitor background. Productive, late infections in all virus groups were confirmed by observing occlusion bodies in infected cells and by examination of SDS-PAGE protein gels for abundant polyhedrin protein.

We were unable to detect any V-CATH proteinase activity in the lysates from AgMNPV-infected T. ni cells at 75 h post-infection (Fig. 5). By contrast, the lysates from AcMNPV-infected T. ni cells and OpMNPV-infected Ld652Y cells showed significant amounts of proteolytic activity.

Our results suggest that V-CATH-like proteinase activity is absent from AgMNPV-2D. To our knowledge, AgMNPV-2D may be the first example of a type I NPV that lacks a v-cath gene. Since the ChiA gene is functionally linked with v-cath (Hawtin et al., 1997; Hom & Volkman, 2000), it could also be presumed that AgMNPV-2D is also lacking a ChiA gene homologue. If the ChiA and v-cath genes are indeed absent from the AgMNPV-2D genome, functional homologues may still exist. A metalloproteinase has been proposed to be the functional homologue of V-CATH in the v-cath gene-lacking baculovirus PsGV (Hashimoto et al., 2000). Alternatively V-CATH and ChiA enzymes may

Fig. 5. Screening AgMNPV for a v-cath homologue. PCR primers were designed for highly conserved regions within the v-cath gene. The sequences of these primers are displayed in the top lines of parts (a) and (b). The corresponding sequences of other group I NPV v-cath homologues are aligned below. Non-homologous sequences are boxed in black. Numbers on the 5' end indicate primer position relative the v-cath gene initiator ATG. (c) The results of PCR amplification using these primers are shown on a 1:0:1 (w/v) agarose/1 × TAE gel (lanes 1–3). No bands were observed above the 1018 bp standard (STD lane). Baculovirus DNA templates are indicated above each lane; AcMNPV (Ac), OpMNPV (Op), AgMNPV (Ag). A control PCR amplification was done with AgMNPV gp64-specific primers (lane 4; see Methods). V-CATH-specific proteinase assays were done using an azocasein-based assay (see Methods). (d) Assays included lysates from baculovirus-infected Tn5B1-4 cells; AcMNPV (●), AcMNPV+v-cath(-) (■) or AgMNPV (▲). Lysates from OpMNPV-infected Ld652Y cells were also included in assays (○). (e) Lysates from uninfected cells [Tn5B1-4 (▲) and Ld652Y (△)] were included as controls. Error bars represent standard deviation of four replicate samples.
simply not be necessary for the propagation of AgMNPV-2D in an insect population.

*Trichoplusia ni* larvae injected with AgMNPV-2D BV died, liquefied and turned black as readily as AcMNPV BV-injected larvae (data not shown). For AcMNPV and other baculoviruses, this pathology has been attributed to the enzymic activities of ChiA and V-CATH (Dai et al., 2000; Hawthin et al., 1997) and is presumed to aid in the dispersal of baculoviruses in the environment. The v-cath and chiA genes are not, however, exclusively linked to host liquefaction. For example, the 25K FP gene of *BmNPV* is also linked to host liquefaction (Katsuma et al., 1999). Host-specific and environment-specific factors may also be at play in the liquefaction or melting of baculovirus-infected insects. Lack of v-CATH activity may be contributing to the success of AgMNPV as biological insecticide. AgMNPV-infected *A. gemmatalis* larvae frequently do not liquefy after death. This characteristic has facilitated the field collection of AgMNPV-infected cadavers in the mass production of baculoviruses, the activities of V-CATH for at least 25 years. When AgMNPV or other baculoviruses are dispersed by human activity, the activities of V-CATH and ChiA become less required. Indeed, the field collection itself may have maintained the selective pressure necessary to prevent recombination of the *ChiA* and v-cath genes from other group I NPVs.

**Conclusions**

In this study, we have examined a genomic region that flanks the gp64 gene of AgMNPV-2D. While there is much similarity between AgMNPV-2D and other group I NPVs it is somewhat striking that AgMNPV-2D appears to be missing ChiA and v-cath genes. Absence of the protease activity is supportive of at least the absence of the v-cath gene. Most certainly the future completion of the AgMNPV-2D genome will clarify this. Future investigations by our group will pursue the functional role of the v-trex gene that is shared between AgMNPV and CjMNPV.

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