VIROLOGY 184, 777–780 (1991)

Insecticidal Effects of an Insect-Specific Neurotoxin Expressed by a Recombinant Baculovirus

SUSUMU MAEDA,*,[1] SANDRA L. VOLRATH,† TERRY N. HANZLIK,*,[2] S. ANDREW HARPER,† KEI MAJIMA,* DARYL W. MADDOX,† BRUCE D. HAMMOCK,*,‡ AND ELIZABETH FOWLER†

*Department of Entomology, University of California, Davis, California 95616; †CIBA–GEIGY Agricultural Biotechnology Research Unit, Research Triangle Park, North Carolina 27709; and ‡Department of Toxicology, University of California, Davis, California 95616

Received April 26, 1991; accepted July 15, 1991

The scorpion *Androctonus australis* has a peptide (AaIT) which selectively targets the insect sodium channel. This mode of action is similar to that of many widely used chemical insecticides. When *Bombyx mori* larvae were infected with a recombinant baculovirus carrying a synthetic AaIT gene, the expressed protein was secreted into the hemolymph and caused symptoms consistent with sodium channel blocking, including tremors and feeding cessation at 40 hr p.i. followed by paralysis and death by 60 hr p.i. Larvae infected with control virus died by 96 hr p.i. These results indicate that foreign genes can be used in recombinant baculoviruses to reduce insect feeding damage and increase the rate of insect kill. © 1991 Academic Press, Inc.

Baculoviruses are major insect pathogens characterized by large circular double-stranded DNA genomes and enveloped rod-shaped virions. They have potential for insect control (*1*) and recently have been proven to be efficient vectors for the expression of foreign genes (*2–4*). The high levels of biologically active molecules produced in infected insect larvae suggest that recombinant baculoviruses carrying an exogenous gene whose product is selectively toxic to insects could be safe and effective insecticides.

The identification of a foreign gene effective for insect control is crucial for the construction of a baculovirus insecticide. Possible candidates include enzymes essential for metabolism, peptide hormones, and insect-specific toxins. Recently, an insect diuretic hormone (*5*) and a juvenile hormone esterase (*6*) have shown some insecticidal and physiological effects on infected insects when baculoviruses carrying these genes were applied to insects; however, the recombinant viruses did not show a strong increase in potency when compared to control viruses.

The insect-specific toxin AaIT from the venom of the scorpion *A. australis* (*7*) consists of a single polypeptide chain of 70 amino acids cross-linked by four disulfide bonds. AaIT affects only insects and has been shown to have no effect on isopods and mammals, even at high doses (*7–9*). Electrophysiological and receptor binding studies using nervous tissue from insects, crustaceans, and mammals have also demonstrated that AaIT exclusively affects the insect nervous system (*10–12*). Furthermore, AaIT is toxic only when it is injected into the body cavity of susceptible insects. AaIT is thus an excellent candidate to improve the efficacy of baculovirus insecticides. In this communication, we describe the acute insecticidal effects of a recombinant baculovirus expressing this protein.

A gene encoding AaIT behind a secretion signal sequence from the silkworm neuropeptide bombyxin (*13*) was synthesized and then inserted into the pBK273 transfer vector of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) under the control of the polyhedrin gene promoter (Fig. 1). Three micrograms of the resulting recombinant plasmid (pBmAaIT) and 2 μg of BmNPV DNA were cotransfected into *B. mori* (BmN) cells by the method described previously (*14, 15*). A recombinant virus, BmAaIT, which lacks polyhedra production was isolated by plaque assay from the cotransfected culture supernatant. BmN cells infected with BmAaIT, BmDH5 (carrying the diuretic hormone gene) (*5*), and a control, BmM14 (polyhedron-deficient virus having a 76 nucleotide deletion in the polyhedrin gene) (unpublished data), produced similar cytopathic effects, suggesting that BmAaIT had no specific cytotoxic effects *in vitro* attributable to AaIT.

The physiological and insecticidal effects of BmAaIT were examined in larvae of the silkworm *B. mori* by the method described previously (*14*). When second instar larvae were injected with 10^5 PFU of virus, they showed dramatic changes in behavior about 40 hr p.i. Larvae infected with BmAaIT showed continuous rotations of the head, dorsal arching, and body tremors (Fig. 2). All larvae stopped feeding 45 to 55 hr p.i. and

[1] To whom requests for reprints should be addressed.

[2] Present address: Division of Entomology, CSIRO, Canberra, ACT 2601, Australia.

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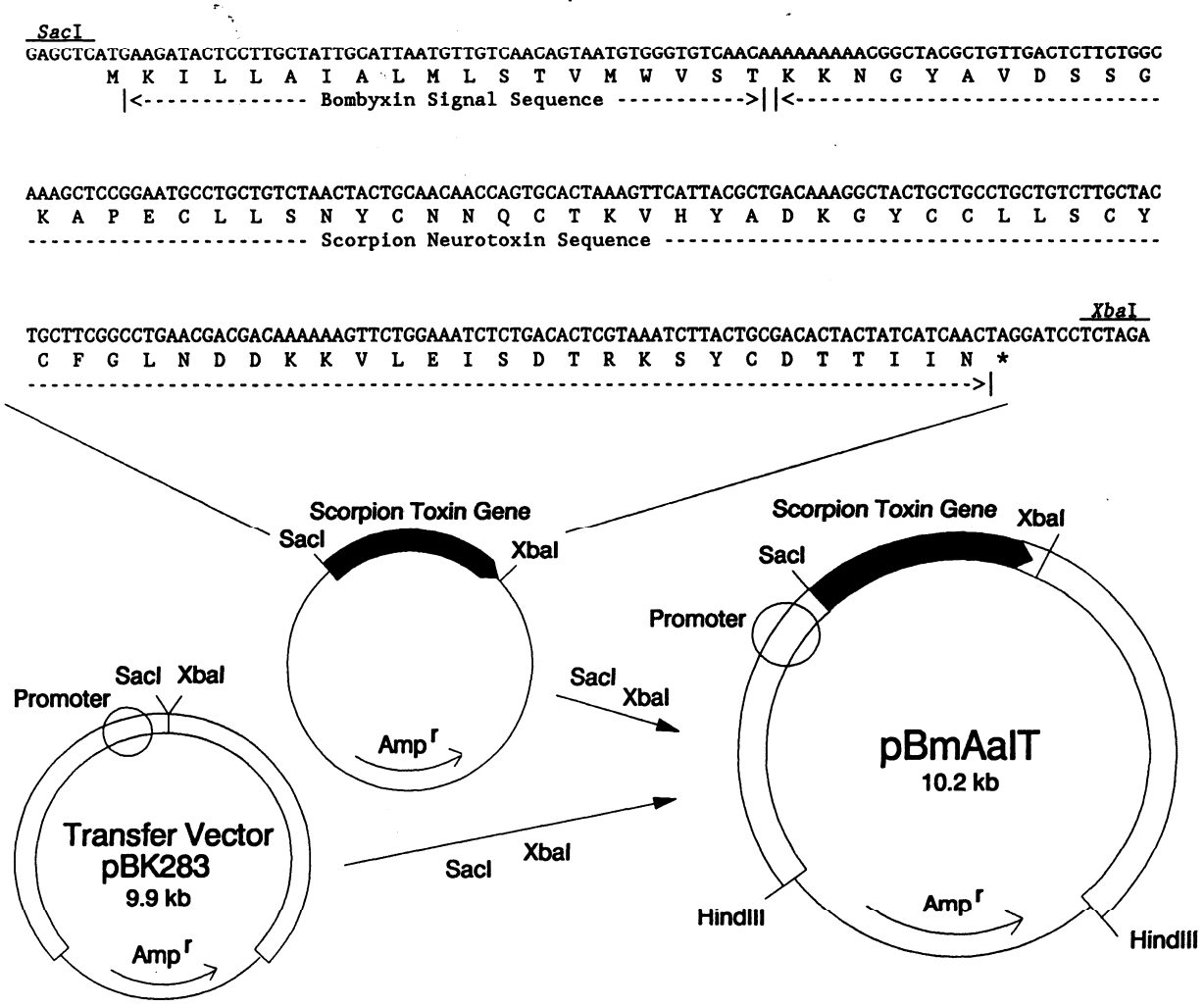

FIG. 1. Construction of a recombinant transfer vector. The scorpion toxin AalT gene was constructed by modifying the gene prepared by Dee *et al.* (*18*) using PCR to insert an isoleucine as the penultimate residue. This corrected the encoded sequence to that published by Darbon *et al.* (*7*). PCR was also used to add a DNA sequence encoding a bombyxin signal sequence (*13*) to the 5′ end of the AalT coding sequence. The inserted gene was sequenced and the *SacI*/*XbaI* fragment was ligated into a *SacI*/*XbaI*-digested BmNPV transfer vector pBK283 (*15*), which placed the chimeric gene under control of the polyhedrin promoter.

did not move, although they were still able to respond to prodding. All larvae died by 60 hr p.i. Similar results were obtained in all *B. mori* instars (2nd-5th) tested. This is approximately a 40% increase in the speed of kill compared to that of the control BmM14 virus (Fig. 3). As reported previously (*5*), infection with BmDH5 virus caused about a 20% increase in the speed of kill compared to that of wild-type virus and a recombinant virus carrying human interferon α, but the larvae infected with BmDH5 did not show any apparent changes in behavior.

To confirm the production of active AalT peptide in infected silkworm larvae, hemolymph removed from BmAalT-infected larvae at 55 hr p.i. was injected into larvae of the blow fly, *Sarcophaga falculata.* Symptoms occurred immediately following injection and were similar to those induced by AalT purified from venom obtained from Sigma using two steps of reverse-phase HPLC. Hemolymph from larvae infected with control BmM14 virus at 55 hr p.i. produced no acute symptoms upon injection. Based upon the dose response in this biological assay, it was estimated that the hemolymph contained about 5 μg/ml of AalT. The presence of AalT in infected 5th instar larvae was also confirmed by immunoblot analysis of hemolymph samples taken at various times p.i. An immunoreactive band which comigrated with authentic AalT was detected in the 24-, 48-, and 55-hr samples (Fig. 4). The amount of

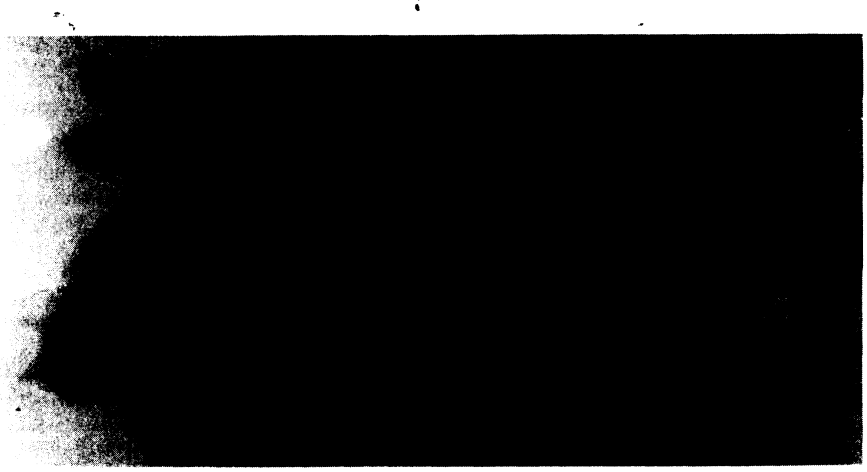

FIG. 2. Effects of toxin expressing (BmAaIT) and control (Bm14) virus on *B. mori* larvae. Fifth instar silkworm larvae injected with about 10^5 PFU of BmAaIT or control BmM14 (marked by M) at 48 hr p.i. Larvae infected with BmAaIT showed dorsal arching and were unable to remain upright.

material in the 55-hr sample was approximately 5 μg/ml, a concentration similar to that estimated from the *Sarcophaga* bioassay. These results indicate that the specific activity of the expressed material is similar to that of AaIT purified from venom. The apparent molecular weight of the AaIT in hemolymph was identical to that of the native protein, indicating that correct cleavage of the heterologous signal sequence in larvae accompanies secretion of the peptide. A recombinant BmNPV construct without the bombyxin signal sequence was also constructed (unpublished data). Larvae infected with this virus showed paralytic symptoms at 60 hr p.i., but the time to death was similar to that caused by the control virus.

In order to correlate the dose and toxicological effect of the BmAaIT virus with the native scorpion toxin, purified AaIT was injected into second instar silkworm larvae (0.6 μl saline per 5–10 mg larva). Doses over 300 ng caused death in most larvae within 24 hr with onset of symptoms at 30 min postinjection. Doses of 12–60 ng caused effects similar to infection with BmAaIT in more than half of the larvae within 24 hr; however, these larvae recovered within 48 hr. Low doses (less than 6 ng) had no apparent effects. The sensitivity of

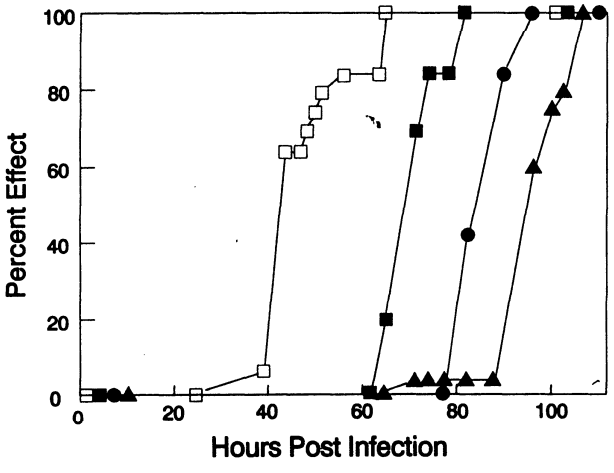

FIG. 3. Mortality and toxicological effects of various recombinant viruses in infected silkworm larvae. Symbols: □, percentage of larvae showing toxic symptoms after injection with the toxin expression virus (BmAaIT); ■, percentage of dead larvae after injection with BmAaIT; ●, percentage of dead larvae after injection with diuretic hormone-expressing virus (BmDH5); ▲, percentage of dead larvae after injection with control (polyhedrin minus) virus (BmM14).

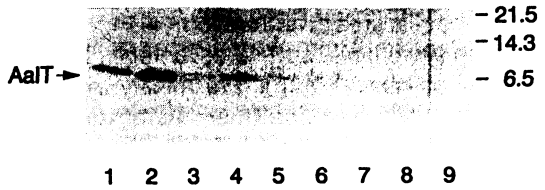

FIG. 4. Immunoblot analysis of hemolymph from fifth instar silkworm larvae infected with BmAaIT. Hemolymph samples taken at various times p.i. were electrophresed on a 10–20% SDS–polyacrylamide gel (Novex), electroblotted to an Immobilon P (Millipore) membrane, and immunostained with a polyclonal rabbit anti-AaIT antibody using an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and *p*-nitroblue tetrazolium. The arrow shows the location of AaIT (7.8 kDa). Lane 1, 10 ng purified AaIT from venom; lane 2, 25 ng purified AaIT; lane 3, 10 ng purified AaIT added to hemolymph (2 μl) from uninfected larvae; lane 4, 25 ng AaIT added to hemolymph from uninfected larvae; lane 5; hemolymph (2 μl) taken 55 hr p.i.; lane 6, hemolymph taken 48 hr p.i.; lane 7, hemolymph taken 24 hr p.i.; lane 8, hemolymph taken immediately p.i.; lane 9, hemolymph from an uninfected larva. Positions of molecular weight markers (trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa; aprotinin, 6.5 kDa) in kDa are shown to the far right.

the silkworm to AaIT is comparable to that of the cutworm, *Spodoptera littoralis,* for which de Dianous *et al.* (*9*) reported an LD_{50} of 130 ng/10 mg body wt. The abnormal behavior of larvae injected with the native AaIT was the same as that observed following infection with BmAaIT. These data also indicate that the quantity of AaIT found in the hemolymph of BmAaIT-infected insects (15 ng/3 μl hemolymph/10 mg larvae/55 hr p.i.) is sufficient to cause the symptoms initially observed, but not enough to kill insects. Continuous expression by the recombinant virus probably leads to sufficient accumulation of AaIT and death. These observations suggest that the *B. mori* baculovirus system is useful for screening genes for pest control and as a new approach for characterizing the effects of neurotoxin molecules in an *in vivo* system. Furthermore, the silkworm is easy to rear, synchronize, and contain in the laboratory while lacking the ability to survive in the field, making it appropriate for recombinant DNA experiments. The use of polyhedrin-negative viruses for laboratory studies also adds an additional safety factor.

Carbonell *et al.* (*16*) reported that a recombinant baculovirus carrying a gene for the insect toxin (BeIT) of the scorpion *Buthus eupeus* preceded by a signal sequence from human interferon α failed to produce sufficient toxin to cause detectable biological activity. The difference between their results and ours might be attributed to the toxin and/or the signal sequence used.

Baculoviruses have long been attractive biological agents for insect control, but one limitation to their use has been the slow rate of kill, resulting in significant crop damage. Our results demonstrate that AaIT expression can significantly increase the insecticidal activity of a baculovirus. The silkworm with the BmNPV expression system has several advantages for screening genes for insect control. Preliminary experiments indicate that oral infection (the natural transmission pathway) of a pest insect, *Heliothis virescens,* by a newly constructed recombinant baculovirus carrying the same AaIT gene and the polyhedrin gene also showed increased speed of kill (19). AaIT is also highly insect specific; 50 mg/kg dose of AaIT produced no effects in mice (*17*). This combination of a protein with specific insecticidal properties with an insect virus of limited host range presents a new and highly selective approach to insect control.

ACKNOWLEDGMENTS

We thank S. G. Kamita for help with the *in vitro* experiments, R. Krenshaw for immunoblot analysis, and L. Harshman, and G. Jen for helpful discussions. Supported by a Ciba–Geigy Corporation grant.

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