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Location of the Dipteran Specificity Region in a Lepidopteran-Dipteran Crystal Protein from *Bacillus thuringiensis*

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Two highly related crystal protein genes from *Bacillus thuringiensis* subsp. *kurstaki* HD-1, designated *cryIIA* and *cryIIB* (previously named *cryB1* and *cryB2*, respectively), were used to study host range specificity. Their respective gene products are 87% identical but exhibit different toxicity spectra; CryIIA is toxic to both mosquito and tobacco hornworm larvae, whereas CryIIB is toxic only to the latter. Hybrids of the *cryIIA* and *cryIIB* genes were generated, and their resultant gene products were assayed for toxicity. A short segment of CryIIA corresponding to residues 307 through 382 was shown to be sufficient for altering host range specificity—i.e., when this region replaced the corresponding segment of CryIIB, the resulting hybrid protein acquired toxicity against mosquitoes. The CryIIA and CryIIB polypeptides differ by only 18 amino acids in this region, indicating that very few amino acid changes can have a substantial effect on the toxicity spectra of these proteins.

The common soil bacterium *Bacillus thuringiensis* synthesizes proteinaceous crystalline inclusions that are toxic to a variety of insects. Several subspecies have been identified to date, and their host ranges can vary substantially. By far the greatest number produce large bipyramidal crystals which are toxic only to lepidopteran larvae (2, 14, 26). A few subspecies that synthesize crystals which are toxic to certain dipteran larvae (mosquitos and black flies; 10) or coleopteran larvae (15) have been identified.

Some of the strains which produce the lepidopteran larvae-specific bipyramidal crystals also synthesize smaller cuboidal crystals which have a somewhat extended toxicity spectrum; they are toxic to mosquito as well as lepidopteran larvae (29). In a previous report (27), we described the cloning and sequencing of two homologous crystal protein genes from *B. thuringiensis* subsp. *kurstaki* HD-1. These genes were previously designated *cryB1* and *cryB2* and have now been renamed *cryIIA* and *cryIIB*, respectively (14). Both genes encode polypeptides of 633 amino acid residues and with molecular masses of ca. 71 kilodaltons, and the deduced amino acid sequences are 87% identical. The *cryIIA* gene product is a major component of the cuboidal crystals from this strain; as shown in this communication, the *cryIIB* gene does not appear to be transcribed at an appreciable level. However, both genes can be expressed efficiently in *Escherichia coli*, and their respective gene products are toxic. Interestingly, the CryIIA and CryIIB polypeptides differ in host range specificity despite their close relatedness. The *cryIIA* product is toxic to both dipteran (*Aedes aegypti*) and lepidopteran (*Manduca sexta*) larvae, whereas the *cryIIB* gene product is toxic only to the latter (27).

To determine which region(s) of the CryIIA and CryIIB crystal proteins is involved in determining host range specificity, we generated hybrid genes. Toxicity assays of the hybrid gene products identified a putative region in CryIIA that is responsible for conferring toxicity to mosquito larvae. Additional support for the location of this region came from the construction of a hybrid gene consisting entirely of *cryIIB* sequences except for a 241-nucleotide segment derived from *cryIIA*. The protein encoded by this gene exhibited the toxicity spectrum of CryIIA—i.e., it was toxic to both test insects.

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MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. *E. coli* MC1000 (5) was used as the host for expressing the cloned genes. Plasmids pUC8, pUC9, pUC118, and pPL703E were used as cloning vectors (8, 24, 25, 27). Plasmid pUC118 was propagated in *E. coli* MV1193 to obtain a single-stranded template for DNA sequencing; M13 phage K07 was used as the helper phage (25). Double-stranded plasmid DNA was prepared by the alkaline sodium dodecyl sulfate (SDS) lysis procedure (3). Single-stranded plasmid DNA templates were prepared as described by Anderson (1). Transformations of *E. coli* were performed by standard methods (18).

Gene designations. A scheme has been proposed for classifying crystal protein genes (*cry* genes) on the basis of structural similarities and host range (14). The *cry* genes are grouped into four major classes and several subclasses. The major classes are class I (Lepidoptera specific), class II (Lepidoptera and Diptera specific), class III (Coleoptera specific), and class IV (Diptera specific). On the basis of this classification scheme, the Lepidoptera-Diptera genes discussed in this paper are designated *cryIIA* and *cryIIB*; these genes were previously named *cryB1* and *cryB2*, respectively (7, 27).

Preparation of RNA from *B. thuringiensis* and *E. coli*. Total RNA was isolated from *B. thuringiensis* subsp. *kurstaki* HD-1 during different stages of development (vegetative growth and stage II, stages III to IV, stages IV to V, and stages V to VI of sporulation) as described by Wong et al. (28). Total RNA was prepared from *E. coli* in a similar fashion, except that passage through a French press was not required for cell breakage; cells were immediately subjected to hot phenol-SDS extractions.

RNA dot analysis. RNA samples (2.5 μg) from *E. coli* and *B. thuringiensis* subsp. *kurstaki* were spotted onto nitrocellulose filter paper (23). An intragenic restriction fragment (0.1 to 0.2 μg) from the *cryIIA* gene (*ScaI* to *SacI* sites, residues 2824 through 3590; see Fig. 2 of reference 27) was labeled with [α-^{32}P]dATP by using the Random Primed labeling kit purchased from Boehringer Mannheim Biochem-

icals and was used as a probe to detect *cryIIA*-specific mRNA. Hybridizations were done in the absence of formamide at 68°C, and washes were performed under stringent conditions (0.2× SSC–0.1% SDS at 68°C; 1X SSC contains 0.15 M NaCl plus 0.015 M sodium citrate).

An oligonucleotide (5′-CGTCCATTCCTTTTGTACGG-3′) complementary to nucleotides 1937 to 1956 (see Fig. 2 of reference 27) was used as a *cryIIB*-specific probe. The oligonucleotide (100 ng) was labeled with T4 polynucleotide kinase (10 U) and [γ-^{32}P]ATP (60 μCi) in a 10-μl reaction mixture (18). Approximately 20 ng was used for each hybridization, which was performed at 42°C; unincorporated nucleotides were not removed prior to hybridization. After overnight incubation, the filters were washed for 1 h at 42°C in each of two solutions: first, 3× SSC, 10 mM sodium phosphate (pH 7.0), 10× Denhardt solution (6), and 5% SDS; second, 1× SSC and 1% SDS. All filters were autoradiographed overnight at −70°C with an intensifying screen.

Plasmid constructions. Recombinant plasmids pWRW30 and pWRW50 were used to construct the plasmids used in this work; both have been described elsewhere (27). All fragments cloned into pUC plasmids were oriented to allow transcription from the *lac*-α promoter. pWRW30 contains a 5.0-kilobase (kb) *Hin*dIII fragment of *B. thuringiensis* subsp. *kurstaki* HD-1 plasmid DNA which harbors the *orf-1*, *orf-2*, and *cryIIA* genes. This plasmid was digested with *Hin*dIII, the ends were filled in with the Klenow fragment, and the plasmid was digested with *Eco*RI. The resulting 4.0-kb *Eco*RI-flush-ended fragment was subcloned into the *Eco*RI-*Sma*I site of pUC118 to generate pWRW10. This plasmid was digested with *Xba*I, treated with S1 nuclease, and recircularized with T4 DNA ligase. A plasmid, designated pWRW11, which had lost the *Bam*HI, *Xba*I and *Sal*I sites of the polylinker system, was identified. pWRW11 was digested with *Bam*HI and *Acc*I, the ends were filled in with the Klenow fragment, and the plasmid was recircularized with T4 DNA ligase to yield pWRW31 (see Fig. 2A). The 2.31-kb *Eco*RI-*Hin*dIII fragment from pWRW31 was subcloned into the *Eco*RI-*Hin*dIII sites of pPL703E to generate pWRW41.

pWRW50 contains a 9.0-kb *Hin*dIII fragment of strain HD-1 plasmid DNA which harbors the *cryIIB* gene. A 3.0-kb *Eco*RI fragment of pWRW50 (bearing *cryIIB*) was subcloned into the *Eco*RI site of pUC9 to generate pWRW27. This plasmid was digested with *Hin*dIII, treated with exonuclease III, digested with S1 nuclease, and cut with *Eco*RI; the resulting *Eco*RI-flush-ended fragments were subcloned into the *Eco*RI-*Sma*I sites of pUC8 to generate pWRW19. The 2.87-kb *Eco*RI-*Hin*dIII fragment from this plasmid was subcloned into the *Eco*RI- *Hin*dIII sites of pUC118 and into pPL703E to generate plasmids pWRW28 and pWRW42 (see Fig. 2A), respectively.

Chimeric plasmid pWRW5 (see Fig. 2A) was constructed as follows. Plasmids pWRW31 and pWRW42 were digested with *Hin*dIII and ligated together, and recombinant clones were selected for ampicillin and neomycin resistance. The resulting chimeras were screened with *Sac*I to identify a plasmid, designated pWRW5, which contained both *cryIIA* and *cryIIB* in the same orientation. Chimeric plasmid pWRW6 was constructed in a similar fashion by using pWRW28 and pWRW41.

The hybrid 15 gene was constructed by digesting pWRW5 with *Bal*I, recircularizing the plasmid DNA with T4 DNA ligase, and transforming *E. coli* MC1000 to yield ampicillin resistant, neomycin-sensitive transformants. Recombinant plasmids were isolated from these clones and screened by digestion with *Eco*RI; this digestion should yield a single fragment of approximately 6.0 kb. The hybrid 5 gene was constructed in a similar fashion by starting with pWRW6; in this case, final screening with *Eco*RI should yield a single fragment of approximately 5.3 kb.

The hybrid 7 and 8 genes were constructed as described above for hybrids 5 and 15, respectively, except that restriction enzyme *Sac*I was used instead of *Bal*I. The hybrid 513 gene was generated by replacing the *Eco*RI-*Bal*I fragment (the 5′ portion of the *cryIIA* gene) of the pUC118 recombinant plasmid containing the hybrid 13 gene with the corresponding *Eco*RI-*Bal*I fragment (the 5′ portion of the *cryIIB* gene) of the hybrid 5 gene by using standard cloning techniques.

DNA sequencing. DNA was sequenced by using the dideoxy chain termination procedure developed by Sanger et al. (21). Single-stranded DNA templates were isolated from the appropriate clones and sequenced by using [α-^{35}S]dATP and the Sequenase DNA sequencing kit purchased from U.S. Biochemical Corp. Synthetic oligonucleotide primers were used to determine the crossover intervals for hybrids 2, 3, 6, 12, 13, 14, and 513.

Preparation of inclusions from *E. coli*. The cuboidal crystal protein genes, oriented to allow transcription from the *lac*-α promoter of the pUC plasmid vectors, were expressed efficiently in *E. coli* MC1000 and accumulated as inclusions. Cells were grown overnight; the inclusions were isolated by sonication and centrifugation and suspended in sterile distilled water as described previously (27).

Insect toxicity assays. Neonate larvae of the tobacco hornworm (*M. sexta*) and mosquito larvae (*A. aegypti*) were kindly provided by Lynn Riddiford and Howard Whisler, respectively, and tested as described previously (19, 22). All assays were done in triplicate and performed in multiwell tissue culture plates (Falcon 3047). For *M. sexta*, eight serial twofold dilutions were made of each inclusion preparation, and 20 μl of each dilution was applied evenly to the diet surface and allowed to dry. Twelve first-instar larvae (placed in separate wells) were tested at each dilution. After 7 days, the numbers of dead larvae were counted.

Toxicities to *A. aegypti* were determined in a similar fashion. Six serial twofold dilutions were made of each inclusion preparation. Ten larvae (approximately second instar) were tested per dilution together in a single well. After the addition of larvae and inclusions, the total volume of each well was brought up to 2 ml and a 5-μl portion of diet (a 0.5% solution of the following mixture: 120 g of flour, 50 g of nonfat milk, 20 g of yeast extract, and 10 g of liver powder) was added to each well. After 3 days, the numbers of dead larvae were counted. The inclusion preparations which showed no detectable toxicity were tested again in the absence of added food—i.e., to grow, the larvae were required to use the added inclusions as a food source.

The concentrations of toxic polypeptides in purified inclusions were estimated by scanning densitometry (202 Ultrascan laser densitometer from LKB Instruments, Inc.) of gels stained with Coomassie blue.

Sequence comparisons. DNA and amino acid sequence analyses were performed on an Apple McIntosh SE computer by using the programs DNA Inspector (Textco), FASTP, and RDF (17). All comparisons were made by using a ktup of 1, and crystal protein sequences were randomly shuffled 50 times when testing for significance with the RDF program. Each pairwise alignment gave a z value of >10 and thus was considered significant.

Reagents. Restriction enzymes were purchased from New England BioLabs, Inc., Bethesda Research Laboratories,

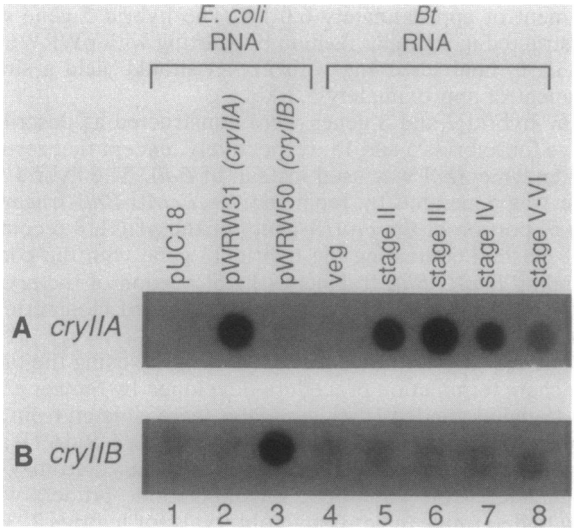

FIG. 1. RNA dot analysis of *cryIIA* and *cryIIB* transcription. Dots 1 to 3, RNAs from *E. coli* MC1000 clones harboring pUC118, pWRW31, and pWRW50, respectively. Dots 4 to 8, RNAs purified from cells of *B. thuringiensis* subsp. *kurstaki* (Bt) HD-1 harvested during vegetative growth and stage II, stages III to IV, stages IV to V, and stage VI of sporulation, respectively. (A) *cryIIA*-specific probe. (B) *cryIIB*-specific probe.

Inc., International Biotechnologies, Inc., and Boehringer. T4 DNA ligase was purchased from Boehringer, and *E. coli* polyperase I (large fragment) was purchased from New England BioLabs. All enzymes were used in accordance with the instructions of the manufacturers. All radiolabeled nucleotides were purchased from New England Nuclear Corp. Oligonucleotides were synthesized on a BioSearch model 8600 DNA synthesizer.

RESULTS

Expression of the *cryII* genes. All crystal protein genes are expressed in *B. thuringiensis* only during sporulation, and all of those examined to date, including *cryIIA* (4), have promoters which differ in sequence from promoters expressed during vegetative growth. In vitro transcription of *cryIIA* requires a *B. thuringiensis* RNA polymerase that is produced early in sporulation and contains a new sigma subunit. These observations suggest that one or more sporulation-specific RNA polymerases regulate the expression of crystal protein genes in vivo.

Approximately 300 base pairs of DNA upstream of *cryIIB* was searched for promoter sequences resembling those preceding other crystal protein genes (4, 28), but none could be found (W. R. Widner, Ph.D. dissertation, University of Washington, Seattle, 1989). Possibly *cryIIB*, like *cryIIA*, is part of an operon, although no substantial open reading frame could be found within the 5′-flanking DNA. To determine whether *cryIIB* is expressed in strain HD-1, we analyzed RNA samples prepared from sporulating cells by hybridization (Fig. 1). As controls, RNAs isolated from *E. coli* MC1000 harboring pUC118, pWRW31, or pWRW50 (Fig. 1, dots 1 to 3) were also analyzed. With a *cryIIA*-specific probe, only RNAs isolated from *E. coli* (pWRW31) and sporulating cells of strain HD-1 hybridized (Fig. 1A, dots 2 and 5 to 8, respectively). Interestingly, when a *cryIIB*-specific probe was used, only marginal levels of hybridization could be detected with any *B. thuringiensis* subsp. *kurstaki* RNA; a strong positive reaction was obtained with RNA from *E. coli* (pWRW50) (Fig. 1B, dot 3). Evidently, the *cryIIB* gene is transcribed very weakly in *B. thuringiensis* subsp. *kurstaki* and/or the *cryIIB* mRNA is extremely unstable. Alternatively, this gene may be expressed poorly because of the growth conditions used, although this explanation seems unlikely. Fortunately, both *cryIIA* and *cryIIB* can be expressed efficiently in *E. coli*, thus allowing both gene products to be tested for toxicity (27).

Isolation of hybrid crystal protein genes. Alignment of the deduced amino acid sequences of these two genes showed that there is a higher density of amino acid differences between residues 300 and 400. Furthermore, if only the nonconservative changes are considered, 9 of the 11 total are located in this region. On the basis of these observations, it seems plausible that sequences responsible for conferring host range specificity would map to this region.

To test this possibility, we isolated and tested several hybrid CryIIA-CryIIB and CryIIB-CryIIA proteins for toxicity against *A. aegypti* and *M. sexta* larvae, with the ultimate goal of identifying a specific segment(s) of the CryIIA polypeptide which determines its specificity for mosquitoes. Hybrid *cryIIA-cryIIB* and *cryIIB-cryIIA* genes were generated by two different approaches, both using the chimeric plasmids pWRW5 and pWRW6 (described in Materials and Methods). Plasmid pWRW5 was constructed by combining plasmids pWRW31 and pWRW42 to form a chimera (Fig. 2A). pWRW31 is a pUC118-derived recombinant which confers ampicillin resistance and harbors the *cryIIA* gene, and pWRW42 is a pPL703E-derived recombinant which confers neomycin resistance and harbors the *cryIIB* gene. Plasmid pWRW6 was constructed in a similar fashion, but the starting plasmids were pWRW28 and pWRW41.

Hybrid genes were generated in vivo via the RecA-dependent homologous recombination system of *E. coli* by using the scheme diagrammed in Fig. 2B. The main advantage of this approach is that a very large number of hybrid genes can be isolated quickly, with relatively little effort. To generate *cryIIA-cryIIB* hybrid genes, we propagated pWRW5 in a RecA$^+$ host. In this genetic background, the plasmid is unstable and resolved through reciprocal recombination between the *cryII* genes to generate two plasmid species (Fig. 2B)—one a pUC118 recombinant containing a hybrid *cryIIA-cryIIB* gene and bearing an ampicillin resistance marker and the other the reciprocal product, a pPL703E recombinant containing a hybrid *cryIIB-cryIIA* gene and bearing a neomycin resistance marker (as well as an *Xba*I site located at the 3′ side of the hybrid gene). Plasmid DNA isolated from a RecA$^+$ strain will contain predominantly parent plasmid pWRW5 and very small amounts of the reciprocal products. To enrich for transformants bearing the pUC118 recombinant containing a hybrid *cryIIA-cryIIB* gene, we digested purified plasmid DNA with *Xba*I and used it to transform *E. coli* MC1000. Both the pWRW5 and pPL703E recombinant plasmids are cut once by *Xba*I, and the pUC118 recombinant plasmid remains intact. Since closed circular DNA transforms *E. coli* with much greater efficiency than does linear DNA, essentially all of the transformants would be expected to harbor the desired product. Using this approach, we generated several *cryIIA-cryIIB* hybrids. The reciprocal *cryIIB-cryIIA* hybrids were obtained in a similar fashion, but the starting plasmid was pWRW6 instead of pWRW5.

A total of 72 hybrid genes were isolated, and 15 of these were selected for analysis (hybrids 1 to 15). To map recom-

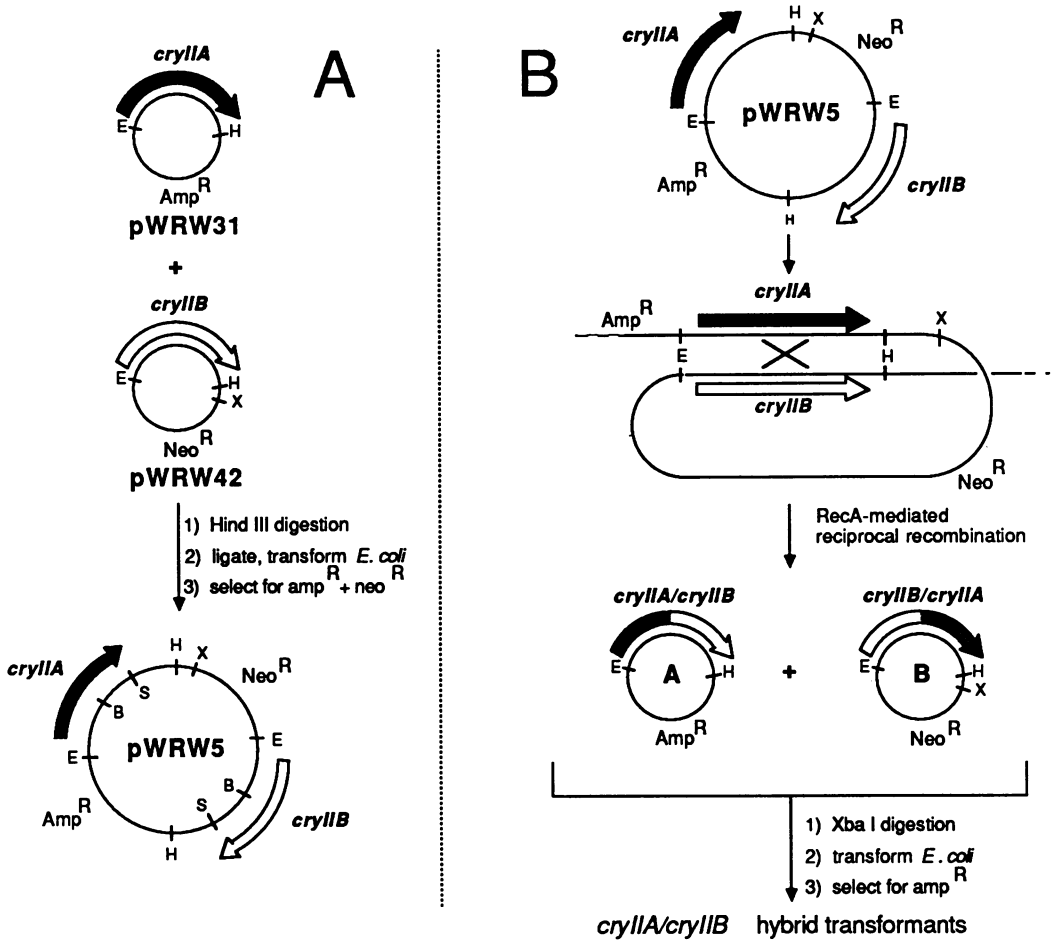

FIG. 2. Strategy for generating hybrid *cryIIA-cryIIB* genes. (A) Construction of chimeric plasmid pWRW5. (B) Generation of hybrid *cryIIA-cryIIB* genes by homologous recombination in *E. coli*. Shaded arrows indicate the *cryIIA*-specific sequences, and nonshaded arrows indicate the *cryIIB*-specific sequences. Restriction enzyme sites are indicated and abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; B, *Bal*I; S, *Sac*I.

binational crossover points, we isolated plasmids from the ampicillin-resistant, neomycin-sensitive transformants and digested them with *Taq*I, *Rsa*I, *Dde*I, *Sca*I, or *Bgl*II. Using this battery of restriction enzymes, we mapped the crossover intervals to within a few hundred base pairs; those for hybrids 2, 3, 4, 6, 12, 13, 14, and 513 were determined more precisely by DNA sequence analysis. Hybrids 5 and 15 and hybrids 7 and 8 were constructed in vitro by using the restriction enzymes *Bal*I and *Sac*I, respectively (see Materials and Methods).

Figure 3 presents diagrams showing the contents of CryIIA and CryIIB sequences in the selected hybrids. From the amino-terminal end, hybrids 1 to 7 (CryIIB-CryIIA hybrids) contain decreasing amounts of CryIIA sequences and increasing amounts of CryIIB sequences; the amounts of CryIIA sequences in hybrids 8 to 15 (CryIIA-CryIIB hybrids) decrease from the carboxy-terminal end. The bar at the top of Fig. 3 shows the locations of the differences in the amino acid sequences of CryIIA and CryIIB, and arrows indicate the positions of nonconservative changes.

Toxicity of hybrid gene products. The growth rate of *E. coli* strains containing cloned *cryIIA* and *cryIIB* genes and hybrid genes was indistinguishable from that of control cultures containing only vector pUC118. Crystalline inclusions comprised of full-length ca. 65-kilodalton polypeptides could be isolated from all strains listed in Fig. 3, although yields varied from strain to strain. The greatest amounts of inclusions were produced by strains containing the *cryIIA* and *cryIIB* genes. Approximately 5-fold lower amounts were produced by most of the strains containing hybrid genes, except for hybrid 14; the amount of inclusions isolated from *E. coli* containing this hybrid was about 100-fold less than that isolated from the strain bearing *cryIIA* (data not shown).

Inclusions were purified from the *E. coli* strains shown in Fig. 3 and tested for toxicity. Because of the variable susceptibilities of different batches of insect eggs used during the course of these experiments, toxicities have been expressed as LC_{50} values (concentration that kills 50% of the larvae) relative to the toxicity of CryIIA (LC_{50} test/LC_{50} CryIIA) to each insect. The LC_{50} values for the latter varied from 50 to 150 ng/cm^2 for *M. sexta* and 50 to 250 ng/ml for *A. aegypti*.

Against *A. aegypti*, hybrid 1 exhibited the same toxicity as CryIIA, and inclusions from the *cryIIB*-containing strain were nontoxic. It should be noted that mosquito larvae exposed to large amounts of CryIIB inclusions in the absence of added nutrients grew significantly, apparently by consuming the inclusions. Inclusions from hybrids 2 to 5 were substantially less toxic than was the CryIIA control, and inclusions from hybrids 6 and 7 were not toxic to

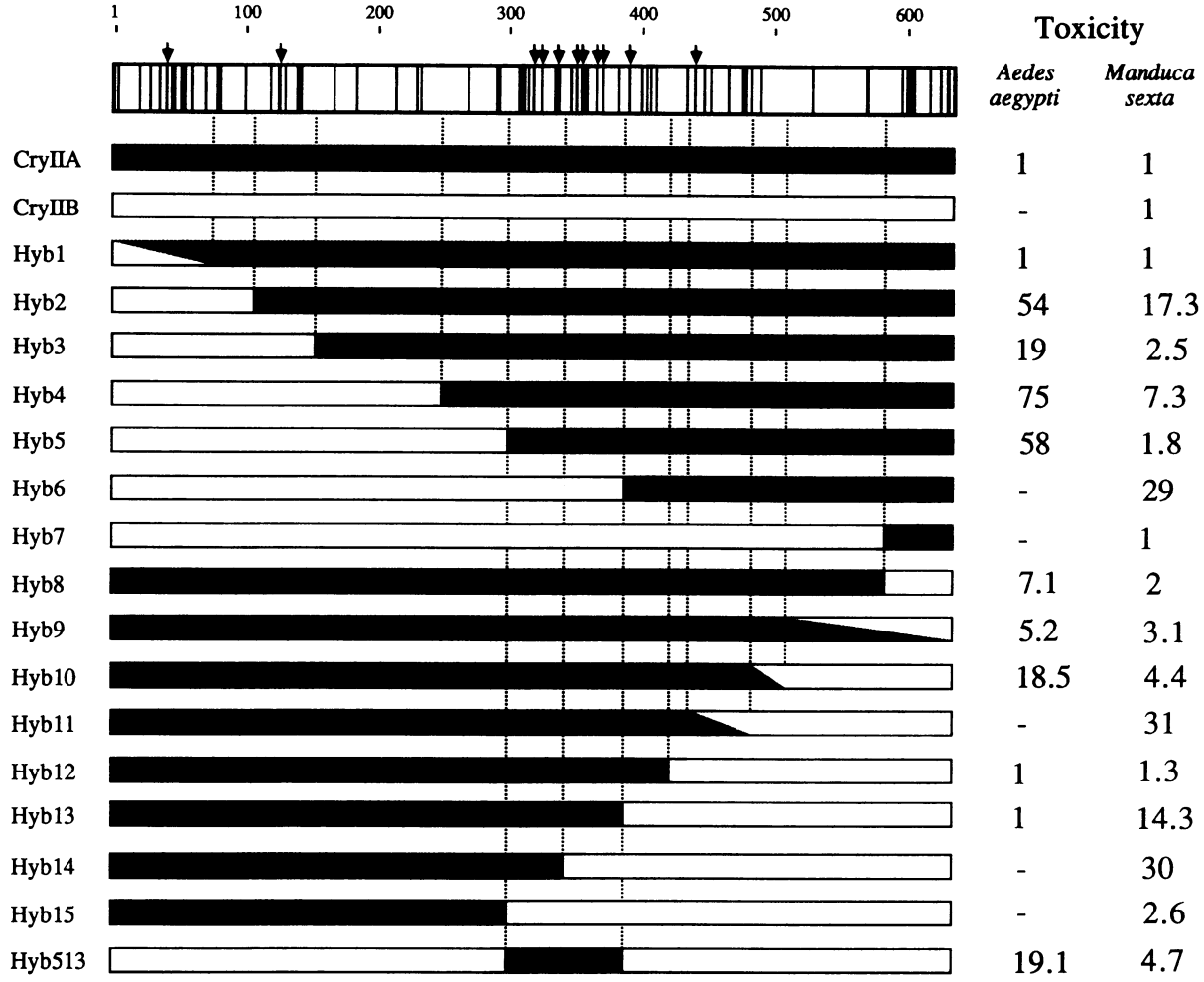

FIG. 3. CryIIA (shaded bar), CryIIB (nonshaded bar), and hybrid gene products (combination of the two patterns) and their toxicities to *A. aegypti* and *M. sexta*. All of the toxicities are relative to that of CryIIA (value of 1); a fivefold difference in toxicity is considered significant. The bar at the top of the figure is a diagram depicting a FASTP alignment of the CryIIA and CryIIB polypeptides; vertical lines represent differences between the two, and arrows above the bar denote the locations of nonconservative changes. Vertical broken lines show locations of the hybrid junctions determined by restriction mapping (hybrids 1, 9, 10, and 11) and DNA sequence analysis (hybrids 2 to 4, 6, 8, 12 to 14, and 513). The dotted lines extend upwards to the alignment diagram to show where the junctions are located with regard to the amino acid differences that exist between the two polypeptides.

mosquito larvae, even when tested at high concentrations (250 μg/ml) in the absence of added food. When tested against *M. sexta*, hybrids 1 and 7 were as toxic as were CryIIA and CryIIB, hybrids 3 to 5 were only slightly less toxic, and hybrid 2 and especially hybrid 6 were substantially less toxic.

Of the CryIIA-CryIIB inclusions, hybrids 12 and 13 were as toxic to *A. aegypti* larvae as was CryIIA, hybrids 8 to 10 were progressively less toxic, and hybrid 11, as well as hybrids 14 and 15, were nontoxic. When tested for toxicity to *M. sexta*, hybrids 8 to 10, 12, and 15 were as toxic or nearly as toxic as was CryIIA or CryIIB, and hybrids 11, 13, and 14 were significantly lower in toxicity.

Except for the results obtained with inclusions from hybrid 11, the absence of mosquitocidal activity in hybrids 6, 7, 14, and 15 suggests that the boundaries of hybrids 5 and 13 delineate the CryIIA sequences that are minimally required for toxicity to mosquito larvae. To determine whether the short segment of CryIIA defined by these boundaries is, in fact, sufficient to influence specificity, we constructed a *cryIIB-cryIIA-cryIIB* hybrid gene in vitro between hybrids 5 and 13 to generate hybrid 513 (see Materials and Methods and Fig. 3). The resulting gene product was toxic to both test insects, indicating that mosquitocidal activity is influenced by residues 307 through 382 of the CryIIA polypeptide. The toxicity of hybrid 513 inclusions to *A. aegypti* was reduced 20-fold relative to that of CryIIA, whereas the toxicity to *M. sexta* was reduced approximately 5-fold.

DISCUSSION

In this study, two highly related crystal protein genes that encode polypeptides possessing different toxicity spectra have been used to study host range specificity. Construction of hybrids between the *cryIIA* and *cryIIB* cuboidal crystal protein genes and toxicity assays of the gene products identified a stretch of 76 amino acids of the CryIIA crystal protein that appears to be important for conferring mosquitocidal activity. A hybrid consisting of predominantly CryIIB amino acids and only the 76-amino-acid segment of CryIIA was toxic to both mosquitoes and *M. sexta*. Since CryIIA and CryIIB differ by only 18 amino acids in this

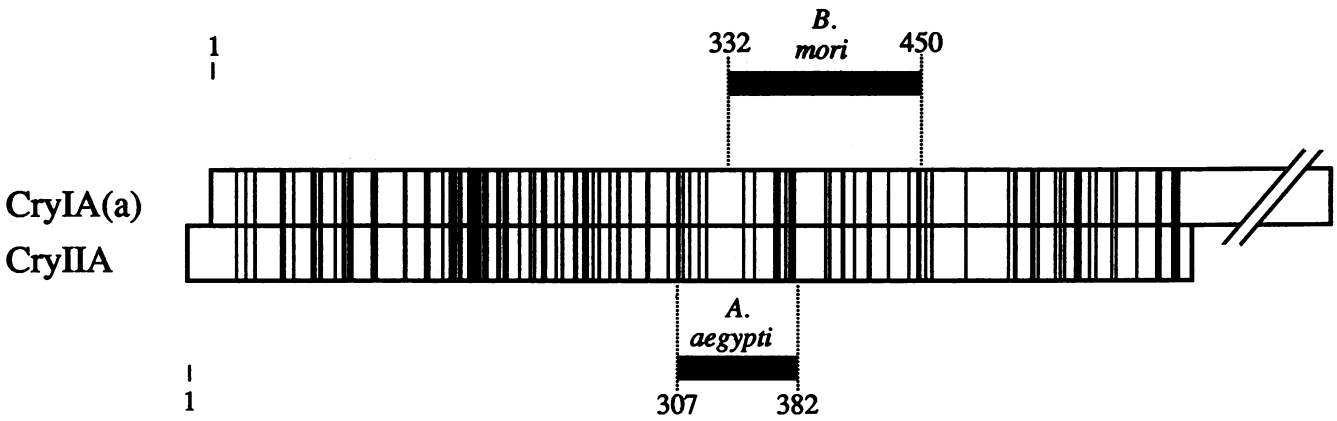

FIG. 4. Host range specificity determinants of the CryIA(a) and CryIIA crystal proteins. The bars show the computer alignment of the two polypeptides; vertical lines indicate identities between the two sequences. Black bars show the positions of the CryIA(a) specificity determinant for *B. mori* and the CryIIA specificity region for *A. aegypti*.

region, it is apparent that very few changes can substantially alter the specificities of these toxins.

It should be noted, however, that sequences outside the putative mosquitocidal region may also be important. Clearly, hybrid 513 inclusions were not as toxic to mosquito larvae as was CryIIA. In addition, toxicities to both test insects were, in most cases, reduced when segments of CryIIA and CryIIB that lie outside of this region were interchanged. For example, hybrids 2 to 5 and hybrids 8 to 11 were considerably less toxic to mosquito larvae than was the CryIIA control, implying that sequences located at both the amino- and carboxy-terminal ends of CryIIA are important for conferring mosquitocidal activity. Haider and Ellar (11) recently proposed a model suggesting that the amino terminus of the proteolytically cleaved CryIA class of toxins interacts with sequences in the carboxy-terminal region to initiate a conformational change and form an active toxin. Perhaps many of the CryII hybrids which are less toxic are impaired in this process.

Alternatively, any reduction in activity may simply be an indirect effect caused by overall perturbations in protein folding. This is a valid possibility, considering that the 76 amino acid changes that exist between CryIIA and CryIIB are distributed throughout the polypeptide chains (Fig. 3). It seems unlikely that all of these changes would be "silent" and have no effect. Undoubtedly, during the evolution of these two toxins, changes that were detrimental to function did arise and some of these were offset by the introduction of compensatory changes located at secondary sites along the polypeptide chains. A potential problem in the generation of these hybrid toxins is the possibility of uncoupling such compensatory changes if they do exist, thereby causing a reduction in toxin activity. Aberrantly folded protein may also be more susceptible to proteolytic degradation in the insect gut, thus causing them to appear less toxic.

The results of this study do not identify a specific region that determines toxicity to *M. sexta*, since CryIIA and CryIIB inclusions are both equally toxic to this insect. However, a comparison of hybrids 5, 6, and 12 to 15 suggests that the segment defined between the hybrid 5 and 12 junctions (amino acids 307 through 410) may be important. Interestingly, this 104-amino-acid segment encompasses the putative mosquitocidal region identified in this report. Perhaps rearrangements occurring within this region result in the formation of hybrid specificity determinants which can no longer interact properly with lepidopteran target cells. Additional experiments, possibly with a lepidopteran species that is susceptible to only one of these toxins, may provide further insight concerning the specificity determinants of the CryIIA toxin—i.e., whether it possesses two independent specificity-determining regions or a single region that is responsible for conferring toxicity to both dipteran and lepidopteran larvae.

As mentioned earlier, *B. thuringiensis* subsp. *kurstaki* HD-1 also produces large bipyramidal crystals which are toxic to certain lepidopteran larvae. These crystals are comprised primarily of three highly related polypeptides which are encoded by the *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* genes (originally called the 4.5-, 5.3-, and 6.6-kb genes; 16). Despite their similarities (ca. 80% identity in amino acid sequence in the toxic portion of the proteins), they exhibit different, although overlapping, toxicity spectra (14). Ge et al. (9) identified a region (residues 332 to 450) of the CryIA(a) crystal protein that is important in specifying toxicity to the lepidopteran insect *Bombyx mori*. Interestingly, when the CryIIA and CryIA(a) polypeptides are aligned, the segment of CryIIA identified in the present report overlaps the *B. mori* specificity determinant (Fig. 4). Since the CryIIA and CryIA(a) polypeptides are only distantly related, it seems plausible that the specificity determinants of most, if not all, crystal proteins (excluding the CytA toxin of *B. thuringiensis* subsp. *israelensis*) are located in or near this region.

The level(s) at which host range specificity is manifested by the CryIIA and CryIIB crystal proteins remains to be elucidated—e.g., toxin processing, toxin binding to a receptor, or perhaps interaction with a physiological target. Hofmann et al. (13) demonstrated that the specificity of at least some crystal proteins is determined at the level of toxin binding to high-affinity receptors on the target cell. Alternatively, Haider et al. (12) showed that the host range specificity of a crystal protein from *B. thuringiensis* subsp. *aizawai* IC1 is determined, at least in part, at the level of proteolytic processing. It seems unlikely that the 76-amino-acid segment identified in the present study contains a proteolytic processing site, since all but one of the crystal proteins characterized to date are processed to yield an active toxin of approximately 60 kilodaltons and processing in this region of CryIIA would yield a polypeptide of considerably lower molecular mass. In fact, it has been shown that the cuboidal crystal protein from strain HD-1 is

processed at the amino-terminal end (20), with little or no processing occurring at the carboxy terminus (27), to generate an active toxin of approximately 51 kilodaltons. It seems more plausible that the 76-amino-acid segment that provides the dipteran toxicity of CryIIA is involved in the binding of CryIIA to a target cell receptor.

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