Transcription Element Responsible for the Brain Cell-Specific Expression of the Bombyxin Gene that Encodes an Insect Insulin-related Peptide

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Transcription Element Responsible for the Brain Cell-Specific Expression of the Bombyxin Gene that Encodes an Insect Insulin-related Peptide

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ABSTRACT—Invertebrate insulin-related peptides play key roles in growth and metabolism. Genes encoding these peptides are specifically expressed in neurosecretory cells. Bombyxin genes of the silkmoth *Bombyx mori* encode insulin-related peptides and are expressed in four pairs of the neurosecretory cells in pars intercerebralis of brain. No regulatory element has been identified to confer the neurosecretory cell-specific expression of bombyxin gene. By promoter-deletion analysis and *in vitro* electroporation, we identified a transcription element essential for the cell-specific expression of bombyxin F1 gene, one of the bombyxin multifamily genes. The element was localized in the region from −170 to −159 bp upstream of the translation start site of the F1 gene. We named the element as BOSE, BOmbyxin gene-Specific Element. No protein that would bind to BOSE was found by searching the transcription factors database. In addition, an activator element responsible for increasing the expression level was identified in the region from −185 to −181 bp. The activator element is similar to the core element in gut-specific enhancer region of the mosquito *Anopeles gambiae* trypsin gene and the element essential for expression of a *Bombyx* chorion gene.

INTRODUCTION

Insulin-like activities are found in various phyla of invertebrates, indicating that insulin-related peptides are common in invertebrates (Kramer, 1985; Smit *et al.*, 1998). The peptides are considered as key hormones for the regulation of growth, metabolism, and longevity (Gregoire *et al.*, 1998; Smit *et al.*, 1998; Kawano *et al.*, 2000). Bombyxin of the silkmoth *Bombyx mori* (Nagasawa *et al.*, 1984; Iwami *et al.*, 1989; Kondo *et al.*, 1996), molluscan insulin-related peptide (Smit *et al.*, 1988, 1998), and locust insulin-related peptide (Lagueux *et al.*, 1990) have been identified as insulin-related peptides. These peptides show remarkable conservation of the basic insulin-like structure and precursor organization. In addition, many bombyxin-related genes with insulin-like domain structure were discovered in various insect species (Kimura-Kawakami *et al.*, 1992; Iwami *et al.*, 1996).

Thirty-two copies of bombyxin gene are present in the *Bombyx* genome and classified into 7 families, A to G, according to the sequence similarities (Kawakami *et al.*, 1989; Iwami, 1990, 2000; Kondo *et al.*, 1996; Yoshida *et al.*, 1997). These genes are clustered on at least two genomic segments

and arranged into three categories: a gene exists singly (single gene), makes a pair with an another gene (gene pair), or makes a triplet with two other genes (gene triplet). All bombyxin genes lack introns (Kawakami *et al.*, 1989; Iwami, 1990, 2000; Kondo *et al.*, 1996; Yoshida *et al.*, 1997). Multiple bombyxin gene copies in the *Bombyx* genome and the lack of introns are in sharp contrast to vertebrate insulin genes that exist in one or two copies per haploid genome and have one or two introns (Steiner *et al.*, 1985).

The expression site is also different between vertebrate insulin genes and invertebrate insulin-related genes. The vertebrate insulin genes are expressed in cells of the gasteroenteromic organ, whereas the invertebrate insulin-related genes are expressed predominantly in neurosecretory cells. In particular, bombyxin genes of all 7 families are expressed exclusively in four pairs of the neurosecretory cells in the brain (Iwami, 1990, 2000; Yoshida *et al.*, 1997). Bombyxin-producing cells (BPCs) are located in the pars intercerebralis (Ichikawa, 1991). The topological specificity led us to identify the element(s) that is responsible for the expression of bombyxin genes. For this end, we introduced a reporter construct that contains the green fluorescent protein (GFP) gene under the control of bombyxin gene promoter into *Bombyx* brain cells. We improved an *in vitro* electroporation method for the introduction of the reporter (Moto *et al.*, 1999). Promoter-deletion experiment was used to analyze the promoter region of bombyxin F1 gene. The bombyxin F1 gene shares a pair with bombyxin B10 gene in an opposite transcriptional

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direction and is an ideal choice for promoter analysis because of its short 5' upstream region (Yoshida *et al.*, 1997). In this study, we identified an activator element that boosts the expression to a high level and a minimal sequence of the BPC-specific transcription element of the bombyxin F1 gene. The sequence of the activator element has similarity with that of previously identified transcription elements, whereas the BPC-specific transcription element is a novel one.

MATERIALS AND METHODS

Animals

Eggs of a racial hybrid of *Bombyx*, Kinshu X Showa, were obtained from Ueda Sanshu (Ueda, Japan). Larvae were reared on an artificial diet, Silkmate (Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under photoperiod of 12 hr light and 12 hr dark. Brains were obtained from day 3, fifth instar larvae.

Reporter genes

The spacer between the bombyxin gene pair B10/F1 was amplified by PCR using a genomic clone 215S as template (Kondo *et al.*, 1996). The PCR thermal profile was 94°C for 30 sec, 62°C for 30 sec, and 74°C for 90 sec. The cycle was repeated 10 times with 2.5 units of KOD dash DNA polymerase (Toyobo, Osaka, Japan) and primers that have *Bam*HI sites and the translation start site of F1 gene. The forward primer has an *Bam*HI site at the 5' region and its sequence is GCGGATCCTCTTCATTGTAATAGATTT. The reverse primer has an *Bam*HI site at the 5' region and the translation start site at the 3' region and its sequence is TAGGATCCAACCAACTTCATGGCTTT. The amplified fragment was digested with *Bam*HI and inserted into the *Bam*HI digested- and CMV- promoter-deleted plasmid pEGFP-N3 (Clontech, USA) in the transcriptional direction of F1 gene. A series of the promoter deletion constructs was prepared using forward primers (Table 1) and inserted into the same vector. Mutated constructs were prepared using forward primers that mutated in the required sites (Table 1). All constructs were verified by sequencing.

Electroporation

Electroporation was done according to Moto *et al.* (1999) with minor modifications. The reporter gene was dissolved in Grace's insect medium (Gibco/BRL, USA), pH 6.6, to a final concentration of 30 μg/ml and an aliquot of the solution (100 μl) was poured onto a sterile electrode chamber (2 mm gap, 450–20BG, BTX, USA). Five *Bombyx* brains were placed on the chamber that was kept on ice during electroporation. Electroporation was carried out by square pulses (60 V, 90 msec, 3 pulses) with a T820 electroporator (BTX). After changing the direction of the electric field, electroporation was repeated. The brains were then cultured in Grace's insect medium supplemented with gentamycin at 25°C for 2 days. Fluorescence of the GFP was observed under a fluorescence microscope (BX-FLA, Olympus, Tokyo, Japan) with U-MNIBA or U-MNUA filter cube.

Immunohistochemistry

Immunohistochemical detection of GFP was carried out essentially as described (Mizoguchi *et al.*, 1987). Briefly, the electroporated brains were washed in PBS (10 mM sodium phosphate, 130 mM NaCl, pH 7.4), fixed in Bouin's solution for overnight, and washed in 70% ethanol. The brains were then soaked in 0.1% deoxycholate/2% Tween 20 in PBS at 4°C for 4 days to facilitate the penetration of antibodies through the brain sheath, and incubated with Living colors peptide anti GFP antibody (Clontech) that was diluted to 1: 100 with the same solution at 4°C for overnight. The brains were washed with the same solution for several times and incubated with 1:1000 dilution of a second antibody, horseradish peroxidase-conjugated anti-rabbit IgG goat serum (Kirkegaard & Perry Laboratories, USA) at 4°C for overnight. Color development was done with 0.05% diaminobenzidine/0.03% hydrogen peroxide in 50 mM Tris-HCl, pH 7.6.

RESULTS

A GFP reporter gene, pB10/F1::EGFP, that contains the whole 5' upstream region of bombyxin F1 gene (Fig. 1A) was introduced into *Bombyx* brains by *in vitro* eletroporation. As shown in Fig. 1B, strong green fluorescence was detected

Table 1. Sequences of forward primers used for deleted and mutated reporter constructs

| Construct	Sequence (from 5' to 3')
−235	ATGGATCCGCCCACGTCAAATCTGG
−190	ATGGATCCAATCTTCAAGAATTTGTGCAAA
−187	ATGGATCCTTCAAGAATTTGTGCAAAACCTAC
−185	ATGGATCCTCAAGAATTTGTGCAAAACCTAC
−184	ATGGATCCCAAGAATTTGTGCAAAACCTACACA
−180	ATGGATCCAATTTGTGCAAAACCTACACACT
−177	ATGGATCCAGAATTTGTGCAAAACCTACACACT
−174	ATGGATCCTGCAAAACCTACACACTGTCG
−172	ATGGATCCCAAAACCTACACACTGTCGAAA
−170	ATGGATCCAAACCTACACACTGTCGAAACAGTT
−169	ATGGATCCAACCTACACACTGTCGAAACAGT
−168	ATGGATCCACCTACACACTGTCGAAACAGT
−146	CAGGATCCACCTAAACAAATAATGTATGCTGAAT
−120	CAGGATCCTATTCATAACAGAGGATCTGA
Mut 1	ATGGATCCAATCTTTGTGAATTTGTGCAAA
Mut 2	ATGGATCCTCAAGCTATTGTGCAAAACCTAC
Mut 3	ATGGATCCAAAGAGACACACTGTCGAAACAGT
Mut 4	ATGGATCCAAACCTGTACACTGTCGAAACAGT
Mut 5	ATGGATCCAAACCTACGTACTGTCGAAACAGT
Mut 6	ATGGATCCAAACCTACACATTGTCGAAACAGTTACCTAAAC
Mut 7	ATGGATCCAAACCTACACACCGTCGAAACAGTTACCTAAAC
Mut 8	ATGGATCCAAACCTACACACTATCGAAACAGTTACCTAAAC
Mut 9	ATGGATCCAAACCTACACACTGTCGTCTCAGTTACCTAAACAAATAAT
Mut 10	ATGGATCCAAACCTACACACTGTCGAAACCTATACCTAAACAAATAAT
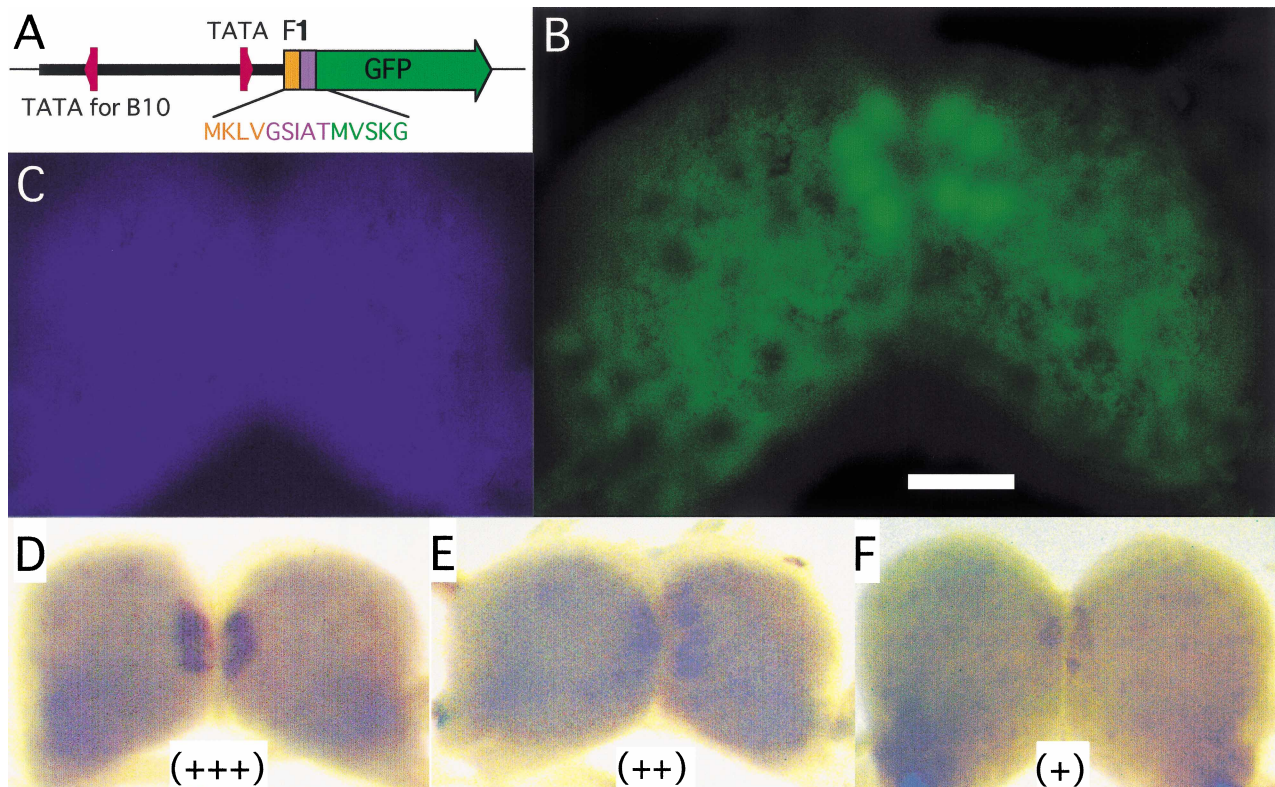
Fig. 1. Electroporation of the reporter gene into *Bombyx* larval brains. (**A**) Schematic representation of the plasmid pB10/F1::EGFP. The plasmid consists of the whole 5' upstream region of bombyxin F1 gene including its translation start codon and the reporter GFP gene. The amino acid sequences of bombyxin F1, GFP, and the bridge between them are shown in orange, green, and purple, respectively. The positions of the TATA-boxes for F1 and B10 genes are indicated with red bar. (**B**) The cell-specific expression of pB10/F1::EGFP in BPCs of a *Bombyx* brain as detected with UV microscope with U-MNIBA cube. Scale bar, 100 μm. (**C**) U-MNUA cube was used to examine autofluorescence of the same brain in (B). The immunodetected levels of GFP expression were graded as high +++ (**D**), medium ++ (**E**), and low + (**F**).

under a UV microscope with U-MNIBA cube in four pairs of the neurosecretory cells in pars intercerebralis of brain that had previously been identified as BPCs (Iwami, 1990, 2000; Ichikawa, 1991). When the fluorescence was detected with U-MNUA cube to differentiate the real GFP expression from the tissue autofluorescence, very weak autofluorescence was observed in the brain (Fig. 1C). The fluorescent signal in BPCs was therefore regarded as fluorescence from the electroporated GFP reporter. The result thus indicated that the reporter is under the control of the bombyxin F1 gene promoter in a BPC-specific manner. Because the autofluorescence varied from brain to brain and sometimes interrupted a trial of quantification of GFP fluorescence by UV microscope, immunodetection of GFP was applied. The GFP expression levels were graded as high (+++), medium (++), and low (+) according to the intensity of immunostaining as shown in Fig. 1D, E, and F, respectively.

A series of promoter-deletion constructs was prepared to identify the minimal region required for the BPC-specific expression of the F1 gene (Fig. 2). High GFP expression level was detected from deletions up to -190 bp upstream of the F1 translation start site (see also Table 2). In some brains, the GFP expression was not identified due to the failure in capturing the reporter gene in BPCs. Medium and low expression levels were detected from the deletion construct −174.

Further deletions up to −146 and −120 bp resulted in abolishment of the GFP expression. These results suggest the presence of an activator element that boosts the expression to a high level and a cell-specific element responsible for the basal expression levels (low and medium) in BPCs. The activator element may locate in the region from −190 to −174 bp, whereas the BPC-specific element may locate in the region from −174 to −146 bp.

To define the minimal sequence of the activator, a series of deletion constructs was prepared in the downstream of −190 bp (Fig. 3). Deletions up to the construct -185 did not affect the high expression level, but the construct −184 resulted in the loss of the high level (Table 2). This result indicates that the 5' start of the activator is at -185 bp. The deletion constructs between −184 and −180 were also expressed in only medium and low levels. Mutations of the three nucleotides from −180 to −178 bp (Mut 2) did not affect the high expression level, whereas mutations in the three nucleotides from −184 to −182 bp (Mut 1) resulted in the loss of the high expression level. Accordingly, the 3' end of the activator is at −181 bp and thus the minimal activator sequence is TCAAG from −185 to −181 bp upstream of F1 translation start site. The loss of the high expression level was observed when the whole promoter construct was electroporated with a double stranded oligonucleotide (AATCTTCAAGAATTTGTGCAAA)
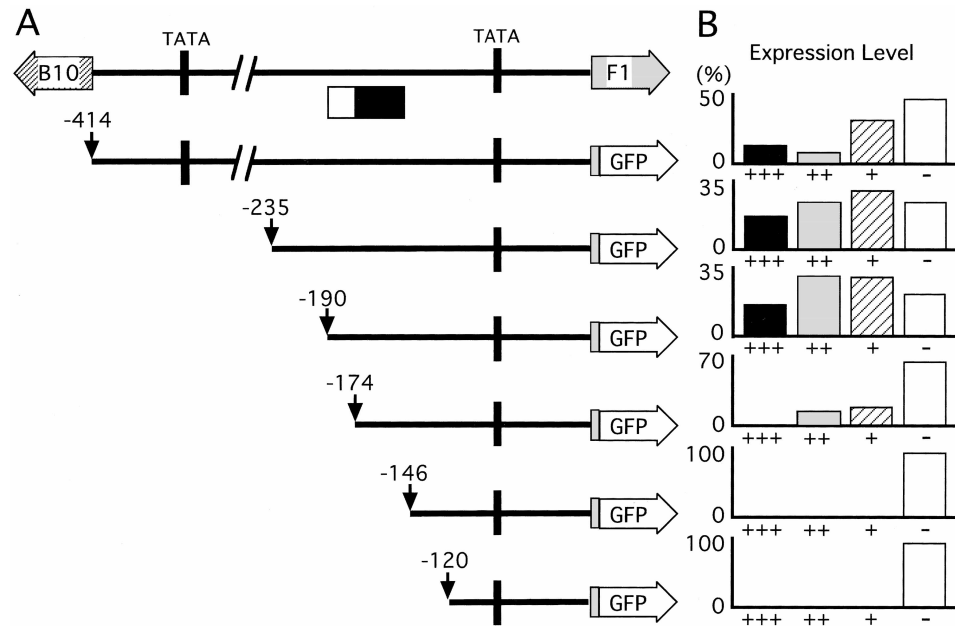
Fig. 2. Promoter-deletion analysis of bombyxin F1 gene. (**A**) The whole promoter region, that is the spacer between the bombyxin gene pair B10/F1 (414 bp), was serially deleted in the F1 transcriptional direction to identify the minimal promoter region that includes the element(s) required for the cell-specific expression of F1 gene. Closed bars show the position of the TATA-box for each gene. Arrows indicate the 5' start of each deletion construct related to the F1 translation start site at +1. Open box represents the region from −190 to −174 bp that may include an activator element. Closed box represents the region from −174 to −146 bp that may include the cell-specific expression element. (**B**) The percentage of brains that showed one of four expression levels related to the total number of electroporated brains is shown against each expression level.

Table 2. Expression patterns of pB10/F1::EGFP and its deleted and mutated constructs. Number of brains that showed one of four expression levels is indicated with the percentage of total brains (shown in parentheses)

Construct	Number of brain (percentage)			
	Level of expression			
	+++	++	+	−
pB10/F1::EGFP	6 (12)	3 (6)	16 (32)	25 (50)
−235	8 (17)	12 (25)	16 (33)	12 (25)
−190	7 (14)	16 (32)	16 (32)	11 (22)
−187	5 (10)	9 (18)	10 (20)	26 (52)
−185	4 (8)	7 (14)	8 (16)	31 (62)
−184	0 (0)	7 (14)	13 (26)	30 (60)
−180	0 (0)	8 (16)	10 (20)	32 (64)
−177	0 (0)	5 (11)	14 (32)	25 (57)
−174	0 (0)	7 (14)	9 (18)	33 (68)
−172	0 (0)	6 (13)	18 (39)	22 (48)
−170	0 (0)	10 (20)	15 (30)	25 (50)
−169	0 (0)	0 (0)	0 (0)	30 (100)
−168	0 (0)	0 (0)	0 (0)	29 (100)
−146	0 (0)	0 (0)	0 (0)	50 (100)
−120	0 (0)	0 (0)	0 (0)	50 (100)
Mut 1	0 (0)	4 (8)	14 (30)	29 (62)
Mut 2	3 (7)	8 (17)	0 (0)	35 (76)
Mut 3	0 (0)	0 (0)	0 (0)	30 (100)
Mut 4	0 (0)	0 (0)	0 (0)	25 (100)
Mut 5	0 (0)	0 (0)	0 (0)	28 (100)
Mut 6	0 (0)	0 (0)	0 (0)	30 (100)
Mut 7	0 (0)	2 (7)	4 (14)	24 (80)
Mut 8	0 (0)	2 (7)	6 (22)	19 (71)
Mut 9	0 (0)	4 (14)	6 (21)	19 (65)
Mut 10	0 (0)	3 (10)	5 (17)	22 (73)

that contains the activator sequence as a competitor.

To determine the minimal sequence of the cell-specific expression element, various deleted and mutated constructs were prepared in the region from −174 to −146 bp (Fig. 4). Deletions up to −170 bp resulted in maintaining the medium and low levels of expression, but the deletion constructs −169 and −168 completely abolished the GFP expression (Table 2), indicating that the 5' start of the cell-specific expression element is at −170 bp. A set of constructs mutated at the sites, −167 to −165 bp (Mut 3), −164 to −163 bp (Mut 4), and −162 to −161 bp (Mut 5), resulted in no GFP expression, indicating that these mutated nucleotides are included in the cell-specific expression element. Reporter constructs with point mutations in the nucleotides at −159 (Mut 6), −158 (Mut 7) or −157 bp (Mut 8) were prepared to determine the 3' end of the element. Only the construct with a mutation at −159 bp (Mut 6) resulted in no GFP expression, indicating that the 3' end of the cell-specific expression element is at −159 bp. Mutations in the nucleotides from −153 to −151 bp (Mut 9) and −149 to −147bp (Mut 10) did not affect the expression levels. Therefore, the transcription element responsible for the basal expression levels (low and medium) in BPCs is a 12 bp segment from −170 to −159 bp with a sequence of AAACCT-ACACAC. We named the BPC-specific element as BOSE, BOmbyxin gene-Specific Element. The shutoff of all expression levels was observed when the whole promoter construct was electroporated with a double stranded oligonucleotide (AAACCTACACACTGTCGAAACAGTT) that contains the BOSE sequence as a competitor. No protein that would bind
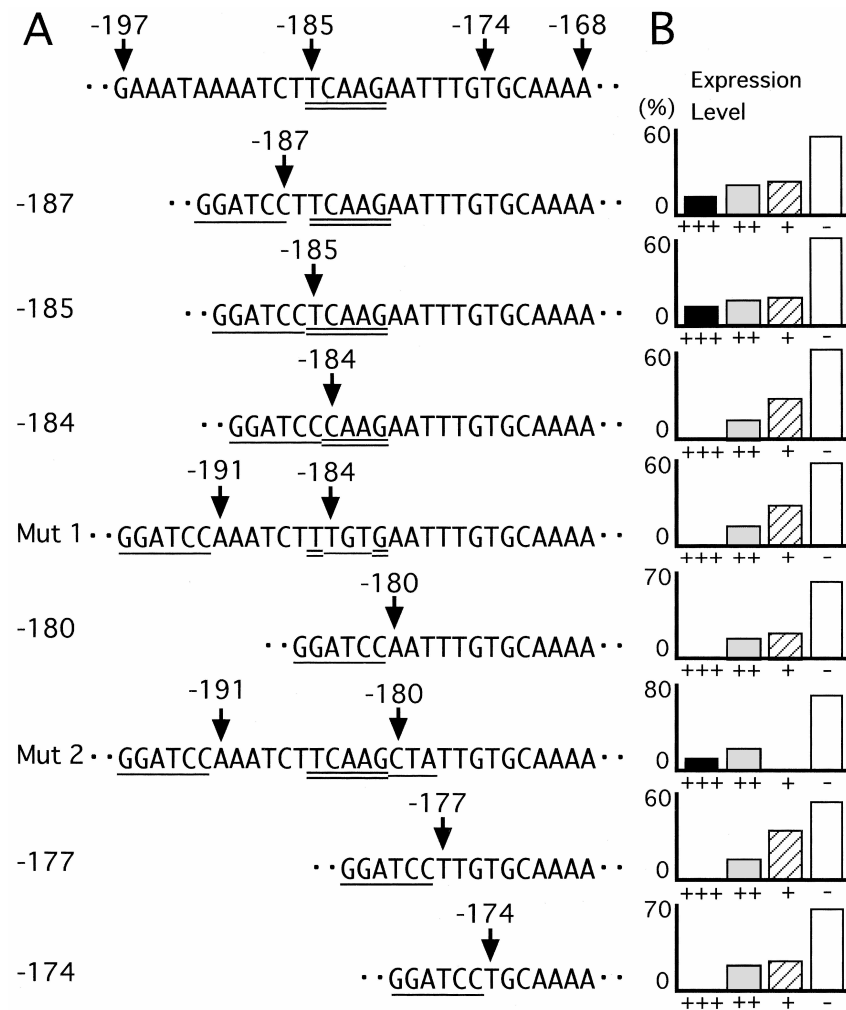
Fig. 3. Activator element in the bombyxin F1 gene promoter. (**A**) Several deleted and mutated constructs were prepared in the region downstream of −190 bp to define the minimal activator element sequence. The activator element is indicated with double-underline. Sequences with underline represent *Bam*HI site or mutated nucleotides and those with double-underline show nucleotides of the activator element. (**B**) Same as for Fig. 2B.

to the element was found by searching the TRANSFAC transcription factors database (Heinemeyer *et al.*, 1998) at TFSEARCH website (Y. Akiyama, http://www.rwcp.or.jp/papia).

DISCUSSION

DNA manipulation coupled with reporter gene assay has increased the understanding about mechanisms that control gene expression. So far, thirty-two bombyxin genes have been identified but there is no information about the mechanism of their expression. The object was to find a reliable method of gene transfer into brain cells. Microinjection is widely used for introducing foreign genes into cells and has been successfully carried out in many kinds of cultured cell lines and embryos. However, microinjection is a time consuming process. Electroporation is an another choice for gene transfer and has been applied successfully in bacteria, yeast, cultured cells, and embryos (Lurquin, 1997; Muramatsu *et al.*, 1998).

We therefore improved the method to be applicable for the gene transfer into intact insect brains (Moto *et al.*, 1999). The transgenic system is widely used successfully in mouse and *Drosophila*, and recently in *Bombyx* (Tamura *et al.*, 2000). However, the efficiency to get transgenic lines in *Bombyx* is too low to do reporter gene analysis. The transgenic system is also time consuming and can not deal with a large number of reporter genes at once. We therefore chose transient expression analysis of the reporter gene.

With the improved electroporation method, we succeeded in identifying two elements responsible for the expression of bombyxin F1 gene. One is an activator with a sequence of TCAAG and the other is BPC-specific expression element, BOSE, with a 12 bp sequence of AAACCTACACAC. The sequence of activator element is similar to that of the core element TYAAGT (Y=C or T) in the gut-specific enhancer region of the mosquito *Anopheles gambiae* trypsin gene (Shen and Jacobs-Lorena, 1998). The similarity of the two elements indicates that *Bombyx* and *Anopheles* may share homologous
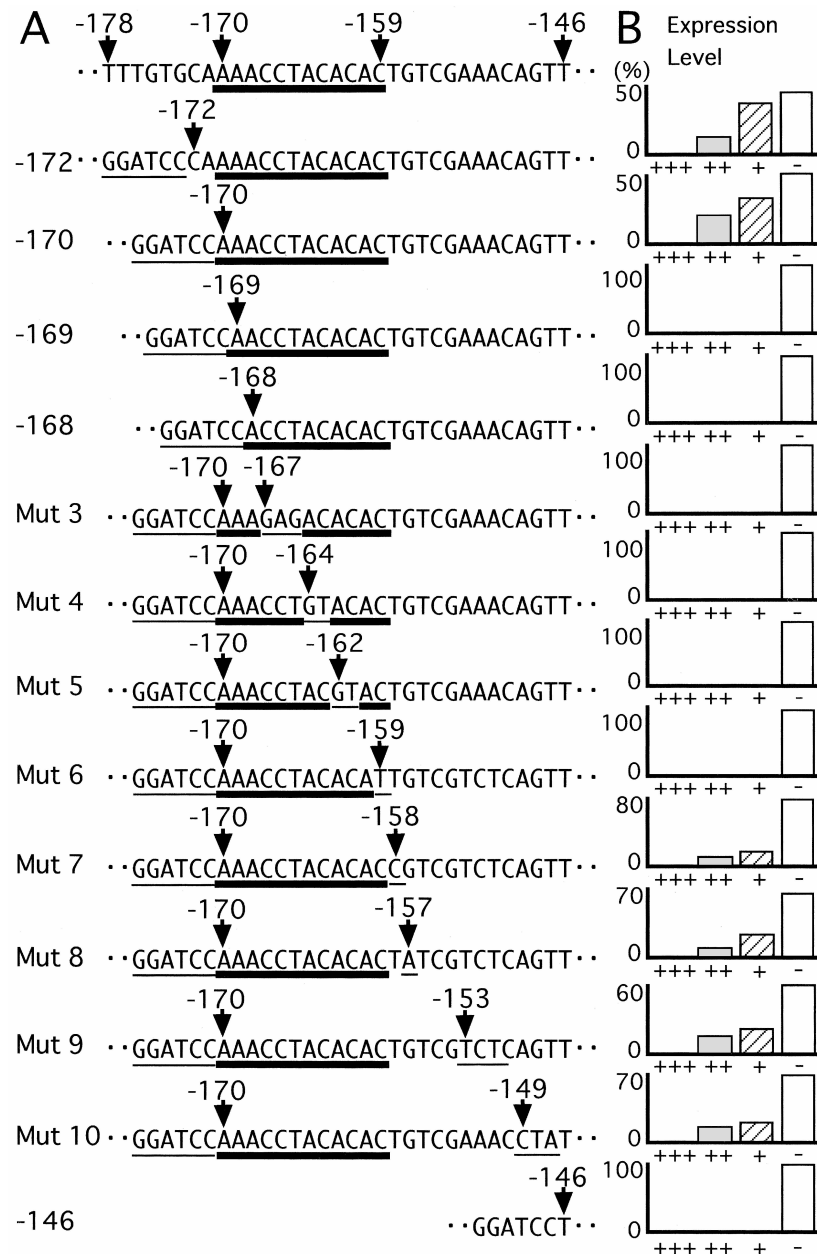
Fig. 4. BPC-specific element in the bombyxin F1 gene promoter. (**A**) Serial deletion of nucleotides from the region from −172 to −146 bp reveals the cell-specific expression element in the region from −170 to −159 bp. The BPC-specific element is indicated in thick underline. Sequences with underline represent *Bam*HI site or mutated nucleotides and sequences with thick underline show nucleotides of the cell-specific element. (**B**) Same as for Fig. 2B.

transcription factor(s) that binds two different genes in different tissues, and that the regulatory machinery of the two genes which are expressed specifically in brain and gut is evolutionary conserved. Similarity was also found between the activator and the element, TCACGT, essential for the expression of the *Bombyx* chorion gene, another multifamily genes organized in divergent pair-based gene organization (Mitsialis *et al.*, 1987; Fenerjian and Kafatos, 1994). The chorion genes are expressed coordinately with strict sex, tissue, and temporal specificity. The sequence of BOSE has no similarity with other known transcription elements and thus BOSE appears to be a novel regulatory element.

The tissue specificity conferred by *cis*-acting regulatory elements was examined for the rat insulin I gene (Dandy-Dron *et al.*, 1995) whose tissue-specific expression is not resulted from any single minimal sequence but from an interaction of multiple sequence elements. In the rat insulin II gene, an element as small as 41 bp is capable of regulating pancreatic temporal and spatial gene expression (Stellrecht *et al.*, 1997). The specific expression of the insulin gene requires the integrated contributions of many factors that bind to a relatively long promoter segment and build a functional transcriptional complex (Ohneda *et al.*, 2000). The complexity of these interactions ensures that transcription complexes can not be com-

pletely replicated in other cell types. In the present study, we demonstrated that a 12 bp element, BOSE, was enough to confer the BPC-specific expression of bombyxin F1 gene. BOSE may represent the binding site of the transcription factor(s) that is essential for the formation of a specific transcriptional complex which is specifically formed in BPCs. Present study first revealed an element involved in neurosecretory cell-specific gene expression and may provide important suggestions for the mechanisms that control such a specific expression in brains of other invertebrates as well as vertebrates.

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