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Location of the *Bombyx mori* specificity domain on a *Bacillus thuringiensis* δ-endotoxin protein

(site-directed mutagenesis/overexpression/biological pesticide)

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ABSTRACT **_Bacillus thuringiensis_ produces different types of insecticidal crystal proteins (ICPs) or δ-endotoxins. In an effort to identify the insect specificity of ICP toxins, two *icp* genes were cloned into the *Escherichia coli* expression vector pKK223-3, and bioassays were performed with purified crystals. The type A protein [from an *icpA1*, or 4.5-kilobase (kb) gene, from *B. thuringiensis* var. *kurstaki* HD-1] was found to be 400 times more active against *Bombyx mori* than type C protein (from an *icpC73*, or 6.6-kb gene, from *B. thuringiensis* var. *kurstaki* HD-244). The type C protein was 9 times more active against *Trichoplusia ni* than the type A protein, while both have similar activity against *Manduca sexta*. To locate the specificity domain of the type A protein for *B. mori*, site-directed mutagenesis was used to introduce or remove restriction enzyme sites, facilitating the exchange of regions of the two genes. The hybrid genes were overexpressed, and purified ICP was used in bioassays. The *B. mori* specificity domain for the ICP A toxin is located in the amino-terminal portion of the hypervariable region between amino acids 332 and 450.**

Bacillus thuringiensis is the most widely used microbial pesticide. The biochemical basis of the pesticide is an insecticidal crystal protein (ICP), which is produced by the bacterium as a 133-kDa protoxin that requires proteolytic cleavage in the insect gut for activation.

B. thuringiensis strains may be classified into five pathotypes, depending on the spectrum of insecticidal activity for their ICPs: (*i*) lepidopteran active, (*ii*) dipteran active, (*iii*) coleopteran active, (*iv*) active against both Lepidoptera and Diptera, and (*v*) no known toxic activity (1). Among the lepidopteran-active strains, differences in insect specificity were first observed by Dulmage (2) and Yamamoto *et al.* (3). In these experiments, whole cultures were used (i.e., crystals and spores); hence, it was not apparent whether insect specificity was due to the ICP, the presence of the spore, or some property of the insect, such as proteolytic activation of the δ-endotoxin. Jaquet *et al.* (4) and Lecadet and Martouret (5) used purified crystals to survey a large number of *B. thuringiensis* crystal proteins for activity against several species of insects. Significant differences were seen in insecticidal specificities. In the above examples, whether whole cultures or isolated crystals were used, the problem of determining whether a particular δ-endotoxin has specificity to a particular insect was confounded by the fact that *B. thuringiensis* strains may harbor more than one δ-endotoxin gene (6); therefore, the specificities of particular genes may be diluted by the products of other δ-endotoxin genes.

DNA sequence analysis of a number of the lepidopteran-active *icp* genes has revealed that they fall into five groups. Three of these exhibit strong homology and have been termed the 4.3-kilobase (kb), 5.4-kb, and 6.6-kb genes (6, 7). Two less-homologous lepidopteran-active *icp* genes have been recently described (8–10). In this paper, we refer to the first three genes as *icpA*, *icpB*, and *icpC*, respectively, following the nomenclature of *icp* for ICP gene (9, 11, 12) and the suggested locus letters *A*, *B*, and *C* (7, 10) for the first three genes sequenced (13–15).

To date, there have been very little quantitative data on lepidopteran-specificity differences between ICPs from pure gene sources. Wilcox *et al.* (16) were the first to recognize the role of an *icp* gene in the specific action against a particular insect by noting that a strain lacking a 5.3-kb gene (*icpB*) showed less activity against *Spodoptera exigua*. Kondo *et al.* (17) demonstrated the specificity of different cloned δ-endotoxin genes (*icpA1* and *icpB1* from *B. thuringiensis* HD-1). In qualitative bioassays with *Escherichia coli* clones of these genes, they observed a significant difference in activity against *Bombyx mori*. Recently, Höfte *et al.* (9) have identified five lepidopteran-active genes that differ in specificity against several insects.

Understanding the biochemical and genetic mechanisms for insecticidal action and specificity of insecticidal proteins would be richly rewarded by revealing a means of synthesizing new biorational insecticides. In this paper, we demonstrate differences in insect specificities between two δ-endotoxin genes, *icpA1* and *icpC73*, against the insects *B. mori*, *Trichoplusia ni*, and *Manduca sexta*. By exchanging regions of these two genes, we are able to locate the region of the *icpA* gene that is responsible for specificity to *B. mori*.

MATERIALS AND METHODS

Origins of δ-Endotoxin Genes, Hosts, and Vectors. *E. coli* containing the *icpA1* gene, ES1 (pES1) (29), was obtained from the American Type Culture Collection as ATCC 31995. The isolation of the *icpC73* gene from *B. thuringiensis* strain HD-244 has been reported (12) as *E. coli* 87-22 (pOS1002). We have sequenced this gene (J. R. Sabourin, J. H. McLinden, and D.H.D., unpublished data) and found it to have exactly the same sequence as the *icpC73* gene from *B. thuringiensis* var. *kurstaki* HD-73 (15). The *E. coli* host JM103 (18) was obtained from R. Swenson (Ohio State University), and cloning vectors M13mp18 (BRL) pUC8 and pKK223-3 (Pharmacia) were obtained from the suppliers indicated.

Site-Directed Mutagenesis and Other Molecular Genetic Techniques. Oligonucleotides were synthesized with an Applied Biosystems model 380 B DNA synthesizer in the Biochemistry Instrumentation Center (Department of Biochemistry, Ohio State University). Single primer site-directed mutagenesis followed the method of Gillam and Smith (19), selection of mutants was by the method of Kunkel

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Abbreviation: ICP, insecticidal crystal protein.

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(20), and screening was by restriction enzyme analysis. Other molecular genetic techniques were as described (18).

Construction of Site-Directed Mutants, Substitution Mutants, and Overexpression Mutants. Fig. 1 illustrates the overall strategy used in this study. Steps in the construction were given as follows:

Step A. To construct parental expression vectors or recipients for site-directed mutants and substitution mutants, the *icpA1* and *icpC73* genes were cut from their original plasmids with *Nde* I, and the ends of the fragment were filled in with the Klenow fragment of DNA polymerase and cloned into the *Sma* I site of a modified pKK223-3, which had its *Eco*RI site removed (by filling-in with the Klenow fragment).

Step B. To prepare for site-directed mutagenesis, the *Hin*cII/*Kpn* I region of the two genes was cloned into M13mp18.

Step C. Oligonucleotide-primed site-directed mutagenesis was used to eliminate and add restriction enzyme sites as follows: (*i*) 5′-GTTCGTGTACGGTAT-3′ was used to remove the third *Eco*RI site from *icpA1*, creating *icpA1* $E_3(-)$. This converted the amino acid Ile-523 of *icpA1* to Val-523 found in *icpC73*. (*ii*) 5′-GAAGAGTTCGCTAGG-3′ was used to remove the first *Eco*RI site from the previously constructed mutant, creating *icpA1* $E_1(-)$, $E_3(-)$. This oligonucleotide was also used to remove the first *Eco*RI site from *icpC73*, creating *icpC73* $E_1(-)$. The removal of the first *Eco*RI site did not alter the amino acids. (*iii*) 5′-GTAACCCTCGAGGCAGAA-3′ was used to add an *Xho* I site to the above two mutants creating *icpA1* X(+), $E_3(-)$, and *icpA1* X(+), $E_1(-)$, $E_3(-)$. The addition of an *Xho* I site changed amino acid 610 from phenylalanine to leucine, which was not a conservative change, but we show below that this change alone did not alter insect toxicity.

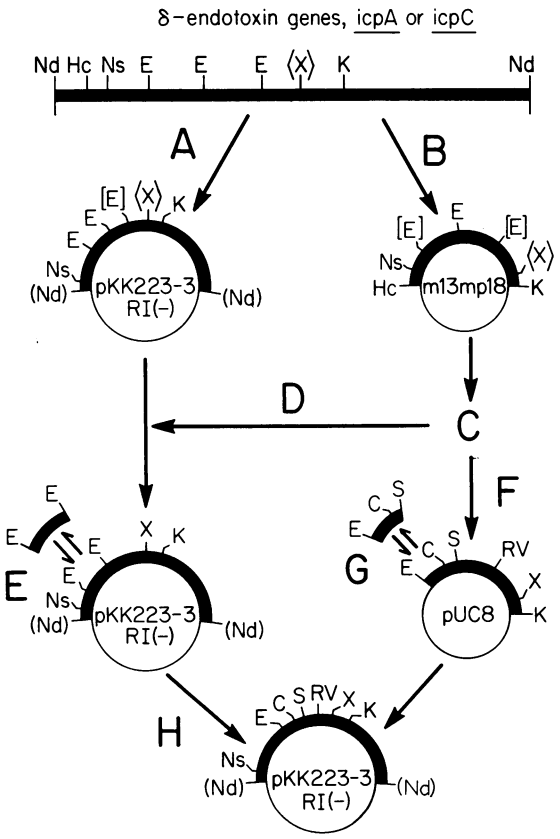

FIG. 1. Construction of substitution mutations and expression plasmids of *icp* genes. C, *Cla* I; E, *Eco*RI; Hc, *Hin*cII; K, *Kpn* I; Nd, *Nde* I; Ns, *Nsi* I; RV, *Eco*RV; S, *Sst* I; X, *Xho* I; [E], removed *Eco*RI sites; ⟨X⟩, added *Xho* I site.

Step D. Site-directed mutations were put into pKK223-3 *Eco*RI(−), in preparation for bioassay, by replacing the *Nsi* I/*Kpn* I portions of the parental genes, cloned in pKK223-3 *Eco*RI(−), with *Nsi* I/*Kpn* I portions of the site-directed mutant genes.

Step E. Exchange of the constant regions was done by exchanging the *Eco*RI_1/*Eco*RI_2 fragments between *icpA1* $E_3(-)$ and *icpC73*.

Step F. Site-directed mutations were put into pUC8 by cloning the *Eco*RI_2/*Kpn* I regions into the equivalent sites in the polylinker.

Step G. Subregions of the variable region were exchanged between the *icpA1* mutant genes and the *icpC73* gene by the scheme shown in Fig. 2.

Step H. Substitution mutations were transferred into expression vectors by cloning the *Eco*RI_2/*Xho* I portions of the genes into a recipient *icpA1* E_1, $E_3(-)$, X(+), or *icpC73* $E_1(-)$ mutant gene cloned in pKK223-3 *Eco*RI(−), yielding the substitution mutants shown in Fig. 2.

Purification of Overexpressed ICP. ICP was expressed to the level of 35–50% of total protein in JM103 using the modified expression vector pKK223-3 as judged by scanning total protein displayed by PAGE with a laser scanner (data not shown; but see ref. 21). ICP was deposited as large diamond-shaped crystals appearing similar in lattice arrangement and overall shape to crystals made in *B. thuringiensis*. ICP was purified from overexpressing cells by sonication and extensive washing with 10% NaBr (22). Final protein preparations used in bioassays were examined by PAGE and shown to be >95% pure ICP. Protein concentrations were determined by BCA protein assay (Pierce) using bovine serum albumin (Sigma) as a standard.

Bioassays. Where LD_{50} values are reported, 10–20 insects were used for each point on the probit plot, and four or five points were used in determination of the slope. LD_{50} values are reported as μg of protein applied to artificial diet or leaf disks. LD_{50} values and 95% fiducial limits were calculated with the PROBIT.SAS program. Controls consisted of *E. coli* containing only the expression vector pKK223-3. Bioassays of both *M. sexta* and *T. ni* were conducted as described by McLinden *et al.* (12). Bioassays of *B. mori* were conducted by pipetting 50 μl of diluted ICP on a mulberry leaf disk (diameter, 1.5 cm) and adding one larva per cup. Ten larvae were used per concentration of ICP protein in mortality assays. *M. sexta* eggs were obtained from Michael Jackson (USDA South Atlantic Area Tobacco Research Laboratory, Oxford, NC). *T. ni* eggs were obtained from Lillian Moug (USDA Western Cotton Research Laboratory, Phoenix, AZ). *B. mori* eggs were obtained from Y. Tanada (University of California, Riverside, CA) and/or purchased from Carolina Biological Supply and reared on mulberry leaves.

RESULTS

Specificity of ICPs. To determine whether ICPs exert selective toxic activity toward particular insects, we cloned individual genes and overexpressed and partially purified their gene products. Quantitative data were sought on the insecticidal specificity of type A and type C ICPs. Table 1 shows the toxicity of purified ICPs from OSU 4101 and OSU 4201, which overexpress the wild-type *icpA1* and *icpC73* genes, respectively. The type A toxin shows greater activity toward *B. mori*, while the type C toxin shows greater activity toward *T. ni*. Both toxins show equally strong activity against *M. sexta*. The clearest difference between the activities of the two ICPs was on *B. mori*, and this insect was chosen for further work.

Location of the Specificity Domain for *B. mori*. We sought to locate the regions of the δ-endotoxin genes responsible for differences in larvicidal specificity by exchanging segments

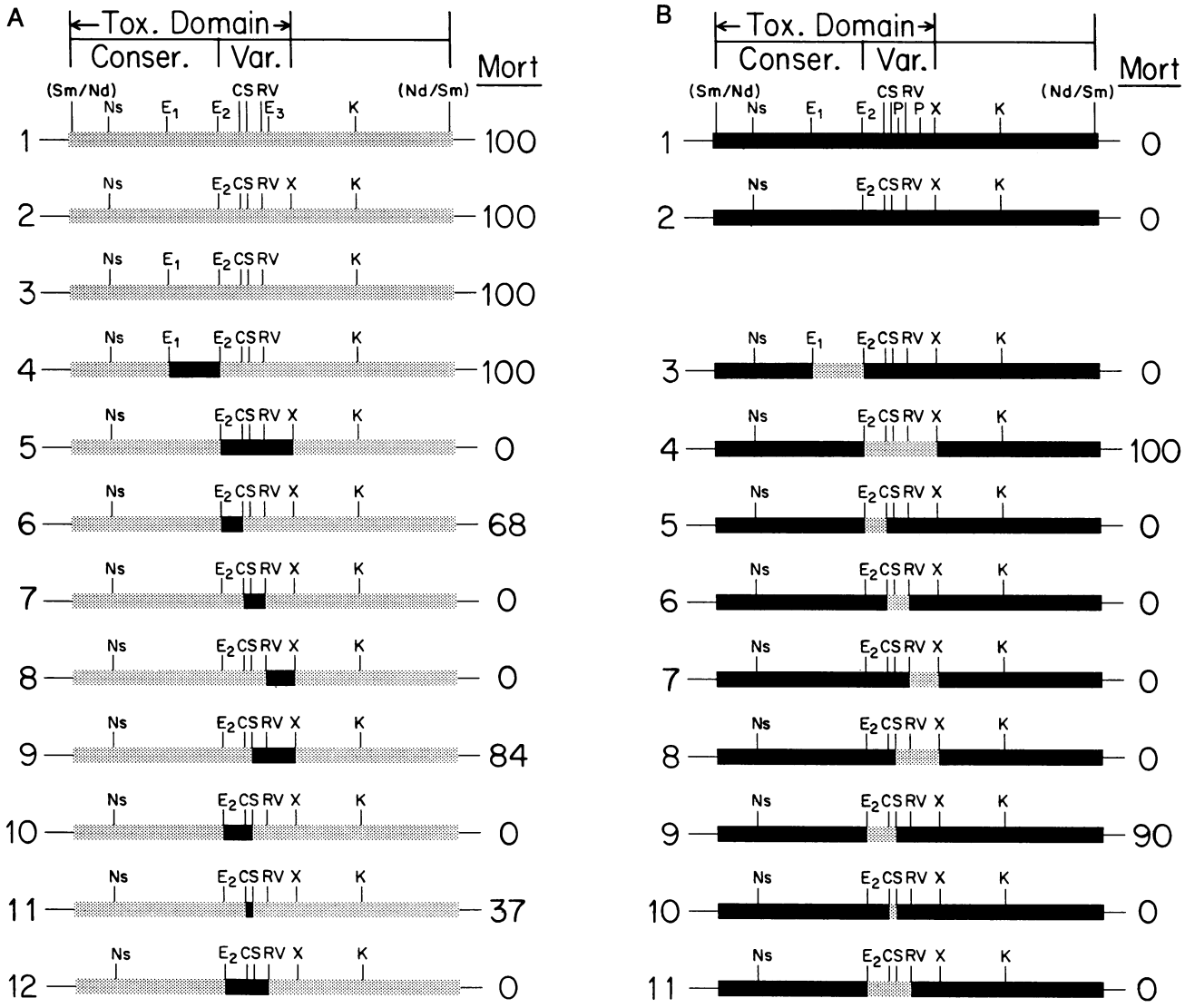

FIG. 2. Substitution mutants cloned onto the expression vector pKK223-3 (modified to remove its *Eco*RI site). The diagram at the top illustrates the toxicity region of the protein (Tox. Domain) and regions of the ICP proteins that share amino acid sequence (Conser.) or are different; i.e., the hypervariable region (Var.). (*A*) OSU 4100 series. Lines: 1, OSU 4101, the original *icpA1* gene; 2, OSU 4102, site-directed mutant of *icpA1* gene with the *Eco*RI$_1$ and *Eco*RI$_2$ sites removed and an *Xho* I site introduced to facilitate exchanges shown in lines 5–12; 3, OSU 4103, site-directed mutant with the *Eco*RI$_3$ site removed to facilitate the exchange shown in line 4; 4, OSU 4104, *Eco*RI fragment from pOS4103 exchanged with corresponding fragment from *icpC73*. 5–12, OSU 4105–4112, DNA fragments from pOS4102 exchanged with corresponding fragments from *icpC73*. (*B*) OSU 4200 series. Lines: 1, OSU 4201, the original *icpC73* gene; 2, OSU 4202, site-directed mutant with *Eco*RI$_1$ site removed to facilitate exchanges shown in lines 4–11; 3, OSU 4103, *Eco*RI fragment from pOS4201 exchanged with corresponding fragment of *icpA1*; 4–11, OSU 4205–4212, DNA fragments from pOS4202 exchanged with corresponding fragments of *icpA1*. MORT, % insect mortality with a dose of 4 μg applied to food disks (mean of three assays, 10–20 insects per assay).

of the *icpA1* gene with corresponding segments of *icpC73*. The resultant substitution mutants are illustrated in Fig. 2. Fig. 2 *A* and *B* (lines 1) illustrate the native *icpA1* and *icpC73* genes, respectively, cloned into the modified expression vector pKK223-3 (*Eco*RI$^-$). Site-directed mutant forms of these genes, wherein restriction enzyme sites were removed or added for purposes of the following exchanges, are illustrated in Fig. 2*A* (lines 2 and 3) and Fig. 2*B* (line 2).

Table 1. Specificity of δ-endotoxins toward three insects

Insect	LD_{50}, μg	
	icpA	*icpC*
M. sexta	0.077 (0.024–0.135)	0.072 (0.062–0.082)
T. ni	2.88 (1.34–5.67)	0.32 (0.12–0.81)
B. mori	0.37 (0.0007–0.729)	>150.0*

M. sexta and *T. ni* assays were done on artificial diet (12). *B. mori* assays were done on mulberry leaf disks. Values in parentheses represent upper and lower fiducial limits.

*A LD_{50} value has not been obtained. The stated amount causes no mortality, no weight loss, and no delay in development.

Exchange of the "conserved region" (Fig. 2*A*, line 4; Fig. 2*B*, line 3) resulted in no measurable effect on the toxicity. Exchange of the complete hypervariable region (Fig. 2*A*, line 5; Fig. 2*B*, line 4) resulted in complete transfer of toxic activity.

Subdivision of the hypervariable region into an amino-terminal half (*Eco*RI$_2$/*Sst* I, yielding OSU 4110 and OSU 4210) and a carboxyl-terminal half (*Sst* I/*Xho* I, yielding OSU 4109 and OSU 4209) yielded interesting results. Transfer of the amino-terminal half of the hypervariable region from *icpA1* to *icpC73* transferred virtually all of the toxic activity (Fig. 2*B*, line 9), while the reciprocal exchange resulted in complete loss of activity (Fig. 2*A*, line 10). We refer to this region as the *B. mori* specificity domain (*Eco*RI$_2$/*Sst* I). Exchange of the carboxyl-terminal half of the hypervariable region *in toto* had no effect on the toxicity of either gene product (Fig. 2*A*, line 9; Fig. 2*B*, line 8).

Further exchanges subdividing the specificity domain (*Eco*RI$_2$/*Sst* I) into subsets (*Eco*RI$_2$/*Cla* I, yielding OSU 4106 and OSU 4206; and *Cla* I/*Sst* I, yielding OSU 4111 and OSU 4211) resulted in reduction of toxicity to *B. mori* with gene products from the *icpA1* background (Fig. 2*A*, lines 6 and 11), while transferring no activity to the *icpC73* background (Fig. 2*B*, lines 5 and 10).

Subdivision of the carboxyl-terminal portion of the hypervariable region (*Sst* I/*Xho* I) had a drastic effect on toxicity to *B. mori*. No toxicity was transferred or retained in either genetic background when this region was split (Fig. 2*A*, lines 7, 8, and 12; Fig. 2*B*, lines 6, 7, and 11), even if the specificity domain from *icpA1* remained intact (Fig. 2*B*, line 11).

DISCUSSION

We have demonstrated that the *B. thuringiensis* δ-endotoxins tested have specific activity to particular insects (Table 1). While this has been known to exist in cases in which the specific activity was broadly different, affecting different orders of insects such as *B. thuringiensis* var. *israelensis*, which affects Diptera, and *B. thuringiensis* var. *tenebrionis*, which affects Coleoptera, specificity by pure ICPs has only recently been quantitatively demonstrated for different species of lepidopterans.

It may be assumed that differences in specificity are due to amino acid differences in proteins. Therefore, we did not expect that the specificity region would lie in the constant region of the gene. Nonetheless, an exchange of a portion of the conserved regions of *icpA1* and *icpC73* was performed to test this simple hypothesis. The conserved region is from amino acid 1 to 283; however, it is convenient to define it as the region between the first two *Eco*RI sites (positioned at amino acid residues 90 and 332), as there are no amino acid differences before the first *Eco*RI site and few differences between *Eco*RI$_1$ and *Eco*RI$_2$. In actuality, there are 10 amino acid differences between these two genes in the rightmost portion of the 725-base-pair fragment bounded by these two *Eco*RI sites. Nevertheless, we observed that the substitution of this fragment did not measurably affect the toxicity of *icpA1* for *B. mori*.

We provide evidence that the first portion of the variable region of the *B. thuringiensis* δ-endotoxin, from residues 332–450, is involved in this specific toxicity. In retrospect, this result is predictable upon inspection of the derived amino acid sequences from *icpA* (13), *icpB* (23, 24), and *icpC* (15) genes, and the knowledge that *icpA* is specific for *B. mori* (ref. 8; Table 1), while the *icpB* (8) and *icpC* genes (Table 1) are specific for *M. sexta*; i.e., $A \neq B$, $A \neq C$, and $B \cong C$ (where *A*, *B*, and *C* refer to the *icp* gene type). Comparison of derived amino acid sequences between *icpA*, *icpB*, and *icpC* shows differences scattered between amino acids 283 and 612, thus defining the variable region. It has been noted (24) that in the region 283–466, $A \neq B$, $A \neq C$, and $B \cong C$, while in the region 466–613, $A \neq C$, $B \neq C$, and $A \cong B$. If some amino acid sequences are necessary for the specific activity of *icpA* against *B. mori* and these are not present in *icpB* or *icpC*, we could deduce that these would be in the region of amino acids 283–466. Our results confirm this prediction and more closely define the *B. mori* specificity domain to amino acids 332–450.

The observation that many of the exchanges of the hypervariable region cause loss or reduction of toxicity, particularly in the carboxyl-terminal portion of the hypervariable region, leads us to predict that this region is composed of several structural domains that are disrupted by the exchanges. The disruption of these hypothetical structures would result in alterations that inactivate the toxin activity.

The predicted secondary structure of the *B. mori* specificity domain of *icpA1* and the corresponding sequences of *icpC73* reveals a potential structural basis for the specificity differences. We have applied the algorithms of Kyte and Doolittle (25) and Chou and Fasman (26) and have observed that the *icpA*-encoded protein has two hydrophilic peaks (Fig. 3*a*), which are superimposed on two β-sheets (Fig. 3*b*) at positions around 70 and 90 in the domain (residues 403 and 423 of the whole protein). The *icpC*-encoded protein has only one hydrophilic peak (Fig. 3*d*), which is superimposed on a single β-sheet about position 90 of the corresponding domain (Fig. 3*e*). Both proteins are predicted to have α-helix helical structures about position 30. The presence of hydrophilic β-sheets suggests surface structures that could interact with midgut cell receptors. A recent report revealed that ICP specificity is correlated with high-affinity cell binding (27). This would suggest that the region we describe here as the specificity domain is involved in receptor binding.

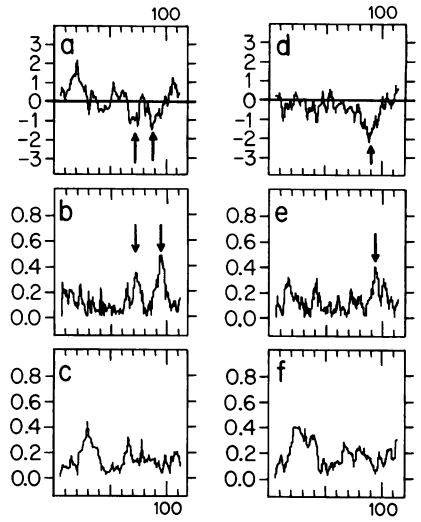

FIG. 3. Predicted secondary structure of the *B. mori* specificity domain of *icpA1* (*a–c*) and the corresponding region of *icpC73* (*d–f*). Numbering of this domain, 0–120, corresponds to 332–452 of the whole protein. (*a* and *d*) Hydrophobicity plots (25). (*b* and *e*) Predicted amphiphilic β-sheets (26). (*c* and *f*) Predicted amphiphilic α-helical conformation (26). Arrows mark hydrophilic peaks (*a* and *d*) and β-sheets (*b* and *e*). In *a* and *d*, the hydrophobic value of each amino acid is plotted.

There are 52 amino acid differences between the *icpA1* and *icpC73* gene products in the region we have identified as the *B. mori* specificity domain: 24 nonconserved, 23 conserved, and 5 misalignments, according to the Dayhoff *et al.* (28) categories of conservative amino acids. We would be interested in knowing whether specificities against other insects lie in this same region and whether alteration of these amino acids by site-directed mutagenesis would improve insecticidal activity against these insects.

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