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Analysis of Active Regions of Insecticidal Proteins of *Bacillus thuringiensis* var. *israelensis*

Ken-ichi YOSHIDA*

ABSTRACT

Among the strains of *Bacillus thuringiensis*, *B. thuringiensis* var. *israelensis* (*BTI*) produces crystalline protein bodies (CPBs) consisting of several kinds of insectcidal proteins, molecular masses of which are 130-kDa, 70-kDa, 28-kDa and so on, and which are toxic to diptera such as mosquitoes and black flies. The insecticidal proteins are solubilized and proteolytically activated, and then break the cells in midgut to kill the insect. Two genes for 130-kDa insecticidal proteins, which we named ISRH3 (CryIVB) and ISRH4 (CryIVA), have been cloned and sequenced. To delineate the mosquitocidal regions of the ISRH3 and ISRH4 proteins, a deletion analysis of these protein genes was done. Based on the evidence that each 130-kDa protein had two mosquitocidal regions, N-terminal and C-terminal ones, and these two regions shared a common part in the center of the 130-kDa proteins, deleted genes on this region were constructed. As the protein products which lacked the central region had reduced activities, the central region could be important for the mosquitocidal activity. The mosquitocidal and non-mosquitocidal truncated gene products of 130-kDa protein genes were also applied to cultured insect cell lines. The mosquitocidal proteins caused the swelling and disruption of the cells, but the non-mosquitocidal proteins did not. therefore, the mosquitocidal fragments of 130-kDa proteins of *BTI* were cytotoxic to two insect cell lines. And an insecticidal fragment of ISRH4 protein actually bound to two insect cell lines.

keywords: *Bacillus thuringiensis* var. *israelensis*, insecticidal protein, mosquitocidal activity, cytotoxicity.

INTRODUCTION

A wide variety of insecticides had been used to control insect damage and to keep sanitary environment. Among them, notable insecticides of the biological control are the insecticidal toxins produced by *Bacillus thuringiensis*: spore preparation of this Gram-positive bacterium has been used as a biological insecticide for more than 30 years.

The strains of *B. thuringiensis* produce intracellular crystalline protein bodies (CPBs) as well as an endospore during the post-exponential phase. The insecticidal activity of *B. thuringiensis* resides in the CPBs, which are composed of several insecticidal proteins, not in chemical compounds. The CPBs are dissolved in the insect midgut and release the insecticidal proteins (protoxins), and then the protoxins are proteolytically fragmented and activated to be toxic fragments (toxins) (Haider *et al.*, 1986). The activated toxins are believed to insert into the membrane of the cell in the midgut

*Department of Biotechnology

and create an ion pore leading to colloid osmotic lysis (Knowles & Ellar, 1987) or perhaps to alter some membrane component to achieve the same effect to kill the host insect.

The *B. thuringiensis* insectcidal toxins are highly specific, in that they are not toxic to other organisms. And different strains of *B. thuringiensis* differ in the target insects. Most of them are active against Lepidoptera, but some strains specifc to Diptera and Coleoptera have been identified (Dulmadge, 1981; Burgess, 1982; Aronson *et al.*, 1986).

At least 40 insecticidal protoxin genes have been cloned from a variety of *B. thuringiensis,* and they have been classified on basis of the target insect species and, as it turns out, the sizes of the encoded protoxins (Table 1, Höfte & Whiteley, 1989; Aronson, 1993). The Cry I types of 130-140-kDa are active almost specifically on Lepidoptera. The CryⅣA and CryⅣB protoxins, on which this study was carried out, are also 130-140-kDa but are active only on Diptera. The CryII protoxins of about 70-kDa are active either on Lepidoptera or in the case of CryIIA, Lepidoptera and Diptera. The CryIII protoxins are about the same size and are specifc for Coleoptera while two other CryⅣD protoxins are dipteran-specific. Protoxins of 130-140-kDa (Cry I and CryⅣ) are processed to 65-70-kDa toxin where those of 70-75-kDa (CryII and CryIII) are processed to 60-65-kDa toxins. The lepidopterous active toxins reside in the N-terminal region of the Cry I type protoxins, while the dipterous toxic fragments of the CryⅣ protoxins were not well identified.

Among the strains of *B. thuringiensis, B. thuringiensis* var. *israelensis* (*BTI*) produces unique CPBs which are specifically toxic to Diptera such as mosquitoes and black flies (Goldberg & Margalit, 1977.) The CPBs of *BTI* are composed of at least five kinds of toxic proteins, which are two 130-kDa (CryⅣA and CryⅣB), two 70-kDa (CryⅣC and CryⅣD) and 27-kDa (CytA) proteins (Yamamoto *et al.*, 1983; Pfannenstiel *et al.*, 1984). As shown in Table 1, the CryⅣA, CryⅣB, CryⅣC and CryⅣD proteins are known to be specifically dipterous active (Visser *et al.*, 1986; Pao-intara *et al.*, 1985). Recently, Sen *et al.* (1988a) and other investigators (Ward & Ellar, 1987; Chunjatupornchai *et al.*, 1988) have suceeded in cloning of the genes for two 130-kDa proteins. And Sen *et al.* (1988a) originally named these two proteins ISRH4 and ISRH3, which are identical to CryⅣA and CryⅣB, respectively (Höfte & Whiteley, 1989). These genes were expressed in *Bacillus subtilis* and *Escherichia coli,* and the insecticidal activity of the gene products were also reported (Sen *et al.*, 1988b; Pao-intara *et al.*, 1988; Ward & Ellar, 1988). It is believed that these two 130-kDa dipterous active proteins are protoxins, which are proteolytically fragmented and activated in the midgut of the insects, larvae of mosquitoes, to become toxic fragments. But toxic fragments of them, which actually work as active toxins, had not been well identified as the lepidopterous active protoxins, and furthermore the interations between the toxic fragments and the target cells had not been monitored, either.

In this paper the author elucidates the insecticidal and cytotoxic activity of the truncated gene product of two 130-kDa insecticidal proteins of *BTI* to delineate the active fragments of them. The aim of this study is to identify the structures and functions which are necessary for their toxicity.

Chapter 1. Insecticidal activity of a peptide containing the 30th to 695th amino acid residues of the 130-kDa protein of *Bacillus thuringiensis* var. *israelensis*

Several investigators have reported cloning of 130-kDa insecticidal protein genes of *Bacillus thuringiensis* var. *israelensis* (*BTI*) and the insecticidal activities of cloned gene products against mosquito larvae (Sekar, 1986; Bourgouin *et al.*, 1986; Angsuthanasombat *et al.*, 1987; Yamamoto *et al.*, 1988). Moreover, Pao-intara *et al.* (1988) have reported the mosquitocidal activity of a 72-kDa N-terminal fragment of a ISRH3-type (CryⅣB) protein. Ward and Ellar (1988) have mentioned the mosquito larvicidal activity of a fragment consisting of the N-terminal 634 amino acid residues of the

Table 1. Insecticidal Protein Genes of *B. thuringiensis*

Gene designation	Host* range	Predicted mol. mass (kDa) of protoxin	Origin of gene: *B. thuringiensis* subspecies
cryIA(a)	L	133.2	*kurstaki* HD1 *sotto*
cryIA(b)	L(D)	131.0	*kurstaki* HD1 *berliner* *aizawai* *alesti*
cryIA(c)	L	133.3	*kurstaki* HD1 *kurstaki* HD73
cryIB	L	138.0	*thuringiensis* HD2
cryIC	L	134.0	*aizawai* *entomocidus* *kenyae*
cryID	L	132.5	*aizawai* HD68
cryIE	L	133.2	*tolworthi*
cryIF	L	133.6	*aizawai*
cryIG	L	130.0	*galleria*
cryIIA	L/D	70.9	*kurstaki HD1* (cryptic)
cryIIC	L	69.5	*shanghai*
cryIIIA	C	73.1	*san diego* *tenebrionis*
cryIIIB	C	75.0	*tolworthi*
cryIVA	D	134.4	*israelensis* *morrisoni*
cryIVB	D	127.8	*israelensis* *morrisoni*
cryIVC	D	77.8	*israelensis* *morrisoni*
cryIVD	D	72.4	*israelensis* *morrisoni*
cytA	D/cytol.	27.4	*israelensis*

***Specified host ranges: L, Lepidoptera: D, Diptera: C, Coleoptera; cytol., cytolytic and hemolytic**

CryⅣB protein. In the case of ISRH4-type (CryⅣA) protein, however, only Ward and Ellar (1987) and Sen *et al.* (1988a) have sequenced its gene, and no one has reported the mosquito larvicidal activity of fragmented ISRH4-type proteins.

In this chapter, the author describes identification of the mosquito larvicidal portion of ISRH4 and detection of the insecticdal activity of a subfragment consisting of the N-terminal amino acid residues of ISRH4.

MATERIALS AND METHODS

(a)Bacterial strains, plasmids and media.

E. coli JM109, a plasmid vector, pUC19 (Yanisch-Perron *et al.*, 1985), and a recombinant plasmid pBGH4 (Sen *et al.*, 1988a), have been previously described. *E. coli* strains were cultured in 2×YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH7.5) containing appropriate chemicals.

(b)Subcloning of the ISRH4 gene.

The plasmid pBGH4, which encodes the 130-kDa protein ISRH4 was partially digested with *Eco*RV and ligated with the *Bam*HI linker (5'-CGGATCCG-3') to construct pBGH4Bam. A 3.5-kb *Bam*HI-*Xba*I fragment of pBGH4Bam was subcloned into the *Sal*I site of pUC19 by filled-in ligation to provide pLH4BX.

(c)Construction of deletion mutant plasmids.

The plasmid pLH4BX was completely digested with *Xba*I and *Kpn*I, and nucleotides were sequentially deleted from the *Xba*I site toward the N-terminus of ISRH4 gene by the method of Henikoff (1984) to construct a series of deletion mutant genes which were fused with the downstream part of *lacZ'* at the newly formed C-termini of truncated ISRH4 genes. The products of deletion mutant genes which were in-frame ligated have the α-fragment of β-galactosidase, which could be enough to exhibit β-galactosidase activity in *E. coli* JM109. The deletion mutant plasmids were introduced into *E. coli* JM109 by the method of Hanahan (1985). They were screened 2×YT agar plates containing 50 μg/ml ampicillin, 0.02% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The deletion mutant plasmid DNAs were extracted from blue colonies to obtain the plasmids carrying the in-frame ligated mutant genes and used for analysis by agarose gel electrophoresis (AGE) and DNA sequencing.

(d)DNA sequencing.

The deletion mutant DNAs which had appropriate lengths were selected by AGE analysis. DNA sequencing was done to identify the deleted amino acids to make sure of the in-frame ligation at junctions of C-termini of the truncated ISRH4 genes and the dowstream part of *lacZ'*. DNA sequences were analyzed by the dideoxy method.

(e)Preparation of protein extracts.

The plasmid pLH4BX and its deletion mutants were introduced into *E. coli* JM109 cells and cultured at 37℃ for 7-8h in 2×YT media containing 50 μg/ml ampicllin and 1mM IPTG. Protein extracts from *E. coli* cells were prepared by the method previously described (Sen *et al.*, 1988b).

(f)Mosquito larvicidal assay.

Mosquito larvicidal activity of the protein extracts was analyzed basically by the procedure of Schnell *et al.* (1984). Protein extracts were diluted in 1ml of 0.1M tris-HCl (pH7.4) and mixed with 10 μl of latex beads (diameter 0.8mm, 10% solids; Sigma). The mixture was left at room temperature for 1h, and then added to 24ml distilled water in which there were 20 third-instar larvae of the mosquito *Culex pipiens pallens*. Mortality was scored after incubation at 26℃ for 24h and 48h.

(g) β-Galactosidase assay.

β-Galactosidase activity was measured by the method of Miller (1972) to show the production of deletion mutant proteins in *E. coli* cells.

RESULTS AND DISCUSSION

(a)Construction of plasmid pLH4BX.

The plasmid pBGH4 (Sen *et al.*, 1988a) encodes the 130-kDa insecticidal protein of ISRH4. The plasmid pBGH4 has two *Eco*RV sites in the region of the gene encoding ISRH4, and one of them is in the position of the 29th codon. The plasmid pBGH4 was partially digested with *Eco*RV and ligated

with a *Bam*HI linker (5'-CGGATCCG-3') providing pBGH Bam, which has a new *Bam*HI site in the 29th codon instead of the original *Eco*RV site. A 3.5-kb *Bam*HI-*Xba*I fragment of pBGH4Bam was subcloned into the *Sal*I site of pUC19 by filled-in ligation to provide pLH4BX (Fig.1). Plasmid pLH BX encoded the N-terminal 13 amino acid residues in the pUC19-borne upstrem part of the α-fragment of β-galactosidase fused with the polypeptide between the 30th and the 1180th amino acid residues of ISRH4. Plasmid pLH4BX, therefore, directed the production of the fused protein in *E. coli* JM109 cells in the presence of 1mM IPTG. This fused protein had no β-galactactosidase activity, because it did not retain the downstream part of the α-fragment of β-galactosidase.

(b)Expression of the ISRH4 gene as a fused protein in *E. coli* cells.

Protein extracts of *E. coli* JM109 cells harboring pLH4BX were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The size of the fused ISRH4 protein produced in *E. coli* was about 130-kDa, and it had almost the same mobility as the 130-kDa proteins of crystals of *BTI* (data not shown). The expression level of the fused ISRH4 gene, however, was rather lower. We expected much more production of the fused ISRH4 protein in *E. coli* cells, because the fused ISRH4 gene must be transcribed under control of a *lac* promoter upon induction by IPTG and because its 3'-flanking region contains an inverted repeat (Sen *et al.*, 1988a) which may act as a transcriptional termination element. Ward and Ellar (1988) tried to produce the CryIVA protein in *E. coli* cells previously, but the expression level of its gene was very low. They thought that translation of the mRNA specific for *cryIVA* gene was limited in *E. coli* cells. The present result could be interpreted similarly.

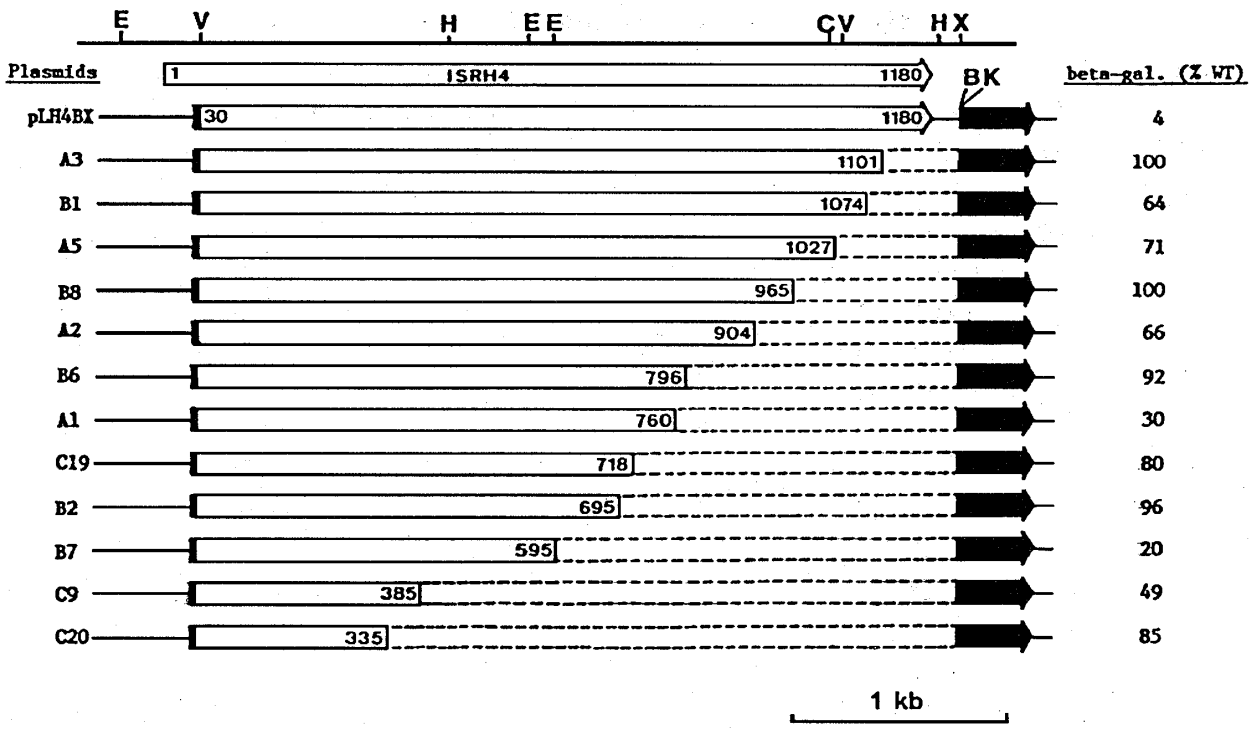

Fig.1 Structure of the Plasmids Encoding the α-Fragment of β-Galactosidase Fused with the Deletion Mutants of ISRH4.

The directions of transcription of the genes are indicated by thick arrows. The solid boxes indicate the stretch of the α-fragment of β-galactosidase encoded by *lacZ'* on pUC19. Dotted lines indicate the deleted nucleotides. The numbers in the thick arrows are the ordinal numbers of amino acid residues of ISRH4. The indication "beta-gal. (% WT)" denotes relative β-galactosidase activities of the deletion mutants with that of the wild type (pUC19) taken as 100. H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; K, *Kpn*I; X, *Xba*I; C, *Cla*I; V, *Eco*RV

(c)Production of deletion mutants of fused ISRH4 proteins.

The author constructed 12 deletion mutants of pLH4BX (Fig.1). All the mutant proteins were fused with the two separate tracts, the upstrem and the downstream parts, of the α-fragment of β-galactosidase, which was encoded by *lacZ'* on pUC19, at their N- and C-termini. By SDS-PAGE and Western blot analysis, fused polypeptides encoded by the seven longer mutant plasmids (pLH4BX-A3, B1, A5, B8, A2, B6, and A1) were detected, but those encoded by the five shorter ones (pLH4BX-C19, B2, B7, C9, and C20) were not (data not shown). The shortest mutant protein (A1) detected by Western blot analysis spanned the 30th through the 760th amino acid residues. The next shorter mutant protein (C19), was between the 30th and 718th amino acid residues, and mutant proteins shorter than C19 could not be detected (data not shown). Therefore a stretch from the 718th to the 760th amino acid residues might be necessary for the immunoreactivity with the IgG against the total crystal proteins of *BTI*. Pao-intara *et al*. (1988) have reported results similar to this about immunoreactivity of an ISRH3-type protein (CryIVB). The essential region for immunoreactivity was included in the C-terminal tracts identical to each other between ISRH4 and ISRH3 in their amino acid sequences, and the C-terminal identical tracts might determine the immunological features of the 130-kDa proteins of *BTI*. To show the existence of deletion mutant proteins in host *E. coli* cells, a β-galactosidase assay was done. The deletion mutant plasmids conferred active β-galactosidase-producing ability upon host *E. coli* JM109 cells, which lack the gene encoding intact β-galactosidase. The results of enzyme assay showed the existence of all the deletion mutant proteins in *E. coli* cells (Fig.1). But the author did not clarify the exact amount of each of the mutant

Table 2. Assay of Mosquito Larvicidal Activity

Test sample* (50 μg/ml)	Average no. dead** 24h	48h	Activity+
JM109/pUC19	0.8	1.7	-
pLH4BX	5.8	7.4	+
pLH4BX(A3)	7.5	11.0	++
pLH4BX(B1)	7.3	10.8	++
pLH4BX(A5)	6.5	7.3	+
pLH4BX(B8)	5.0	8.0	+
pLH4BX(A2)	6.8	9.3	++
pLH4BX(B6)	6.5	8.0	+
pLH4BX(A1)	8.3	11.0	++
pLH4BX(C19)	6.0	8.8	++
pLH4BX(B2)	5.8	9.8	++
pLH4BX(B7)	2.1	4.6	±
pLH4BX(C9)	1.8	3.7	±
pLH4BX(C20)	2.2	3.7	±

* Names of host *E. coli* and plasmids are shown. Protein extracts were mixed with latex beads to make it easy for mosquito larvae to eat the proteins. The mortality of the 3rd instar larvae of mosquito *Culex pipiens palens* was scored after 24 h and 48 h. The final concentration of the protein extracts were 50 μg/ml.

** Average no. dead: The numbers of dead larvae per 20 larvae are shown.

+ Mosquito larvicidal activity is shown symbolically; ++ > + > ± > -.

proteins in the protein extracts.

(d)Mosquito larvicidal activity of fused ISRH4 and its deletion mutant proteins.

The results of the assay for mosquito larvicidal activity are summarized in Table 2. The fused ISRH4 protein, which lacked the N-terminal 29 amino acid residues of intact ISRH4, had retained the mosquito larvicidal activity (pLH4BX). Therefore the N-terminal 29 amino acid residues could be removed without a significant loss of the mosquito larvicidal activity.

The nine longer deletion mutant proteins encoded by pLH4BX-A3, B1, A5, B8, A2, B6, A1, C19, and B2 had mosquito larvicidal activities, and they were generally more toxic than the fused ISRH4 protein. The 130-kDa proteins are protoxins, which are proteolytically activated to become smaller active toxins (Lilley *et al.*, 1984). By the deletion of C-terminal region, the 130-kDa insecticidal proteins of *BTI* might become smaller activated forms as they are activated by the proteolysis in the midgut of insects. Therefore, the insecticidal deletion mutant proteins might be activated without proteolysis to become more toxic than the full-length fused ISRH4 protein. The shortest insecticidal deletion mutant protein (B2) spanned the 30th and the 695th amino acid residues. Therefore, amino acid resdues between the 696th and the 1,180th could be removed without a significant loss of the mosquito larvicidal activity. And the mutant proteins which were shorter than B2 (mutants B7, C9, and C20; see Fig.1) lost the mosquito larvicidal activity. Since the mutant B7 which spanned the 30th through the 595th amino acid residued was not toxic, the 100 amino acid residues between the 596th and the 695th were necessary for the mosquito larvicidal activity of ISRH4. These results demonstrated that the mosquito larvicidal protion of ISRH4 was included in the region between the 30th and the 695th amino acid residues.

(e)Insecticidal domains in ISRH3 and ISRH4.

The amino acid sequence in the N-terminal region from the 29th to the 58th of ISRH4 is homologous with those of ISRH3 and the CryⅢA protein of *B. thuringiensis* var. *san diego* known to be toxic for coleoptera (Chestukhina *et al.*, 1988). The homologous amino acid sequence might be important for the insecticidal activity, therefore, the homologous stretch was not removed to construct the fused ISRH4 protein gene. But Pao-intara *et al.* (1988) have reported that the N-terminal 38 amino acid sequnce of the ISRH3-type protein could be removed without a loss of insecticidal activity. The N-terminal 38 amino acid residues contained the N-terminal homologous region which was retained in the fused ISRH4 protein. It is considered that the N-terminal homologous region is not important for the toxicity, and therefore, a few more amino acid residues can be removed beyond the 30th amino acid residue to get the insecticidal portion of ISRH4.

Several investigators have reported that there is an insecticidal domain in the N-terminal half of the 130-kDa protein (CryIA) of *B. thuringiensis* var. *kurstaki* and other varieties (Schnepf & Whiteley, 1985; Adang *et al.*, 1985; Wabiko *et al.*, 1985; Nagamatsu *et al.*, 1984), which are toxic to lepidopterans. In the case of the 130-kDa proteins of *BTI*, there is an mosquito larvicidal domain in the N-terminal half of the 130-kDa protein as postulated with CryIA of *B. thuringiensis* var. *kurstaki* (Pao-intara *et al.*, 1988; Ward & Ellar, 1988), and Sen *et al.* (1988b) have reported that there is another insecticidal domain in the C-terminal half of ISRH3 (CryⅣB). Therefore, there might be two mosquito larvicidal domains in ISRH3, and the C-terminal half of ISRH4 (CryⅣA) may also be insecticidal. The N- and C-terminal insecticidal fragments of the ISRH3-type (CryⅣB) protein overlapped in a region of about 100 amino acid residues in length. Ward and Ellar (1988) have reported that there is a homologous region (only 10 amino acid residues in length) in the 130-kDa proteins of *BTI*(CryⅣA and CryⅣB) and Cry I A, and that the overlapping regions of 100 amino acid sequences contain the homologous region. Moreover an insecticidal N-terminal half of ISRH4 (CryⅣA) also contained the homologous region of 10 amino acid residues. The homologous region

of 10 amino acid residues might be important for the insecticidal activity of 130-kDa proteins of *B. thuringiensis*. The active center relevant to the insecticidal activity might consist of the homologous region as a core and its N-terminus- or C-terminus-flanking region of the 130-kDa protein.

Chapter 2. Importance of the central region of 130-kDa insecticidal proteins of *Bacillus thuringiensis* var. *israelensis* for their activity *in vivo* and *vitro*

The CPBs of *Bacillus thuringiensis* var. *israelensis* (*BTI*) consist of several proteins, the molecular masses of which are 130-kDa, 70-kDa, 28kDa, and so on (Yamamoto *et al.*, 1983; Pfannenstiel *et al.*, 1984). Among them, the 130-kDa (Visser *et al.*, 1986; Pao-intara *et al.*, 1988) and 70-kDa (Lee *et al.*, *1985*) protein subunits were reported to have specific toxicity toward dipterans, the 28-kDa protein subunit being cytotoxic for both insect and mammalian cells, and also hemolytic (Thomas & Ellar, 1983; Armstrong *et al.*, 1985; Yu *et al.*,1987).

Sen *et al.* (1988a) previously reported the cloning and sequencing of the genes for the 130-kDa proteins, ISRH3 and ISRH4. The author and his colleagues delineated one of the mosquitocidal regions of ISRH4 by a deletion analysis, which suggested that the central region of the 130-kDa protein might be important for the insecticidal activity (Yoshida *et al.*, 1989). Recently, Ge *et al.*(1989) located the region responsible for the insecticidal specificity toward *Bombyx mori* in the center of the 130-kDa proteins (Cry I A(a)) of *B. thuringiensis* var. *kurstaki*. In the case of the 130-kDa proteins of *BTI*, the central region might contain the same type of determinant of selective insecticidal activity.

In this chapter, the author reports the delinetion of the mosquitocidal regions of the two 130-kDa proteins (ISRH3 and ISRH4), and demonstrates that the 130-kDa proteins were inactivated by deletion of the amino acid residues in the central regions. On the other hand, we have found that cultured lepidopteran cells, TN-368 (Hink, 1970), from the ovary of the cabbage looper, *Trichoplusia ni*, became swollen and disrupted on treatment with solubilized CPBs of *BTI* in spite of the insecticidal specificity of CPBs. So it was examined whether or not the 130-kDa proteins of CPBs were involved in the cytotoxicity toward TN-368 cells.

MATERIALS AND METHODS

(a)Bacterial strains, plasmids, and media.

BTI4Q1, which was obtained from the *Bacillus* Genetic Stock Center, Ohio, USA, was cultured in PY medium (1% tryptone, 0.5% yeast extract, 0.5% Nacl, pH7.0) or GNB medium (1% tryptone, 0.5% beef extract (dry powder), 0.1% glucose, 0.2% Nacl, pH7.0) at 30℃. (Himeno *et al.*, 1985a) *E. coli* JM109, plasmid vectors, pUC13 and pUC19 (Yanisch-Perron *et al.*, 1985), and recombinant plasmids, pUCH3 and pUCX1' (Sen *et al.*, 1988b) have already been described. The recombinant plasmids pLH4BX and pLH4-B2 encoding truncated ISRH4 genes under the control of *lac* promoter were constructed in the previous study (Yoshida *et al.*, 1989). *E. coli* strains were cultured in 2× YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH7.5) containing appropriate chemicals.

(b)Construction of the recombinant plasmid pUAH3.

The 3.5-kb *Eco*RI fragment of pUCH3 (Sen *et al.*, 1988b), which encodes the reading frame for ISRH3, was subcloned into the *Acc*I site of pUC13 by filled-in ligation to provide pUAH3 (Fig.3-1A), which encoded the ISRH3 gene transcribed under the control of the *lac* promoter from pUC13. By this filled-in ligation, the second codon of ISRH3 was connected to the downstream of N-terminal 10 amino acid residues of the α-fragment of *lacZ'* (Ullman & Perron, 1970) in frame.

(c)Construction of C-terminal deletion mutants of pUAH3.

After plasmid pUAH3 had been digested with *Bam*HI and *Sac*I, nucleotides in the C-terminal region of the ISRH3 gene were sequentially removed by the method of Henikoff (1984), followed by self-ligation. Then JM109 was transformed with these ligated plasmids. The trasformants with β-galactosidase activity, which indicated the in-frame ligation of the truncated ISRH3 gene and downstream part of *lacZ'*, were selected to yield pUAH3-C6 and C7 (Fig.3-1B). Their plasmids were isolated and analyzed by DNA sequecncing to locate the deletion end points as previously described (Yoshida *et al.*, 1989).

(d)Deletion of the central region of 130-kDa protein genes.

A recombinant plasmid, pUAH3G, was constructed, which carried the engineered ISRH3 gene lacking the tract between the blunt ended *Acc*I and *Xba*I sites in the coding region. A *Bam*HI linker (5'-CGGATCCG-3') was inserted in place of the deleted site. A recombinant plasmid, pLH4G, was constructed, which carried the engineered ISRH4 gene lacking the nucleotides between the *Hinc* II and blunt ended *Acc* I sites in the coding region. In both cases their reading frames were not shifted by the internal deletion introduced in them. Plasmids pUAH3G and pLH4G were digested with *Cla* I and *Sac* I, filled in and then ligated with *Bgl* II linker DNA (5'-GCAGATCTGC-3') to yield pUAH3Glac and pLH4Glac, respectively, which carried the truncated 130-kDa protein genes fused with the downstream part of *lacZ'*.

(e)Purification of CPBs and preparation of protein extracts.

CPBs were purified from *BTI*4Q1 cells by the method previously described (Himeno *et al.*, 1985a). Protein extracts of *E. coli* cells were prepared by the method previously described (Sen *et al.*, 1988b).

(f)Western blot analysis.

Protein extracts were electrophoresed on 12.5% SDS-polyacrylamide gel and then transferred to a nylon membrane (Nihon-pall). The membrane was treated with antibodies against CPBs of *BTI* and signals were obtained by a reaction catalyzed by horseradish peroxidase. The details were given previously (Yoshida *et al.*, 1989).

(g)Assay of mosquitocidal activity.

Protein preparations were mixed with latex beads and then incubated at room temperature for an hour (Schnell *et al.*, 1984). The mortality of the 3rd instar larvae of the mosquito *Culex pipiens pallens* was scored after 48h. The concentration of the crude protein extracts was 50 μg/ml.

(h)Culture method for TN-368 cells and assaying of the cytotoxicity.

The culture method for the cell line, TN-368, was previously described (Himeno *et al.*, 1985b). To estimate the effects of the solubilized CPB proteins and 130-kDa proteins of *BTI* on the cells, a cell suspension containing approximately 1.0×10^4 cells/ml was subcultured in 96-well microtiter plates (Falcon) (200 μl/well), the plate being incubated at 28℃ for 3 days. Then the cells were rinsed gently with PBS (without Ca^{2+} or Mg^{2+}, adjusted to pH6.0 with 1N HCl), followed by the addition of the protein preparations in PBS. During further incubation of the cells at 28℃, the response of the cells was observed under a phase-contrast microscope and recorded photographically after 2h.

RESULTS

(a)Construction of deletion mutant plasmids and expression of the genes coding for 130-kDa proteins.

To produce the engineered ISRH3 and ISRH4 in *E. coli* cells, the inducible expression system of the *lac* promoter and cloning sites on the pUC plasmid vectors were used.

A recombinant plasmid, pUAH3, which encoded the N-terminal 10 amino acid residues of the α-fragment of β-galactosidase fused with the polypeptide of ISRH3, was constructed (Fig.2). Transcription of the fused ISRH3 gene proceeded under the direction of the *lac* promoter in pUC13.

Two deletion mutants of pUAH3 (pUAH3-C6 and C7 in Fig.2) were constructed. The N- and C-termini of these truncated ISRH3s were fused with the upstream and downstream parts, respectively, of the α-fragment of β-galactosidase, (Fig.2).

The plasmid pUCX1' encoded a fusion-protein of the C-terminal 575 amino acid residues of ISRH3 and the N-terminal 15 amino acid residues of the α-fragment of β-galactosidase from pUC13 as reported previously(Sen *et al.*, 1988b). The plasmid pLH4BX encoded the N-terminal 13 amino acid residues in the pUC19-borne upstream part of the α-fragment of β-galactsidase fused with the polypeptide between the 30th and the 1180th amino acid residues of ISRH4, and pLH4-B2, which was derived from pLH4BX, encoded the N-termimal toxic fragment of ISRH4 fused with the downstream part of lacZ' (Fig.2) as reported previously (Yoshida *et al.*, 1989).

A truncated gene for the C-terminal half of ISRH4, pLH4C (Fig.2), spanning downstream from the central *Hinc* II site, was newly constructed. This pLH4C showed no β-galactosidase activity, as pLH4C did not carry the downtream part of the α-fragment of β-galactosidase (Fig.2).

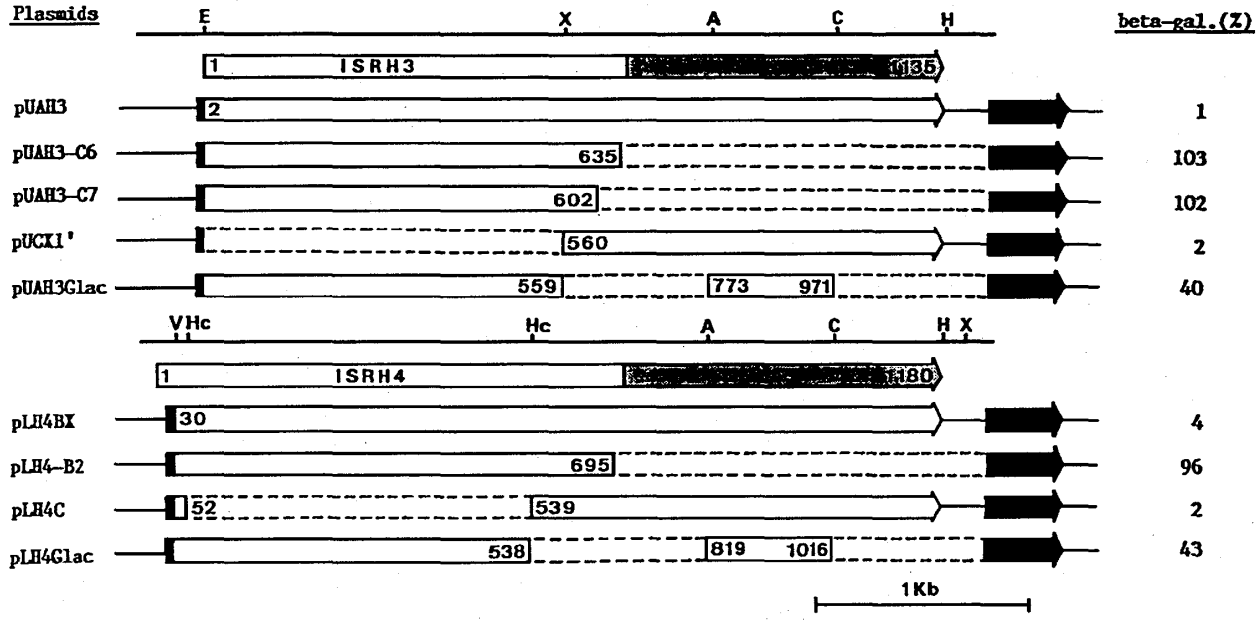

Fig.2 Structures of the Plasmids Encoding the α-Fragment of β-Galactosidase Fused with the Deletion Mutants of ISRH3 and ISRH4. The direction of transcription of the genes is indicated by thick arrows. The solid arrows and boxes indicate the stretch of *lacZ'* encoding the α-fragment of β-galactosidase from pUC vector. Dotted lines indicate the deleted nucleotides. The numbers in the thick arrows are the ordinal numbers of amino acid residues of ISRH3 or ISRH4. The shadowed boxes indicate the C-terminal tract common to ISRH4. "beta-gal.(%)" denotes the relative β-galactosidase activities of the deletion mutants, with that of the wild type (pUC13 for pUAH3 and its dervatives or pUC19 for pLH4BX and its derivatives) taken as 100. The β-galactosidase assay was done by the method of Miller (1972). A, *Acc* I ; C,*Cla* I ; E, *Eco*R I ; H, *Hind*III; Hc, *Hinc* II; V, *Eco*R V, X, *Xba* I .

The plasmid pUAH3Glac was derived from pUAH3 and carried the truncated ISRH3 gene laking the sequences between the *Xba* I and *Acc* I sites and downstream from the *Cla* I site (Fig.2). The plasmid pLH4Glac was derived from pLH4BX and carried the truncated ISRH4 gene lacking the sequences between the central *Hinc* II and *Acc* I sites and downstream from the *Cla* I site (Fig.2). Their truncated genes were connected to the downstream part of *lacZ'* in frame. And in both cases, their reading frames were not shifted by the internal deletions introduced in them.

The production of engineered toxin proteins in *E. coli* JM109 cell harboring recombinant plasmids, pUAH3, pUCX1', pLH4BX and pLH4C, was confirmed by Western blot analysis (Fig.3). The signal strength of the main bands on the Western blot was measured densitometrically to estimate the experssion ratios. For the product of pUAH3, several bands were detected which might have been caused by degradation of the product, and the strongest band at the position of 130-kDa was measured (lane 7 in Fig.3). The ratio of the signal strength of pUAH3/pUCX1' was 7/4 (lanes 7 and 8), and that of pLH4BX/pLH4C was 5/3 (lanes 9 and 10). Based in these ratios and the expected molecular mass of the engineered toxin proteins, their molar ratio in the crude extract was estimated to normalize the results of the bioassays.

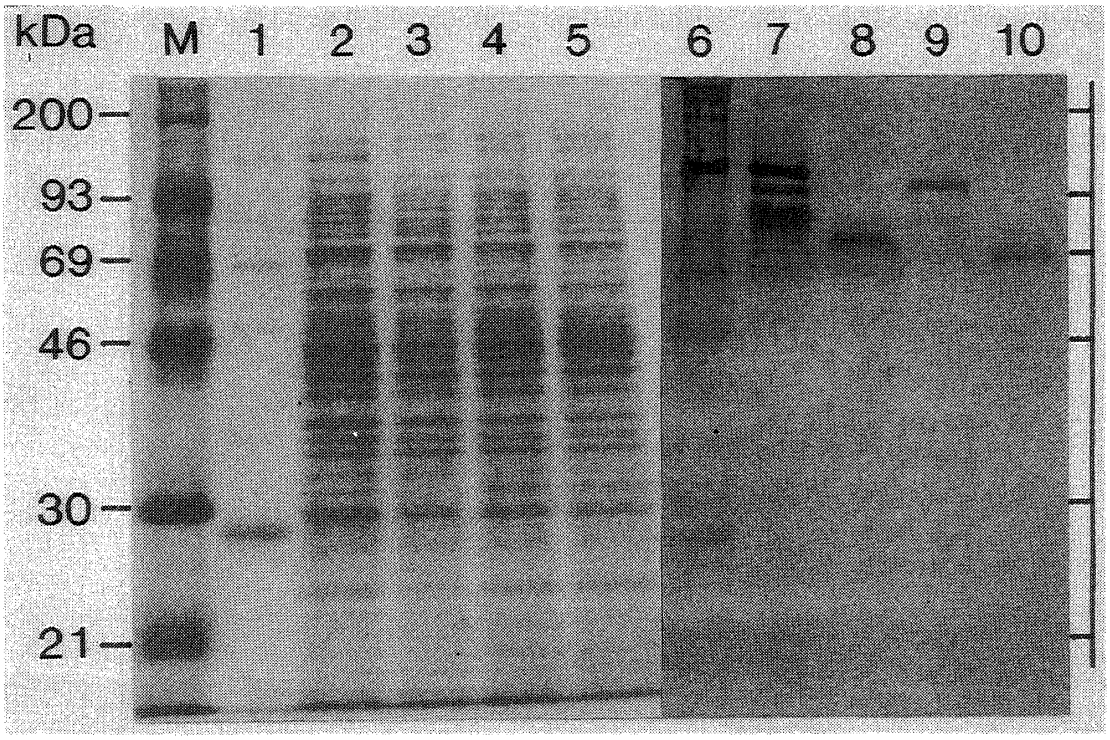

Fig.3 Production of Deletion Mutant Proteins in *E. coli* Cells.
Protein extracts of *E. coli* cells harboring appropriate plasmids were analyzed by SDS-PAGE and Western blotting as described previously. An SDS-polyacrylamide gel electrophoregram (lanes 1-5) and the corresponding Western blot (lanes 6-10)are shown. Lanes 1 and 6 contained the crystal protein of *BTI*4Q1 (10 μg). Each of lanes 2-5 and 7-10 contained about 50 μg of protein extract; *E.* coli JM109 carrying pUAH3 (lanes 2 and 7), pUCX1' (lanes 3 and 8), pLH4BX (lanes 4 and 9), and pLH4C (lanes 5 and 10). lane M contained the size-marker proteins, and a size-marker scale is given on both sides of the panel.

The production of other engineered toxin proteins was confirmed by an alternative procedure, since they lacked the essential regions for immunoreactivity. The 615th to 678th amino acid residues of ISRH3 and the 719th to 760th amino acid residues of ISRH4 are necessary for immunoreactivity for the antibody raised against the CPB of *BTI* (Pao-intara *et al.*, 1988;Yoshida *et al.*, 1989). Therefore, these deletion mutants lacking these regions were fused with the downstream part of the α-fragment of β-galactosidase, the production of the truncated toxin proteins in the *E. coli* cells carrying pUAH3-C6, -7, pLH4-B2, pUAH3Glac, and pLH4Glac was confirmed indirectly by assaying the activity of β-galactosidase (Fig.2). The β-galactosidase activity of these mutants was used for normalization of the bioassay results.

Though pUAH3, pUCX1', pLH4BX, and pLH4C had weak β-galactosidase activity (Fig.2), this was not used for the normalization. Because the engineered toxic protein genes were not fused with the downstream part of *lacZ'*, the products did have β-galactosidase activity. Therefore, their weak β-galactosidase activity was due to read-through in transcription and translation.

(b)Mosquitocidal activity of 130-kDa proteins.

The bioassay for mosquitocidal activity was done using a single dose (50 μg/ml of the crude extracts). Even at this concetration of the crude extract of *E. coli*, nearly 10% of larvae for negative controls (pUC13 and pUC19) were dead (data not shown) and only 30-50% were killed by the toxic extracts, therefore it was impossible to raise the concentration of the samples and to assay using a full range of doses. The reaults of assaying for mosquitocidal activity are summarized in Fig.4. The mortalities were corrected for the control mortality, and normalized on basis of the estimated molar ratios or the β-galactosidase activity.

The recombinant plasmid, pUAH3, carried the engineered ISRH3, which lacked the first methionine residue and was fused with the N-terminal part of the α-fragment of β-galactosidase.

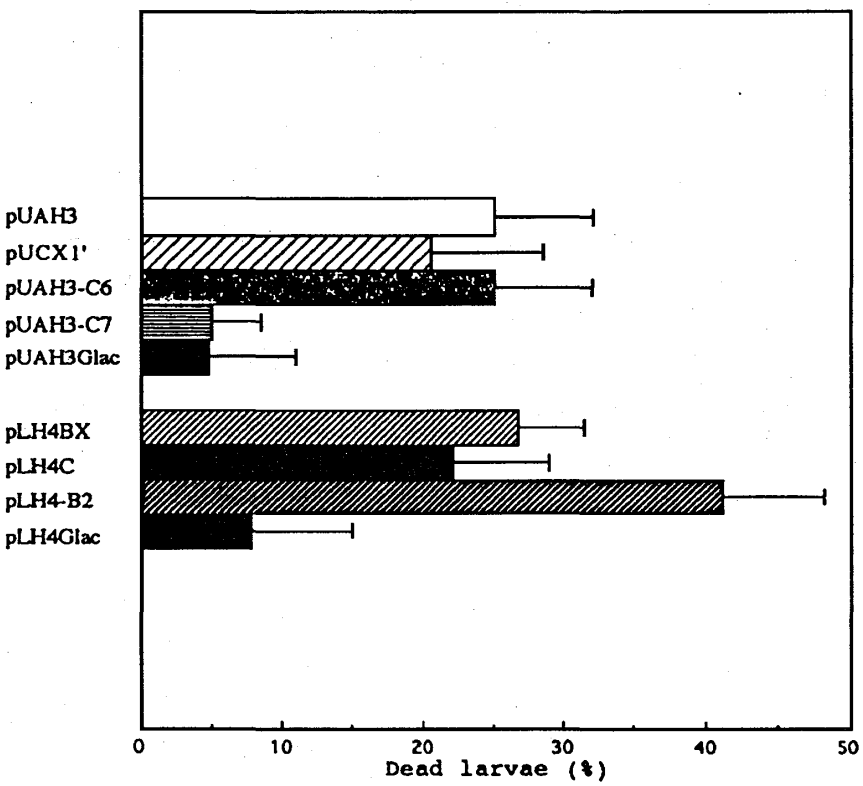

Fig.4 Insecticidal Activity of the Engineered 130kDa Proteins.

The names of recombinant plasmids are shown in left side of the panel. The mortality of the 3rd instar larvae of the mosquito, *Culex pipiens pallens*, was measured after 48h. The final concentration of the crude protein extracts was 50 μg/ml. The products of pUC13 and pUC19 were used as negative controls for pUAH3 derivatives and pLH4BX derivatives, respectively. The mortalities were corrected for the negative controls, and normalized. The mortality of pUCX1' product was normalized to pUAH3 product, and that of pLH4-B2 was normalized to pLH4BX product on basis of the molar ratios estimated from signal strength on the Western blot (Fig.2) and their expected molecular weights. The mortalities of pUAH3-C7 and pUAH3Glac products were normalized to pUAH3-C6 product, and that of pLH4Glac product was normalized to pLH4-B2 on basis of the β-galactosidase activity. The data present the average percentages + mean errors for eight indepedent assays.

The recombinant ISRH3 retained the mosquitocidal activity. The truncated ISRH3 proteins lacking the C-terminal regions beginning from Val636 carried by pUAH3-C6 retained the insecticidal activity. Another truncated ISRH3 protein lacking the C-terminal region beginning from Ser603 (pUAH3-C7) did not have mosquitocidal activity (Fig.4). Thus, amino acid residues between positions 636 and 1135 could be removed without a significant loss of the mosquitocidal activity. Also, the internal region of ISRH3 consisting of 33 amino acid residues, from Ser603 through Ser635, was essential for the mosquitocidal activity. The active C-terminal half of ISRH3 (pUCX1') contained the internal region of 33 amino acid residues (Fig.2 and Sen *et al.*, 1988b), suggesting that two mosquitocidal regions exist in ISRH3. These two toxic regions overlapped in the central region of 76 amino acid residues (Fig.2), in which the essential region of 33 amino acid residues was contained.

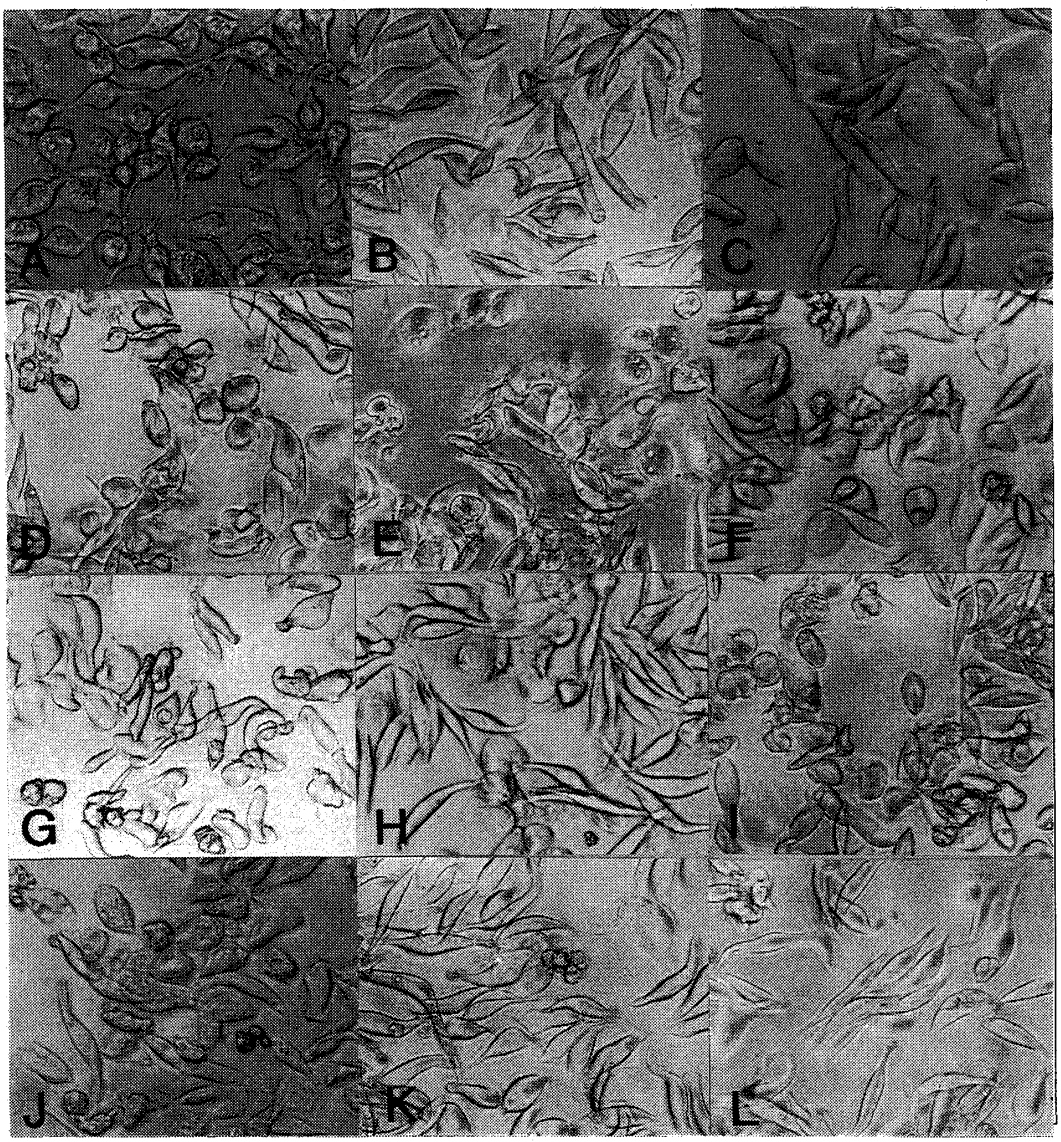

Fig.5 Light Micrographs Illustrating the Morphological Changes of TN-368 Cells Induced by Deletion-mutants of 130kDa Proteins.

TN-368 cell were incubated with protein extracts (3mg/ml)for two hours. The protein extracts were prepared from CPBs of *BTI* or *E. coli* JM109 cells carrying plasmids; CPBs of *BTI* as a positive control (A, 15 μg/ml), pUC13 (B) and pUC19 (c) as negative controls for pUAH3 and pLH4BX derivatives, respectively, pUAH3 (D), pLH4BX (E), pUAH3-C6 (F), pLH4-B2 (G), pUAH3-C7 (H), pUCX1' (I), pLH4C(J), pUAH3Glac (K), and pLH4Glac (L).

The C-terminal half of ISRH4, pLH4C, which spanned Thr539 through Glu1180 (Fig.2), was also mosquitocidal (Fig.4). The N-terminal region between Pro30 and Ile695 of ISRH4 (pLH4B2) was mosquitocidal (Fig.4). Therefore ISRH4 was also proved to have two mosquitocidal regions that overlapped in the central region between positions 539 and 695 (Fig.2), in which the essential region of 100 amino acid residues from Gln596 to Ile695 was contained (Yoshida *et al.*, 1989).

To confirm the importance of the central regions containing the essential region in the 130-kDa mosquitocidal proteins, he constructed deletion mutants, pUAH3Glac and pLH4Glac, lacking both the internal 200-300 amino acid residues, which included the central region, and the C-terminal 164 amino acid residues (Fig.2). The products proved not to be mosquitocidal (Fig.4). The toxicity of pLH4-B2 and pLH4Glac products seemed to be rather higher than that of pLH4BX. This might be due to the fact that he did not normalize the results of pLH4BX and pLH4-B2 and that the result of pLH4Glac was normalized to pLH4-B2, because the pLH4BX product has no β-galactosidase activity while pLH4-B2 and pLH4Glac products were not immunoreactive. Therefore, the actual

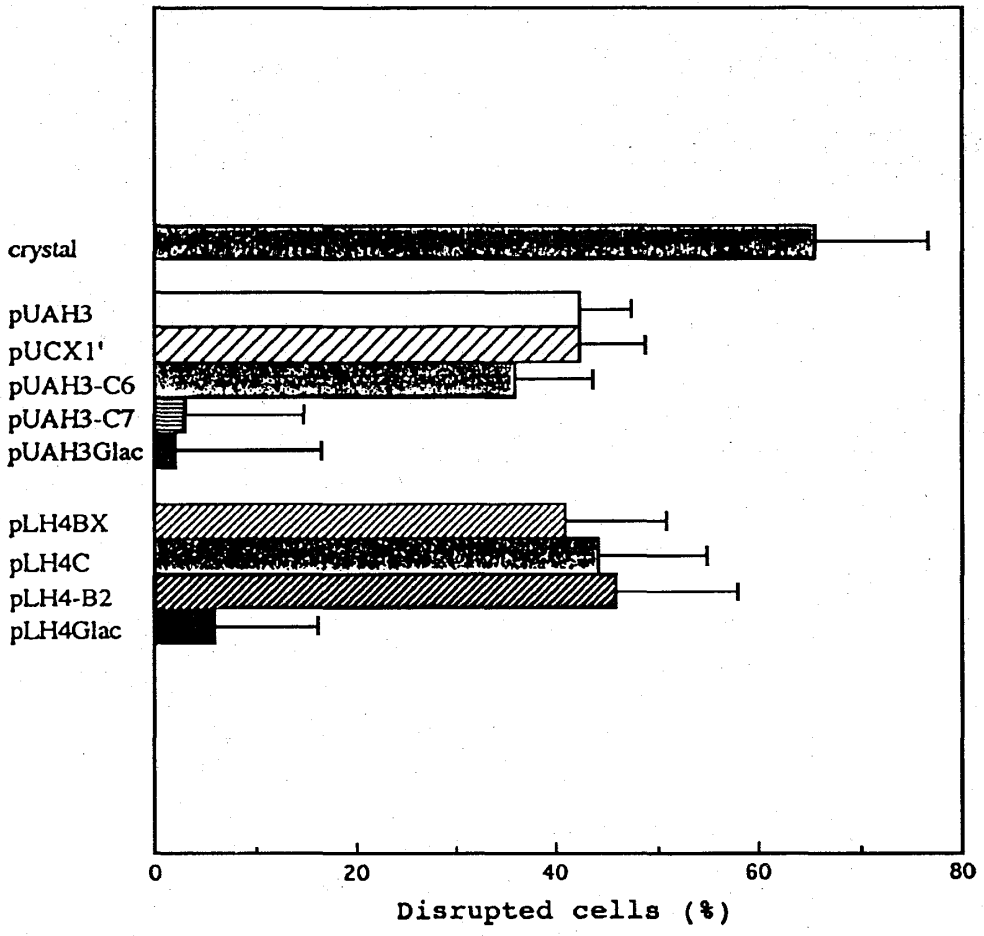

Fig.6 Morphological Changes of TN-368 Cells Induced by Gene Products of the Engineered 130-kDa Proteins.

TN-368 cells were incubated with protein extracts prepared from the *E. coli* cells carrying the recombinant plasmids shown in the left side of the panel (3mg/ml) for 2h, and the percentages of disrupted cells were calculated. The products of pUC13 and pUC19 were used as negative controls for pUAH3 and pLH4BX dervatives, respectively. The percentages were corrected for the negative controls and normalized similarly to the *in vivo* assay (Fig.4). The percentage of cells disrupted by solubilized CPBs of *BTI* (crystal) was corrected for the negative control (pUC13). The data present the average percentages ± mean errors for four independent assays.

concentration of pLH4-B2 product might be higher than that of pLH4BX, and the results of pLH4-B2 and pLH4Glac might be overestimated.

(c)Cytotoxicity of the 130-kDa proteins.

We examined the cytotoxicity of the solubilized CPBs of *BTI* toward a cultured insect cell line, TN-368, which were derived from a lepidopteran. Contrary to the prediction based on the insecticidal spectra of the toxin the cells were swollen and disrupted (Fig.5, panel A).

To examine whether or not the 130-kDa proteins included in the solubilized CPB proteins were concerned with the cytotoxicity, the 130-kDa proteins and their deletion mutants were applied to TN-368 cells. The bioassay for cytotoxicity was also done using a single dose (3mg/ml of the crude extract). Because the proteins in the crude extracts precipitated at the concentration over 3mg/ml at pH6.0, it was impossible to raise the protein cocentration. Even below this high protein concentration, about 60% of the cells were disrupted, while nearly 20% were disrupted in the controls. Therefore assays using a full range of doses were impossible. The cells treated with the mosquitocidal proteins were disrupted (panels D, E, F, G, I, and J in Fig.5, but the cells treated with non-mosquitocidal proteins were not panels H, K, and L in Fig.5) as well as the negative controls (panels B and C).

To assay the responsiveness of cells to the engineered toxin proteins, the ratio of the cells that were disrupted by mosquitocidal or non-mosquitocidal protein was calculated (Fig.6). The results were normalized like those of the assay for mosquitocidal activity. The engineered proteins retaining the mosquitocidal activity, such as shown in Fig.4, were significantly toxic for the TN-368 cells, but the non-mosquitocidal proteins were not. The toxicities of pLH4-B2 and pLH4Glac were high. This was coincident with the *in vivo* results and might be due to the same reason.

DISCUSSION

(a)Expression of the 130-kDa protein genes in *E. coli* and mosquitocidal regions of the 130-kDa proteins of *BTI*

Even when the cloned genes encoding 130-kDa proteins and their deletion mutants were placed under the control of the *lac* promoter, the amounts of produced proteins were not enough for the formation of inclusion bodies in *E. coli* cells. Because of the inefficiency in producing the engineered toxic proteins, it was impossible to do bioassay using the purified engineered proteins. Therefore, the *in vivo* and *in vitro* bioassays using the crude extracrs were done. Ward and Ellar (1988) had produced inclusion bodies of the CryⅣB protein in *E. coli* cells and the CryⅣA protein in *B. subtilis* cells. In our experiments, the ISRH3 (CryⅣB) protein was rather more efficiently produced in *E. coli* cells than the ISRH4 (CryⅣA) protein, judging from the results of Western blot analysis. Adams *et al.* (1989) and Visick and Whiteley (1991) have reported that the production in *E. coli* of the 28-kDa protein subunit (CytA) of CPB of *BTI* increased by the 20-kDa minor protein subunit. It is possible that unknown factors such as the 20-kDa protein might be necessary for effcient production of the ISRH4 protein in *E. coli* cells.

Some investigators have described the N-terminal mosquitocidal region of CryⅣB protein. Ward and Ellar (1988) reported the mosquitocidal activity of the polypeptide of the N-terminal 634 amino acid residues. Pao-intara *et al.* (1988) delineated the mosquitocidal region of the polypeptide between the 39th and 677th amino acid residues. In the present experiments, the polypeptide between the 2nd and 635th amino acid residues was fully mosquitocidal. Stretches of these mosquitocidal regions were slightly different from one another. Based on the results of comparison of the three, the C-terminal border of the mosquitocidal region of the ISRH3 protein would be around the 634th amino acid residue. On the other hand, in the case of the ISRH4 (CryⅣA) protein, the polypeptide between the

30th and 695th amino acid residues is the mosquitocidal region (Yoshida *et al.*, 1989). Therefore, both types of 130-kDa mosquitocidal proteins of *BTI* contain mosquitocidal regions in their N-terminal halves.

The C-terminal halves of ISRH3 and ISRH4 were also mosquitocidal (pUCX1' and pLH4C in Fig.4). The N-terminal and the C-terminal toxic regions overlapped in a region about 100 amino acid residues long in the center of the 130-kDa proteins. Deletion of the overlapping region reduced the mosquitocidal activity of both 130-kDa proteins. Ge *et al.* (1989) have described the region responsible for the insectidal specificity toward *Bombyx mori* in the center of the 130-kDa protein of *B. thuringiensis* ver. *kurstaki* (Cry I A(a)). The central regions of 100 amino acid residues in the 130-kDa proteins of *BTI* appear to be important role in the insecticidal activity as well as the central region of the Cry I A(a) protein. The crystal structure of an insecticidal protein has been analyzed (Li *et al.*, 1991) and the conserved amino acid sequence blocks were compared among insecticidal proteins (Höfte & Whiteley, 1989). The central regions of *BTI* 130-kDa proteins presented here correspond to domain III of Li's definition and to blocks 4 and 5 of Höfte's definition.

(b)Cytotoxicity of the 130-kDa proteins of *BTI* toward lepidopteran cells.

The solubilized CPBs of *BTI* were toxic towerd TN-368 cells and the mosquitocidal fragments of the 130-kDa proteins were also toxic toward the cells (Fig.5 and 6). Though the CPBs of *BTI* were highly toxic toward dipterans, but not toward lepidopterans (Goldberg & Margalit, 1977), they were toxic toward lepidopteran cells. Moreover, these results showed that the 130-kDa proteins, which had been reported to be responsible for the selective toxicity toward dipterans (Visser *et al.*, 1986; Pao-intara *et al.*, 1988) are also toxic toward the leidopteran cells. But the *in vitro* toxicity of the constructs which produced almost the full length of the 130-kDa proteins was not so high as the *in vivo* toxicity and was supposed to be caused by the degraded proteins that were seen in the Western blot (Fig.3), because the 130-kDa proteins (protoxins) were proteolytically activated to show the toxicity. These results imply that the TN-368 cells have receptors for the toxic fragmements of 130-kDa mosquitocidal proteins of *BTI*.

The receptors for the toxic fragments of 130-kDa insecticidal proteins of *B. thuringiensis* are included in the cell membrane so as to able to react with the toxic proteins (Hofmann *et al.*, 1988; Knowles & Ellar, 1986). The receptors might determine the insecticidal specificity. Van Rie *et al.* (1990a) reported that the resistance of a laboratory-selected *Plodia interpunctella* strain to CPBs of *B. thuringiensis* var. *kurstaki* was correlated with a reduction in the affinity of the membrane receptors to CPBs, and that the resistant strain was still sensitive to other CPBs of *B. thuringiensis* var. *entomocidus* HD110. Therefore, it is conceivable that if insect cells carry high-affinity receptors for a certain toxic protein, the toxic protein would be highly toxic toward the insects, and *vice versa*. According to this hypothesis, the affinity of receptors for the toxic fragents of 130-kDa proteins of *BTI* in the cell membrane of TN-368 cells is high enough to react with the toxic proteins under the *in vitro* assay conditions used.

But Wolfersberger (1990) reported that the toxicity of Cry I A did not correlate with its binding affinity, which was against this hypothesis, and there could be some other factors that determine the insecticidal specificity of toxic proteins of *B. thuringiensis*. The *in vitro* assay conditions, which we developed, must be different from the conditions *in vivo*. The CPBs of *B. thuringiensis* are solubilized and digested in the midgut of the host insects, and then attack the target cells *in vivo*. But the insectcidal proteins of *BTI* were already solubilized in *E. coli* cells and partially degraded by proteolysis under the *in vitro* assay conditions. If the insecticidal specificty were determined by the ability of the host insects to digest the CPB proteins, the undigested CPB proteins would not be toxic *in vivo*. It is also possible that the specificity of the receptors was highly restricted *in vivo*. Considering

these possibilities, the cabbage looper, *Trichoplusia ni*, might be resistant to the CPBs of *BTI* because of an inability to digest them or of the highly restricted specificity of the receptors. Anyway, the cells derived from *T. ni* were proved to respond to the toxic fragments of 130-kDa proteins of *BTI*. This suggests that the *in vitro* specificity of *B. thuringiensis* toxins is not as strict as predicted from the observed *in vivo* phenomena.

Chapter 3. Binding of an engineered 130-kDa insecticidal protein of *Bacillus thuringiensis* var. *israelensis* to insect cell lines.

The insecticidal proteins of *Bacillus thuringiensis* are proteolytically activated, and then break the cells in the midgut to kill the insect (Haider *et al.*,1986), and they also toxic to the cultured insect cell lines (Thomas and Ellar, 1983). Some of them, which were especially toxic to Lepidoptera, were suggested to bind to the midgut cells of the host insects through the putative receptor (Hofmann *et al.*, 1988). It was suggested that the receptor was a determinant of the insecticidal specificity (Van Rie *et al.*, 1990b).

In the case of insecticidal proteins of *B. thuringiensis* var. *israelensis* (*BTI*), Hofmann *et al.*, (1986) demonstrated that the CPB proteins (mixture of CryⅣA, CtyⅣB, CryⅣD and CytA proteins) labeled with fluorescein isothiocyanate bound to two types of cell cultures unspecifically, one of which was derived from Lepidoptera and the other from Diptera. Though the 130-kDa insecticidal proteins of *BTI* were reported to be specifically toxic to Diptera, it has not been determined whether this protein may bind to the insect cells specifically.

In this chapter, the author shows that an N-terminal insecticidal fragment of ISRH4 (CryⅣA) protein binds to two insect cell lines nonspecifically.

MATERIALS AND METHODS

(a)Bacterial strains, Plasmids and media.

E.coli JM109 (Yanisch-Perron *et al.*, 1985) and MC4100 (Silhavy *et al.*, 1984) carrying plasmids were cultured in 2×YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH7.5) containing appropriate chemicals. The plasmid pMC1403 (Casadaban *et al.*, 1980), pJG200 (Germino & Bastia, 1984), pLH4B-A3, pLH4BX-B2 and pLH4BX-B7 (Yoshida *et al.*, 1989) were previously described.

(b)Preparation of the protein extracts.

E. coli MC4100 carrying recombinant plasmids were cultured in 10ml of 2×YT containing 50 μg/ml ampicillin and 1 mM IPTG, and harvested by centrifugation (10,000rpm, at 4℃). Cells were washed with 1M NaCl and suspended in 200 μl of 0.1M β-mercaptoethanol and disrupted by shaking with zirconium beads (0.5mm in diameter) for 3 min using a mini-beadbeater (Biospecproducts). After centrifugation, the supernatant solutions were used as protein extracts.

(c)Insect line cells and method of cell-culture.

The cell strain TN-368 was derived from ovary cells of *Tricoplusia ni*, and established by Hink (1970). The culture methods were previously described (Himeno *et al.*, 1985). The cell strain NIAS-AeAl-2 was derived from the first instar larvae of *Aedes albopictus* (Mitsuhashi, 1981). The cells were cultivated in MTCM-1103 (Mitsuhashi, 1981) medium supplemented with 3% fetal bovine serum. The cells in 5ml of the medium were cultivated in a plastic flask (25cm^3) for tissue culture at 28℃ for 4-6 days. The medium was removed gently and the cells were suspended in the new medium. The cell suspension containing approximately 1.0×10^4 cells/ml was subcultured into a 96-well microtiter plate (Falcon) (200 μl per well), and the plate was incubated at 28℃ for 3 days.

(d)Method of binding assay and color reaction.

The medium in the wells of the microtiter plate was gently removed, the cells were washed with

PBS (pH6.0), and the protein extracts diluted (3 mg/ml) with PBS (pH6.0) was applied to the cells. The cells were further incubated at 28℃ for 2hr and washed twice with PBS (pH6.0). Two hundred μl of the reaction solution (Miller, 1972) (125 mM sodium phosphate buffer (pH6.0),0.5mM $MgSO_4$, 0.1mM $MgSO_4$, 0.05mM β-mercaptoethanol and 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was poured into the well and incubated until the blue color developed (30min to several hours). The color development was stopped washing the cells with PBS(pH6.0).

RESULTS AND DISCUSSION

(a)Expression of the truncated gene of ISRH4 fused with β-galactosidase.

To construct an assay system, interaction between insect cell lines and truncated gene products of ISRH4 fused with β-galactosidase (β-gal) was examined. The recombinant plasmids, pM4B2 and pM4B7 (Fig.7), were constructed from pLH4-B2 and -B7, respectively (Yoshida *et al.*, 1989). The plasmid pM4B2 encoded an dipterous active fragment of ISRH4 fused with β-gal derived from pMC1403 (Casadaban *et al.*, 1980) and pM4B7 encoded an inactive one.

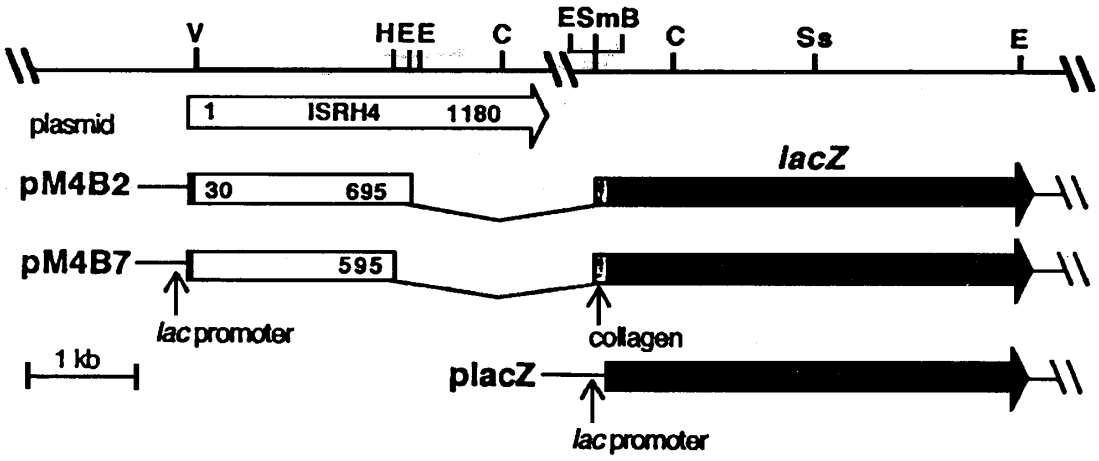

Fig.7 Schematic Organization of the Genes Encoding Truncated ISRH4 Fused with β-Galactosidase.

The names of recombinant plasmids are indicated at the left side. The directions of transcription of the genesare indicated by thick arrows. The solid boxes indicate the stretch of the β-gal encoded by *lacZ* derived from pMC1403. The shadowed boxes indicate the stretch of a collagen linker (Germino & Bastia, 1984). The numbers in the thick arrows are the ordinal numbers of amino acid residues of ISRH4. B, *Bam*H I ; C, *Cla* I ; E, *Eco*R I ; H, *Hind*III; S, *Sal* I ; Sm, *Sma* I ; Ss, *Sac* I ; V, *Eco*R V .

The protein extracts prepared from *E. coli* cells carrying the recombinant plasmids were electrophoresed on an SDS-polyacrylamide gel and analyzed by immunoblotting with antibody against β-gal (Fig.8). The intact fused proteins were produced, but the majority of the each fused protein was decomposed. Therefore the amount of intact fused proteins in the protein extracts was too small to measure (approximatey 0.1-0.2% of the total protein), and the amounts of intact products of pM4B2 and pM4B7 in the extracts were estimated to be nearly equal, judging from their signals on the immunoblot.

(b)Toxicity of the truncated ISRH4 fused β-galactosidase.

An assay of insecticidal activity was done as previously described (Yoshida *et al.*, 1989) and the product of pM4B2 was mosquitocidal but that of pM4B7 was not (data not shown).

The TN-368 cells were typically susceptible to CPBs of *BTI* and active fragments of ISRH4

(Yoshida *et al.*, 1993). As shown in Fig.9, TN-368 cells treated with the insecticidal fuesd protein were disrupted and stained after the color reaction catalyzed by β-gal (pane B), which gave a blue color produced by hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and those treated with the inactive fused protein or the placZ product were not stained (panels C and A, respectively). Moreover, the cells treated with a mixture of solubilized CPBs of *BTI* and purified β-gal which was about ten-fold more concentrated than the placZ product (pane A) were disrupted but not stained (panel D). Because this reaction occurred in the region where the β-gal located, the β-gal activity found in the stained cells would indicate that the β-gal was anchored to the cell through the fused insecticidal fragment of ISRH4 protein. NIAS-AeA1-2 cells (Mitsuhashi, 1981), which were derived from a larva of mosquito, *Aedes albopictus*, were treated with the fused proteins (Fig.9). While the cells treated with insecticidal proteins were disrupted (panels F and H), the others were not disrupted, though the shape of them was not changed so obviusly as that of TN-368 cells (panels E and G). And those treated with the insecticidal fused protein were stained after the color reaction (panel F), and the others were not stained (panels E, G and H). Therefore, the active fragments of ISRH4 were toxic and bound to the NIAS-AeA1-2 cells. These results suggested that a dipterous active truncated 130-kDa protein bound to these two insect cell lines unspecifically, but the difference in the affinity was not measured in the experiment, because the production of fused protein and the activity of the color reaction were too weak.

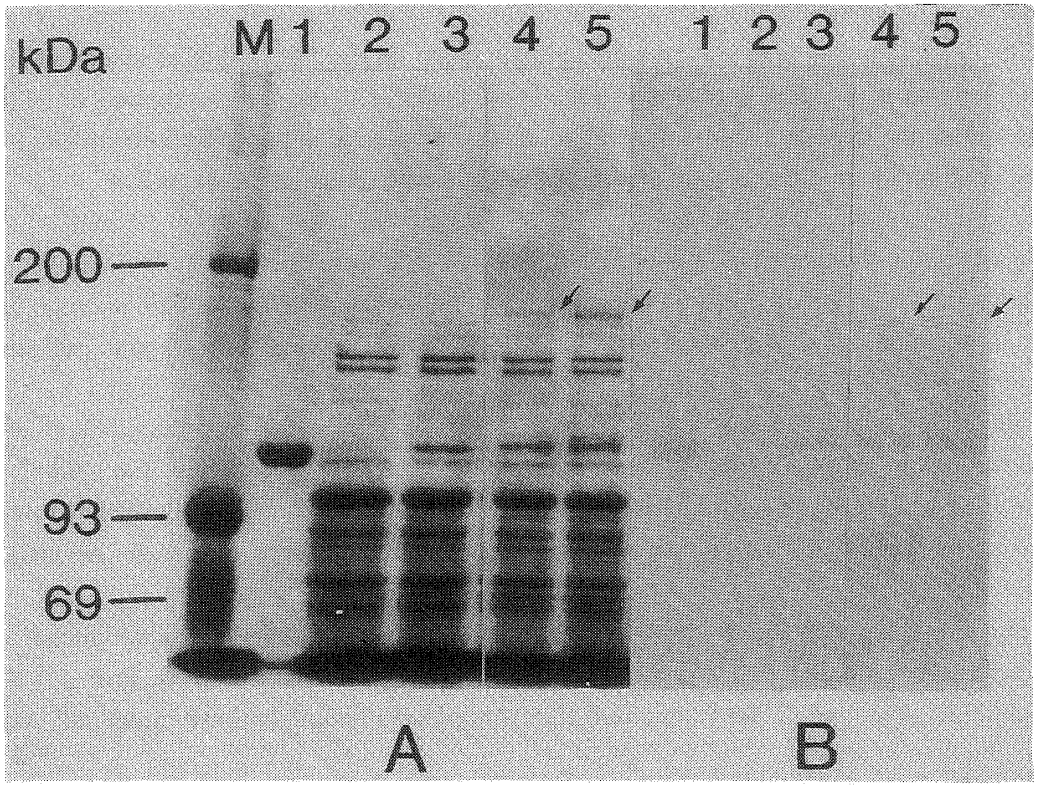

Fig.8 Expression of the Genes for Truncated ISRH4 Fused with β-Galactosidase.

An SDS-PAGE (panel A) and a corresponding immunoblot using rabbit antibodies to β-galactosidase (panel B) are shown. Lane 1, β-gal (2 μg). Each of the lanes 2 to 5, contained 50 μg of protein extract from *E. coli* MC4100 carrying pMC1403 (lane 2), placZ (lane 3), pM4B2 (lane 4) and pM4B7 (lane 5). The small arrows indicate the positions of the intact fused proteins. M contains the standard

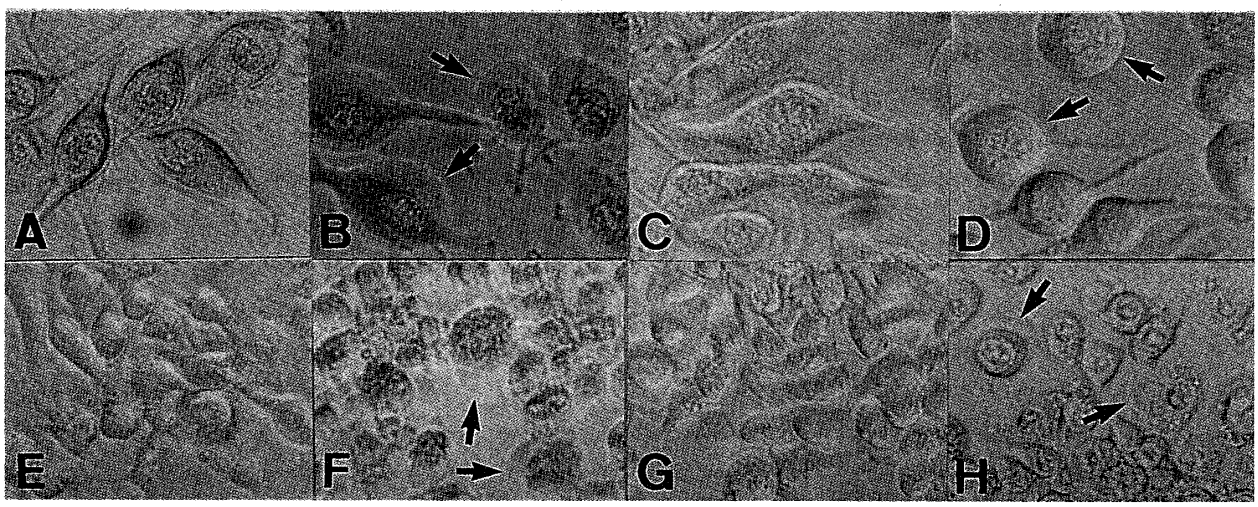

Fig.9 Two Insect Cell Lines After the Color Reaction.
Panels A to D are the photographs of the TN-368 cells after the color reactions, and panels E to H are those of NIAS-AeA1-2 cells. The cells were treated with the protein extracts from *E. coli* MC4100 cells carrying placZ (A and E) as negative controls, pM4B2 (B and F), and pM4B7 (C and G). The cells were treated with solubilized CPBs of *BTI* (15 μg/ml) and β-gal (15 μg/ml)(D and H). Typically disrupted cells were indicated with arrows.

SUMMARY

To clarify the important primary struture and function of the two 130-kDa insecticidal proteins, ISRH3 and ISRH4, of *Bacillus thuringiensis* var. *israelensis*(*BTI*), the author carried out the deletion analysis of the genes and the *in vivo* and *in vitro* assays of insecticidal activity and cytotoxicity of the engineered gene products. The results of this study are summarized as shown below.

1. The two genes for the 130-kDa insecticidal protein of *BTI* were fused with *lacZ'* and expressed in *Escherichia coli*. The deletion analysis revealed that the 130-kDa proteins of *BTI* had two insecticidal regions, N-terminal and C-terminal ones. And the central region of them was essential for the insecticidal activity.
2. The insecticidal and non-insecticidal truncated 130-kDa proteins were applied to a cultured lepidopteran cell line, TN-368. The insecticidal proteins disrupted the cells, but the non-insecticidal proteins did not. Therefore, the insecticidal regions of the 130-kDa proteins were also cytotoxic to TN-368 cells. And the central region of them was also essential for the cytotoxicity.
3. An insecticidal truncated ISRH4 protein linked to the β-galactosidase was produced in *E. coli*. This engineered protein was insecticidal and toxic to two insect cell lines, TN-368 and NIAS-AeA1-2. Moreover, it was suggested that this protein bound to these insect cell lines.

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