A Novel Lipopolysaccharide Response Element in the Bombyx mori Cecropin B Promoter∗

Received for publication, November 29, 1999, and in revised form, February 28, 2000

Kiyoko Taniai†‡ and Shuichiro Tomita¶

From the †Laboratory of Biological Defense, Department of Insect Physiology and Behavior and the ‡Department of Insect Genetics and Breeding, National Institute of Sericultural and Entomological Science, Tsukuba 305-8634, Japan

Cecropin B is one of the major antibacterial peptides in the silkworm, Bombyx mori. Transcription of the cecropin B gene (CecB) occurs rapidly after bacterial invasion. Using 235 base pairs (bp) of the CecB promoter region, a κB-related protein and two additional DNA-binding complexes (designated F2BPI and F4BP) were identified in nuclear extracts from immunized larval fat body by the electrophoretic mobility shift assay (EMSA) (1). Further EMSA analyses indicated that the F2BPI-binding site was CATTa, and that F2BPI translocated into the nucleus after infection. In a cell line, NISES-BoMo-DZ, 235 bp of CecB promoter linked to a reporter luciferase was activated 6-fold by stimulation with lipopolysaccharide (LPS), which is a major trigger of CecB expression in larvae. Truncation of the F2BPI-binding site from the promoter reduced the activation 2-fold. Deletion of either of two κB motifs also reduced promoter activation 2-fold. Elimination of both the F2BPI-binding site and the κB motifs resulted in the complete loss of LPS inducibility. These results indicate that the F2BPI-binding site is an LPS-responsive cis-element that is necessary for full activation of CecB.

Insects have developed an effective innate immune system consisting of humoral and cellular responses. Rapid induction of several antimicrobial peptides in the hemolymph is a major humoral defense against microorganisms (2, 3). Five different peptides have been isolated from the hemolymph of Bombyx mori larvae immunized by bacterial injection: cecropin A, cecropin B, cecropin D, lebocin, and moricin (4, 5). The expression of these genes occurs simultaneously in fat body cells and hemocytes within a few hours of bacterial infection (6, 7). Triggers activating the antibacterial cecropin B gene (CecB) have been characterized in detail (6). Various species of LPS, Lipid A (the lipid part of the LPS core), 2-keto-3-deoxyoctonate (a saccharide in the LPS core), peptidoglycan (PG), and lipoteichoic acid (LTA) from bacteria induced the gene expression strongly. However, laminarin, zymozan, and scyzophillan, all of which contain mainly β-1,3-glucan, or spores of Beauveria bassiana never induced any gene expression. β-1,3-glucan is a common cell wall component of fungi. These observations indicate that B. mori distinguishes bacteria from fungi and expresses CecB in a bacteria-specific response.

The structure of CecB has been analyzed to elucidate the bacteria-specific gene activation mechanism (1). At least four copies of the genes exist in each individual. Two cloned genes, CecB1 and CecB2, revealed 90% identity with the upstream region spanning 800 bp, suggesting that the genes are regulated by the same transcription factors. In the proximal region of the promoters, two κB-like decaemer motifs, three GATA sites and one mammalian type II interleukin-6 response element (IL-6RE) were found. The electrophoretic mobility shift assay (EMSA) identified three different DNA-binding proteins that bind to 235 bp of the CecB1 promoter. One of the proteins is probably a κB-related factor because competition with a κB-like sequence inhibited the binding (1). We designated the other proteins F2BPI and F4BP.

κB motifs and a GATA site have been identified in most insect immune-inducible protein genes (8, 9). The induction mechanisms of antimicrobial peptide gene expression have been well studied in Droso phila melanogaster. NF-κB-related factors of the Rel family (Dorsal, Dif, and Relish) (10–12) and a GATA site-binding protein (Serpent) (13) play major roles in the induction. Several other mammalian cis-elements have been reported in insect immune-inducible promoters, such as both type I and type II IL-6REs and the interferon response element (14, 15). The presence of the nuclear factors for these elements was suggested in the D. melanogaster dpterin promoter using the DNase I footprinting assay (16). However, the function of the elements has not been verified.

In this study, we characterized one of the DNA-binding proteins, F2BPI. We found that the F2BPI-binding site consists of two CATTa in the CecB promoter region. Furthermore, we explored a B. mori cell line that responds to bacterial cell wall components. Transfection assays using these cells revealed that the F2BPI-binding site is necessary for full activation of the CecB1 promoter by LPS.

EXPERIMENTAL PROCEDURES

Insects, Nuclear and Cytoplasmic Extracts, and EMSA—B. mori larvae (Tokai × Asahi strain) were reared on an artificial diet (Nohon Nosanko) at 26 °C and were used at day 3 of the fifth instar. The fat bodies were dissected from control larvae, or from larvae injected with autoclaved Escherichia coli (10⁷ cells/larva), and incubated at 26 °C for 4, 6, or 12 h. A nuclear extract was prepared by the method of Koba yashi et al. (17), and a cytoplasmic extract was prepared as described (18). EMSA using 32P-labeled F2 or F2S DNA (8 fmol) and competitor (800 fmol) was done by essentially the same method as described previously (1).

Oligonucleotide Probes and Competitors—Oligonucleotides were synthesized in a DNA synthesizer (model 392, Applied Biosystems). F2 lipopolysaccharide; EMSA, electrophoresis mobility shift assay; PG, peptidoglycan; LTA, lipoteichoic acid; IL, interleukin; IL-6RE, IL-6 response element; SL2, Schneider line 2; GM-CSF, granulocyte/macrophage colony-stimulating factor; bp, base pair(s); β-gal, β-galactosidase; F2BPI, F2-binding protein I.

* This work was supported by the project “Development of Effective Animal Genome Analysis Techniques and the Application of Useful Genes” of the Ministry of Agriculture, Forestry, and Fisheries, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Laboratory of Biological Defense, Dept. of Insect Physiology and Behavior, National Institute of Sericultural and Entomological Science, Tsukuba 305-8634, Japan. Tel.: 81-298-38-6154; Fax: 81-298-38-6028; E-mail: taniai@nises.affrc.go.jp.

‡ The abbreviations used are: CecB, cecropin B gene; LPS, lipopolysaccharide; EMSA, electrophoresis mobility shift assay; PG, peptidoglycan; LTA, lipoteichoic acid; IL, interleukin; IL-6RE, IL-6 response element; SL2, Schneider line 2; GM-CSF, granulocyte/macrophage colony-stimulating factor; bp, base pair(s); β-gal, β-galactosidase; F2BPI, F2-binding protein I.
A specific competitor was mixed at the indicated excess level. C12 mic (EMSAs were done using the F2S probe and nuclear (are underlined), and N0, N4, N6 and nuclear (tion and time course of F2BPI. EMSAs were done using the F2S probe.

Laminaria digitata was obtained from Wako Pure Chemical. Basic vector (Promega). A minimum promoter region (279 to 999-TCATTAACTGGGAGAGC-3 dTTP. F2S DNA probe was prepared by annealing two oligonucleotides, shown in Fig. 1A.

DNA probe was prepared by annealing of two oligonucleotides, 5'-ATCTCTATCTCGGGCAGCATATTCCAGGTAACCTTATA and 5'-TAAAGGTATGATCCCTC-3', and then the gap was filled using Klenow fragment in the presence of [α-32P]dATP, dCTP, dGTP, and dTTP. F2S DNA probe was prepared by annealing two oligonucleotides, 5'-TCACTTAACCTGAGACCTTGGGAGTTAATGGGATTTAATTTGAGGGGATTAACTTTTA-3' and 5'-TAATGCTCTCCCAGT-3.

The sequences of the upper strands of the competitors are indicated below the EMSA photograph. Bold letters indicate IL-6RE, italics indicate the xB-like motif, and the probable F2BPI-binding sites are underlined. F2BPI localizes to the cytoplasm of the fat body. EMSAs were done using the F2S probe and nuclear (N12) and cytoplasmic (C12) extracts from larval fat body of 12-h immunization. The specific competitor was mixed at the indicated excess level, C, localization and time course of F2BPI. EMSAs were done using the F2S probe and nuclear (N0, N4, N6, and N12) or cytoplasmic (C0 and C12) extracts. The numbers after N and C indicate hours after immunization.

Luciferase activity was measured using a luciferase assay system (Promega). All assays were done in triplicate. B, F2BPI localizes to the cytoplasm of the fat body. EMSAs were done using the F2S probe and nuclear (N12, N4, N6, and N12) or cytoplasmic (C0 and C12) extracts. The numbers after N and C indicate hours after immunization.

Cell Lines and Triggers—B. mori BmN4 and NISES-BoMo-DZ (19) and Spodoptera frugiperda SF9 were provided by Dr. Shigeo Imanishi, Department of Insect Genetics and Breeding, of our institute. D. mela-
nogaster Schneider line 2 (SL2) cells were a gift from Dr. Kumiko Tei, Department of Insect Genetics and Breeding, of our institute. D. melano-
nogaster Schneider line 2 (SL2) cells were a gift from Dr. Kumiko Tei, Department of Insect Genetics and Breeding, of our institute. D. melano-
nogaster Schneider line 2 (SL2) cells were a gift from Dr. Kumiko Tei, Department of Insect Genetics and Breeding, of our institute.

Determination of the Binding Sequence of F2BP—Two nuclear proteins with differing migration patterns were bound to the F2 DNA fragment in EMSA (Fig. 1A, lane 2). F2 contains a xB-like motif, GGGATTAACT, and an IL-6RE, CTCGGA. The fast migrating band is probably the xB-like protein described previously. We designated the slow migrating complex F2BPI (F2-binding protein 1). To determine the F2BPI binding sequence, we used four different competitors in EMSA. Competitor F2S, which is a partial sequence of F2 (Fig. 1A, lane 4), inhibited F2BPI binding. Competitor M1, which has two point mutations in IL-6RE, also inhibited F2BPI binding (Fig. 1A, lane 5). Thus, the binding site of F2BPI is not identical to IL-6RE. In competitor M2, two CATA were replaced by GCGG and CACAG, respectively. As shown in lane 6, M2 could not inhibit the formation of the F2BPI complex. Therefore, CATA is an important sequence for F2BPI binding. The Cx oligo, which contains a xB-like motif from the CecB promoter region, did not inhibit F2BPI binding, but it inhibited the formation of the fast migrating complex (Fig. 1A, lane 7).

Cellular Localization of F2BPI—The cellular localization of
F2BPI was analyzed by EMSA. Using F2S probe, one distinct band shift was observed in both nuclear and cytoplasmic extracts from 12-h immunized larvae (Fig. 1B). This band was inhibited by a specific competitor, F2S. In the cytoplasmic extract, another faint band was observed at a higher position than F2BPI, and F2S also inhibited this band. Similarly, two bands were observed in normal cytoplasmic extract (Fig. 1C, C0). No band shift was observed in normal nuclear extract (Fig. 1C, N0). The intensity of F2BPI was similar in nuclear extracts from 4 and 6 h, but it declined slightly at 12 h. These results indicate that F2BPI is localized in the cytoplasm and then translocates to the nucleus upon infection.

*NISES-BoMo-DZ Respond to Various CecB Triggers*—No other *B. mori* cell line that responds to bacterial challenge has been reported. To develop a promoter assay system of immune-related genes using culture cells, we screened four cell lines, NISES-BoMo-DZ, BmN4, SF9, and SL2 cells. To test the cell lines, a reporter plasmid (pC235) carrying luciferase under the control of 235 bp of the *CecB1* promoter was transfected and analyzed with and without LPS stimulation. After incubation with LPS, luciferase activity increased 6-fold in NISES-BoMo-DZ and 2-fold in SF9 cells (Fig. 2A). The luciferase activities in BmN4 and SL2 cells were not changed after LPS addition. The induced activity of the *CecB1* promoter in NISES-BoMo-DZ increased in an LPS dose-dependent manner (Fig. 2B). The other triggers for *CecB* expression in larvae, PG, and LTA also increased luciferase activity in a dose-dependent manner (Fig. 2B). On the other hand, the cells did not respond to laminarin, in which β-1,3-glucan is a major component (Fig. 2B). As laminarin cannot induce *CecB* expression in larvae, the response of NISES-BoMo-DZ is similar to that of larvae. The responses suggest that this cell line can be used as a model system to analyze gene regulation of antibacterial peptides in *B. mori*.

**F2BPI-Binding Site Is Necessary for Full Activation of CecB1 Promoter**—To examine the functions of the F2BPI site, kB motifs, and GATA sites in the activation of the *CecB1* promoter, we transfected NISES-BoMo-DZ cells with the reporter plasmids containing different lengths of wild-type and mutated *CecB1* promoters (Fig. 3). As shown in Fig. 4, the luciferase activity generated by pC790, pC479, pC235, pC200, pC151, pDG1, and pDG2 (all of these plasmids contain the F2BPI site, kB motifs, and two GATA sites) did not differ significantly. Induction of luciferase activity by LPS was raised 4–6-fold. The induction with pC123 lacking the F2BPI site was increased only 2-fold. Deletion of either of the kB motifs also decreased the level of induced activity. The induction with pDc1 and pDc2 was 2-fold. Deletion of both kB motifs further reduced the induction level to 1.8-fold. Elimination of both the F2BPI site and the kB motifs (pDFkkG) resulted in the complete loss of LPS inducibility of the promoter. Most of the plasmids except pDc1, pDcG, and pDFkkG produced basal activity that was about 10-fold the activity of the control vector without *CecB1* promoter.

**DISCUSSION**

We identified NISES-BoMo-DZ as a useful cell line for assaying promoter activity of immune-inducible genes. We tested whether endogenous *CecB* is expressed in the cells by Northern blot analysis and found that the cells were incapable of expressing *CecB* with or without LPS (data not shown). Nevertheless, this cell line recognizes bacterial cell wall components and possesses the cellular signaling pathway(s) to activate exogenous *CecB* promoter constructs. Using this cell line, the F2BPI site was identified as a cis-element necessary for the full response to LPS (compare pC151 with pC123, Fig. 4). In addition, both kB motifs are functional and required for full activation of the *CecB* promoter. The plasmid (pDFkkG) without kB motifs still has LPS inducibility, suggesting that the F2BPI site is capable of inducing the *CecB* promoter independently of the kB motifs. F2BPI and the kB motifs seem to contribute equally to LPS inducibility. The basal activities of the promoters with the F2BPI site and the kB motifs suggest that NISES-BoMo-DZ cells are constitutively stimulated in medium without the addition of bacterial factors. No other site spanning 790 bp of the promoter region was identified as important for LPS inducibility. In our system, deletion of any one GATA site did not affect promoter activity at all.

We determined that the probable F2BPI site consists of two CATTAs, although we did not identify the binding sequence precisely. Further experiments should determine whether both

---

**Fig. 3. Nucleotide sequences of the mutant CecB1 promoters.** The promoter sequence around possible cis-elements and the 5’-end of the promoter in pC150 and pC123 are depicted. The kB-like 1 motif can be oriented in either direction. Dashes indicate the deletion of a nucleotide.

**Fig. 4. Both the F2BPI-binding site and the kB-like motifs are required for LPS-inducible CecB1 promoter activity.** Schematic structures of the transfected plasmids are shown on the left. Transient expression levels of luciferase activity under the control of different lengths of wild-type or deletion mutant promoters of *CecB1* are represented by white (basal activity) and gray (induced activity) bars. T-bars indicate the S.D. of at least three independent experiments. Each value was corrected by ß-gal activity with co-transfected pA5-LacZ.
CATTA are required or whether one is enough for CecB1 promoter activation. The CATTA appears as a mammalian immune-related cis-element in CLEO, the conserved lymphokine element 6, and in the promoter of IL-4, IL-5, and human granulocyte/macrophage colony-stimulating factor (GM-CSF) (21, 22). The function of the CATTA has been demonstrated using the GM-CSF promoter (23). In this case, CATTT was also functional. The CATTA(T) repeat in the promoter was required for cell expression in T-lymphocytes and several leukemia cells. Because GM-CSF gene expression is induced by LPS in macrophages (24), CATTA(T) also could be an LPS response element of this gene. If so, CATTA(T) is a common LPS response cis-element in mammals and insects, in addition to the b motif and GATA site.

We examined the 5’ upstream region of other LPS-inducible genes for the presence of the CATTA(T) motif. In all, two CATTA are conserved in CecB1 and CecB2, and one is conserved in CecA1 and CecA2. Other b. mori genes and other genes from four different insect species contain one to seven copies of CATTA(T) on both or either strand in the proximal promoter region, although the number and position vary (Table I). The wide distribution of CATTA(T) suggests that this sequence is a common LPS response element in insect immune-inducible genes.

Acknowledgments—We thank Dr. Ylva Engström for critical reading of the manuscript.

REFERENCES

1. Tanai, K., Kadono-Okuda, K., Kato, Y., Yamamoto, M., Shimabukuro, M., Chowdhury, S., Xu, J., Kotani, E., Tomino, S., and Yamakawa, M. (1995) Gene 163, 215–219.
2. Boman, H. G. (1995) Annu. Rev. Immunol. 13, 61–92.
3. Natori, S. (1994) in Antimicrobial Peptides (Boman, H. G., ed.) pp. 123–134, John Wiley & Sons, Chichester, UK.
4. Teshima, T., Nakai, T., Uehi, Y., and Shiba, T. (1987) Tetrahedron 43, 4513–4518.
5. Yamakawa, M., and Tanaka, H. (1999) Dev. Comp. Immunol. 23, 281–289.
6. Tanai, K., Furukawa, S., Shono, T., and Yamakawa, M. (1996) Biochem. Biophys. Res. Commun. 226, 783–790.
7. Furukawa, S., Tanaka, H., Nakazawa, H., Ishibashi, J., Shono, T., and Yamakawa, M. (1999) Biochem. J. 340, 265–271.
8. Hultmark, D. (1995) Trends Biochem. Sci. 20, 178–183.
9. Engström, Y. (1997) in Molecular Mechanisms of Immune Responses in Insects (Brey, P. T., and Hultmark, D., eds) pp. 213–244, Chapman & Hall, London.
10. Petersen, U.-M., Bjerklund, G., Ip, Y.-T., and Engström, Y. (1995) EMBO J. 14, 3146–3158.
11. Gross, I., Georgel, P., Kapppler, C., Reichhart, J.-M., and Hoffmann, J. A. (1996) Nucleic Acids Res. 24, 1238–1245.
12. Dushay, M. S., Åsling, B., and Hultmark, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10343–10347.
13. Petersen, U.-M., Kadalayli, L., Lebow, S., and Lander, R., and Engström, Y. (1999) EMBO J. 18, 4013–4022.
14. Reichhart, J.-M., Meister, M., Dimarcq, J.-L., Zachary, D., Hoffmann, D., Ruiz, C., Richards, G., and Hoffmann, J. A. (1992) EMBO J. 11, 1469–1477.
15. Yanano, Y., Matsumo, M., Sasahara, K., Sakamoto, E., and Morishima, I. (1998) Biochem. Biophys. Res. Commun. 252, 267–274.
16. Georqel, P., Meister, M., Kapppler, C., Lemaire, B., Reichhart, J.-M., and Hoffmann, J. A. (1995) Biochem. Biophys. Res. Commun. 197, 508–517.
17. Kobayashi, A., Matsui, M., Kubo, T., and Natori, S. (1995) Mol. Cell. Biol. 13, 4049–4056.
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, A. J., and Struhl, K. (eds) (1995) Current Protocols in Molecular Biology, Ch. 12.1, Greene Publishing Associates/Wiley-Interscience, New York.
19. Imanishi, S., Cho, E.-S., and Tomita, S. (1999) J. Appl. Entomol. 34, 259–266.
20. Magne, A., Julien, G., Prudhomme, J.-C., and Couble, P. (1997) J. Mol. Biol. 265, 266–274.
21. Miyata, K., Shikomi, K., and Kato, T. (1997) Mol. Cell. Biol. 17, 5894–5901.
22. Thomas, M. A., Mordvinov, V. A., and Sanderson, C. J. (1999) J. Biol. Chem. 274, 3531–3539.
23. Nimer, S., Fraser, J., Richards, J., Lynch, M., and Gasson, J. (1990) Mol. Cell. Biol. 10, 6084–6088.
24. Rao, P., Falk, L. A., Dougherty, S. F., Sawada, T., and Pluznik, D. H. (1997) J. Immunol. 159, 3531–3539.
25. Tanai, K., Isihi, T., Sugiyama, M., Miyashita, M., and Yamakawa, M. (1990) Biochem. Biophys. Res. Commun. 220, 594–599.
26. Furukawa, S., Tanai, K., Ishibashi, J., Hara, S., Shono, T., and Yamakawa, M. (1997) Biochem. Biophys. Res. Commun. 238, 768–774.
27. Kani, A., and Natori, S. (1980) Mol. Cell. Biol. 10, 6114–6122.
28. Kotani, E. (1995) Structure and Function of Hemocytin, a Bombyx mori Humoral Lectin, Which Is Homologous with the Mammalian von Willebrand Factor. Doctoral dissertation, Kyoto Institute of Technology.
29. Ochiai, M., and Ashida, M. (1999) J. Biol. Chem. 274, 11854–11858.
30. Gudmundsdottir, G. H., Liddham, D. A., Asling, B., Gan, R., and Boman, H. G. (1991) J. Biol. Chem. 266, 11510–11517.
31. Xanthopoulos, G. J., Lee, J., Faye, I., and Boman, H. G. (1988) Eur. J. Biochem. 172, 371–376.
32. Sun, S.-C., Asling, B., and Faye, I. (1999) J. Biol. Chem. 274, 6644–6649.
33. Sun, S.-C., Lindstrom, I., Lee, J.-Y., and Faye, I. (1991) Eur. J. Biochem. 196, 247–254.
34. Lindstrom-Dinnetz, I., Sun, S.-C., and Faye, I. (1995) Eur. J. Biochem. 230, 920–925.
35. Wang, Y., Wiltott, E., and Kanost, M. R. (1995) Infect. Mol. Biol. 4, 113–123.
36. Kylin, P., Samakovlis, C., and Hultmark, D. (1990) EMBO J. 9, 217–224.
37. Trystelius, Y., Samakovlis, C., Kimbrell, D. A., and Hultmark, D. (1992) Eur. J. Biochem. 204, 395–399.
38. Dimarcq, J.-L., Hoffmann, D., Meister, M., Bulet, P., Lanot, R., Reichhart, J.-M., and Hoffmann, J. A. (1994) Eur. J. Biochem. 221, 201–209.
39. Charlet, M., Lagueux, M., Reichhart, J.-M., Hoffmann, D., Braun, A., and Meister, M. (1996) Eur. J. Biochem. 241, 699–706.
40. Kobayashi, A., Hira, H., Kubo, T., Ueno, K., Nakanishi, Y., and Natori, S. (1989) Biochim. Biophys. Acta 1009, 244–250.
41. Kani, A., and Natori, S. (1989) FEBS Lett. 256, 199–202.
A Novel Lipopolysaccharide Response Element in the *Bombyx mori* Cecropin B Promoter

Kiyoko Taniai and Shuichiro Tomita

*J. Biol. Chem.* 2000, 275:13179-13182.

doi: 10.1074/jbc.275.18.13179

Access the most updated version of this article at http://www.jbc.org/content/275/18/13179

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 11 of which can be accessed free at http://www.jbc.org/content/275/18/13179.full.html#ref-list-1