Choristoneura fumiferana nucleopolyhedrovirus encodes a functional 3′–5′ exonuclease

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The Choristoneura fumiferana nucleopolyhedrovirus (CfMNPV) encodes an ORF homologous to type III 3′–5′ exonucleases. The CfMNPV v-trex ORF was cloned into the Bac-to-Bac baculovirus expression-vector system, expressed in insect Sf21 cells with an N-terminal His tag and purified to homogeneity by using Ni-NTA affinity chromatography. Biochemical characterization of the purified V-TREX confirmed that this viral protein is a functional 3′–5′ exonuclease that cleaves oligonucleotides from the 3′ end in a stepwise, distributive manner, suggesting a role in proofreading during viral DNA replication and DNA repair. Enhanced degradation of a 5′-digoxigenin- or 5′-32P-labelled oligo(dT)30 substrate was observed at increasing incubation times or increased amounts of V-TREX. The 3′-excision activity of V-TREX was maximal at alkaline pH (9–5) in the presence of 5 mM MgCl2, 2 mM dithiothreitol and 0·1 mg BSA ml−1.

The Choristoneura fumiferana nucleopolyhedrovirus (CfMNPV) infects the Eastern spruce budworm, C. fumiferana, one of the most destructive insects in North American forests. Cf114, one of the 146 ORFs identified in the double-stranded CfMNPV DNA genome, encodes a 232 aa homologue of group III 3′–5′ exonucleases, known as three-prime repair exonucleases (TREXs), which are abundant in mammalian cells (Mazur & Perrino, 1999, 2001; J. de Jong, H. A. M. Lauzon, C. Dominy, A. Poloumienko, E. B. Carstens, B. M. Arif & P. J. Krell, unpublished results). Homologues of the CfMNPV trex (v-trex) are also present in C. fumiferana DEF nucleopolyhedrovirus, although only as a partial ORF lacking the ExoIII motif at the carboxyl end (68% identity in the overlapping region), and in Anticarsia gemmatalis MNPV (67·5% amino acid identity), but have not yet been found in other baculoviruses (Slack et al., 2004; J. de Jong, H. A. M. Lauzon, C. Dominy, A. Poloumienko, E. B. Carstens, B. M. Arif & P. J. Krell, unpublished results). The mammalian TREX proteins are structurally closely related to the bacterial ε-subunit of DNA polymerase III (Scheuermann & Echols, 1984), Exol (Lehman & Nussbaum, 1964) and ExoX (Viswanathan & Lovett, 1999), which have proofreading (Scheuermann & Echols, 1984) and repair (Lahue et al., 1989; Razavy et al., 1996; Bzymek et al., 1999) functions. A unique feature of the TREX proteins is that they function as independent enzymes rather than as subunits of the DNA polymerase complex, as for other type III exonucleases (Mazur & Perrino, 2001). V-TREX contains three conserved TREX motifs, EXOI, EXOII and EXOIII, that form the exonuclease active site (Bernad et al., 1989; Slack et al., 2004). EXOIII of V-TREX contains the signature sequence, HXAXXD (named Exoe), of TREX type 3′–5′ exonuclease, another unique feature of the TREX family (Barnes et al., 1995; Strauss et al., 1997; Taft-Benz & Schaaper, 1998). To determine the functionality of the CfMNPV V-TREX, we cloned the Cf114 ORF with an N-terminal His tag into the Autographa californica MNPV Bac-to-Bac baculovirus expression-vector system. The recombinant V-TREX protein was purified by using Ni-NTA affinity chromatography. Both 5′- or 3′-digoxigenin (DIG)-labelled oligonucleotide and 5′- or 3′-32P-labelled oligonucleotide substrates were used to detect exonuclease activities. Our results demonstrated for the first time that the CfMNPV V-TREX possesses 3′-excision activity and that this activity is optimal under alkaline conditions in the presence of Mg2+, dithiothreitol (DTT) and BSA.

The full-length Cf114 (v-trex) ORF was amplified by PCR with forward primer 5′-ACTAGTCCATGGGCCCCATT-C-3′ and reverse primer 5′-CAGTCTAGTATTGGATACCC-3′, containing BamHI/XbaI sites (Sigma), digested with BamHI and XbaI and inserted in-frame into the pFastBac HT donor vector (Invitrogen), downstream of the 6 × His tag, by T4 DNA ligase and the sequence was confirmed (Guelph Molecular Supercentre, University of Guelph, Canada). This construct was transformed into DH10Bac competent cells containing a bacmid with a mini-attTn7 target site and a helper plasmid. Subsequent recombinant baculovirus particles were obtained by transfection of recombinant bacmid DNA into Sf21 cells using Cellfectin reagent (Invitrogen). Exponentially growing Sf21...
cells at a density of 0.5 x 10^6 cells ml^-1 were infected with recombinant baculovirus at an m.o.i. of 0.1, grown at 27 °C in complete Grace’s medium and harvested at 24, 48, 72 and 96 h post-infection. Infected cells were lysed in lysis buffer [20 mM phosphate (pH 8·0), 300 mM NaCl, 1% Nonidet P-40, 5 mM 2-mercaptoethanol] and soluble and insoluble cell proteins were fractionated by three freeze–thaw cycles and centrifugation (10 000 g, 30 min). Production of recombinant V-TREX was analysed by 15% SDS-PAGE and identified by Western immunoblotting using a Penta-His HRP conjugate kit (Qiagen). The His-tagged V-TREX protein had an apparent molecular mass of 28 kDa, similar to the expected 30 kDa, and was detected in both soluble and insoluble fractions at 48–96 h post-infection (Fig. 1a and b). About 50% of the V-TREX was recovered as soluble protein by 72 h post-infection, whereas the amount of insoluble V-TREX protein increased above 50% when cells were harvested at 96 h post-infection (Fig. 1a).

At 48 h post-infection, V-TREX protein was purified by affinity chromatography from the soluble fractions. Soluble cell extracts were also prepared from Sf21 cells infected with wild-type (wt) AcMNPV, lacking His-tagged V-TREX, at 48 h post-infection, to rule out contamination by cellular nucleases or the endogenous baculovirus alkaline nuclease (Li & Rohrmann, 2000; Mikhailov et al., 2003). The two preparations were applied separately onto an Ni-NTA agarose affinity column (Qiagen) that had been equilibrated with buffer A [20 mM phosphate (pH 8·0), 500 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, 20 mM imidazole, 5 mM MgCl₂]. After washing out unbound proteins with buffer B (buffer A plus 1 M NaCl), the bound proteins were eluted into 1 ml fractions with 2 ml of each of the four elution buffers [20 mM phosphate (pH 8·0), 10% glycerol, 5 mM 2-mercaptoethanol, 5 mM MgCl₂], containing 50, 200, 300 and 400 mM imidazole, respectively. Most of the recombinant V-TREX was recovered in fraction 4, as determined by 4–12% pre-cast Bis-Tris acrylamide gel (Invitrogen) electrophoresis and Western immunoblotting (Fig. 1c). Coomassie blue staining showed that V-TREX was purified close to homogeneity (Fig. 1d). The migration rate of V-TREX relative to markers in a NuPAGE Bis-Tris acrylamide gradient gel lacking SDS was different from that in the 15% linear SDS-acrylamide gel (Fig. 1a and b). The purified V-TREX and the corresponding fraction derived from cells infected with wt AcMNPV (control sample) were concentrated and dialysed against nuclease-free PBS, respectively. Initial protein concentration (Bradford, 1976) was 0.44 mg ml^-1 for the purified V-TREX and 0.10 mg ml^-1.

**Fig. 1.** Expression and affinity chromatography of His-tagged V-TREX protein. Cells were harvested at the indicated times post-infection and soluble and insoluble proteins were resolved by 15% SDS-PAGE (a, b). The His-tagged V-TREX protein was identified by immunoblotting using Penta-His antibody–HRP conjugate (a) and analysed by Coomassie blue staining (b). Soluble proteins extracted from Sf21 cells 48 h post-infection were eluted from an Ni-NTA column with an imidazole gradient (c, d). Fractions 1–8 were run on a 4–12% Bis-Tris acrylamide gel (Invitrogen) and purified V-TREX was detected by immunoblotting using Penta-His antibody–HRP conjugate (c). Polypeptide composition of the fractions was analysed by Coomassie blue staining (d). The migration position of the V-TREX protein on the two different types of gel systems is indicated by arrows.
for the control sample. Glycerol (10%, v/v) was added to each sample for storage at −80 °C. The yield of the purified V-TREX protein was 0.3 mg from ten 75 cm² flasks of infected Sf21 cells. The control sample showed a few weak protein bands by Coomassie blue staining, but none cross-reacted with the anti-His–horseradish peroxidase (HRP) conjugate based on Western immunoblot (data not shown).

For biochemical characterization of V-TREX, we first used 5′- and 3′-DIG-labelled oligo(dT)₃₀ (referred to as dT₃₀) (Synthegen) as substrates for the nuclease activity assays (Kruchen & Rueger, 2003). Later, dT₀ (Sigma) labelled with ³²P at the 5′ or 3′ end, generated by standard methods with [γ-³²P]ATP or [α-³²P]dATP (PerkinElmer), respectively, was used. The ³²P-end-labelled substrates were purified by using Sephadex G-25 mini quick spin oligo columns (Roche) and exonuclease activities were assayed by using both DIG- and ³²P-labelled oligonucleotides in parallel.

To determine whether the affinity-purified, His-tagged V-TREX had nuclease activity, standard nuclease reactions (50 µl) containing 25 mM Tris/HCl (pH 9.5), 5 mM MgCl₂, 2 mM DTT, 0.1 mg BSA ml⁻¹, 10 ng purified V-TREX µl⁻¹ and 0·8 ng 5′- or 3′-DIG-labelled dT₃₀ µl⁻¹ were set up. For radioisotope-labelled substrates, 5 ng V-TREX µl⁻¹ and 12 nM 5′- or 3′-³²P-labelled dT₃₀ were used. Reactions were done at 30 °C and 5 µl aliquots were withdrawn at various times (Fig. 2a–d). Reactions were stopped with 3·5 µl 6 x loading dye containing 95% formamide and boiled for 3 min. Samples were analysed on a 20% denaturing acrylamide gel with 7 M urea, followed by autoradiography for the ³²P-labelled samples and by dry-blot transfer onto a nylon membrane and detection by antibody and chemiluminescence substrate of the DIG-labelled bands. The laddering of bands in Fig. 2(a and c) is suggestive of a 3′-excision activity. By using ³²P-labelled dT₃₀, only full-length dT₃₀ was observed and that decreased with time during the assay (Fig. 2b and d). This suggested that the enzyme cleaved from the 3′ end and, moreover, did not exhibit 5′-exonuclease activity. Unlike the alkaline exonuclease of herpes simplex virus type 1, V-TREX did not also have endonuclease activities (Hoffmann & Cheng, 1979; Bronstein & Weber, 1996; Mikhailov et al., 2004). The 3′-excision rate of V-TREX was much faster with the 5′-³²P-labelled dT₃₀ (Fig. 2c) than with the 5′-DIG-labelled one (Fig. 2a). Moreover, the level of digestion using 3′-DIG-dT₃₀ was lower than that using 5′-DIG-dT₃₀. Even digestion using the 5′-³²P-labelled substrate was lower than has been reported for the mammalian TREX proteins (Mazur & Perrino, 2001) and phage T4 DNA polymerase (Promega), which has 3′-5′ exonucleolytic activity in a pH 7·5 buffer. Complete degradation of dT₃₀ was obtained with 0·02 U T4 DNA polymerase µl⁻¹ after 5 min incubation at 30 °C with either 5′-DIG- or 5′-³²P-labelled substrates (data not shown).

Taken together, these results indicated that this slower excision rate of V-TREX could not be attributed only to the presence of the 3′- or 5′-end DIG. For example, the preferred substrate for V-TREX may be mispaired 3′ termini, as for the mammalian TREX proteins (Mazur &
Perrino, 2001), rather than dT_{30}. Furthermore, our reaction conditions for V-TREX might not be optimal and/or viral or cellular cofactor(s) may also be needed for maximum function.

Protein derived from Sf21 cells infected with wt AcMNPV and eluted from Ni columns in the same fraction as V-TREX served as a negative control, to exclude the possibility of contamination with endogenous viral or cellular nucleases. Even by using a concentration equivalent to the highest amount of V-TREX protein used (10 ng µl⁻¹), we could not detect any degradation of either the 5'- or the 3'-DIG- or 32P-labelled dT_{30} for the wt virus control protein preparation (Fig. 2e and f) under parallel assay conditions.

The gradual digestion of the 5'-labelled substrate with time (Fig. 2a and c) indicated that V-TREX either degrades oligonucleotides in a processive manner, but only very slowly, or it acts in a distributive fashion, where dissociation and reassociation of the enzyme with substrate are the limiting factors. To evaluate this, substrate-competition experiments were performed with unlabelled oligonucleotide substrate as a competitor to trap any dissociated V-TREX. 5'-32P-labelled dT_{30} (12 nM) and purified V-TREX protein (5 ng µl⁻¹) were pre-incubated for 15 min at 4 °C in the absence of MgCl₂. The reaction was initiated by addition of 5 mM MgCl₂ with or without unlabelled dT_{30} (1-2 µM) and incubated at 30 °C for the times indicated (Fig. 2g). Addition of 100-fold excess cold dT_{30} retarded the degradation of the labelled substrate, compared to the reaction without the competitor (Fig. 2c). This indicated that V-TREX might dissociate from the oligonucleotide substrate before completing digestion, suggesting that V-TREX acts in a distributive manner.

V-TREX activity under different enzyme concentrations was also investigated under standard assay conditions for 60 min. Limited cleavage of the 5'-DIG-labelled dT_{30} occurred with 10 ng V-TREX µl⁻¹ and was almost complete with 20 ng µl⁻¹ (Fig. 3a), whereas digestion of 5'-32P-labelled substrate started at 1 ng V-TREX µl⁻¹ (Fig. 3b). Increased amounts of V-TREX enhanced the degradation of the 5'-labelled substrate in both cases (Fig. 3a and b). The lower activity with DIG-labelled dT_{30} may have been due to the terminal DIG label, which might affect enzyme-substrate interactions, as discussed above. Consequently, the terminal DIG-labelling system does not seem to be suitable for analysis of the polarity and kinetics of V-TREX function (Fig. 2a and b). Nevertheless, DIG-labelled dT_{30}
is more stable and convenient than $^{32}$P-labelled dT$_{40}$ and was still valid for studying the enzymic activity. V-TREX activity increased at alkaline pH with an optimum of 9.5, dropping dramatically at pH 10 (Fig. 3c). Furthermore, Mg$^{2+}$ was required, with maximal activity at 5 mM and an inhibitory effect by 50 mM (Fig. 3d). NaCl was not required for V-TREX activity and concentrations of 5 mM NaCl or more were inhibitory, with no activity at 150 mM (Fig. 3e). Addition of as little as 0·01 mg BSA ml$^{-1}$ effectively stabilized V-TREX activity, whereas increases in V-TREX activity were not significant with higher amounts of BSA (Fig. 3f).

In conclusion, we demonstrated that the CfMNPV V-TREX protein is a functional 3′→5′ exonuclease that cleaves oligonucleotides in a distributive manner. Based on the functional aspects of other members of the TREX families, the baculoviral V-TREX may play important roles in proofreading and DNA repair during CfMNPV DNA replication.

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References


