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Author(s)	Kojima, K.; Oritani, K.; Nakatsukasa, T.; Asano, S.; Sahara, K.; Bando, H.
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Ecdysone response element in a baculovirus immediate early gene, *ie1*, promoter

K. Kojima^{a,b}, K. Oritani^b, T. Nakatsukasa^b, S. Asano^b, K. Sahara^b, H. Bando^b

^a Silk-Materials Research Unit, National Institute of Agrobiological Sciences, 1-2 Owashi, Tsukuba Ibaraki, 305-8634, JAPAN

^b Department of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, N9 W9 Sapporo, 060-8589, JAPAN

corresponding author

Hisanori Bando, e-mail: hban@hokudai.ac.jp, Tel/Fax: +81-11-706-3348

Abstract

A computer-assisted analysis identified tentative target sequences for regulatory proteins including ecdysone-inducible factors such as BmFTZ-F1 and Broad-Complex Z4 (BR-C Z4) in the *ie1* promoter of BmNPV. A transient expression experiment using BmN cells and a series of truncated *ie1* promoter constructs demonstrated that the activity of the *ie1* promoter responded to alpha-ecdysone and 20-hydroxyecdysone, which required a tridecameric nucleotide stretch (*ie1*EcRE, 5'-GTGTTATCGACCT-3') homologous to the ecdysone response element reported for *Drosophila* (*Dm*EcRE). RT-PCR demonstrated the expression of BmEcR and BmUSP, which are required as ecdysone-specific activators for EcRE-mediated activation, in BmN cells. Furthermore, the *ie1*EcRE-mediated response was confirmed by using a recombinant BmNPV possessing a luciferase gene under the control of the *ie1* promoter with or without *ie1*EcRE. This is the first report of an ecdysone response element in a baculoviral gene promoter. These results also suggested that the regulation of the *ie1* by ecdysone may militate viral replication at least under certain conditions during natural infections *in vivo*.

Key words

BmNPV, *immediate-early 1*, ecdysteroids

Abbreviations

Ecdysone response element: EcRE, nuclear polyhedrosis virus: NPV,

1. Introduction

Baculoviruses, with their large (88 - 150 kbp), double-stranded supercoiled DNA genome and enveloped virions, are efficient vectors for the expression of foreign genes in insect and mammalian cells, and have great potential for agricultural pest control (Miller, 1995). The complete genome sequences of various baculoviruses (AcMNPV, BmNPV, OpMNPV and so on) have been determined and the coding regions for about 130 genes, including tentative genes of unknown function, were identified (Ahrens et al., 1997; Ayres et al., 1994; Gomi et al., 1999; Yamagishi et al., 2003). The expression of baculovirus genes in infected cells indicates the existence of at least three classes of genes: early (immediate-early and delayed early), late, and very late genes, and is coordinately regulated in a sequential order (Friesen and Miller, 1986; Lu and Miller, 1995; Yamagishi et al., 2003). These observations suggested that a number of *cis*-acting elements and *trans*-acting factors are involved in the regulation of viral gene expression. The transcription of early genes involving viral DNA replication and regulation of early and late gene expression require only host RNA polymerase II and its associated transcription factors. Though a large part of the regulatory mechanism of the baculovirus gene expression cascade is still obscure, studies so far imply that an immediate early gene (*ie1*) product, IE1, plays a key role both in the regulation of early gene expression (Kovacs et al., 1991; Olson et al., 2002; Passarelli and Miller, 1993), and in DNA replication (Olson et al., 2002). These studies suggest that the regulation of IE1 expression could be a key step in the optimal regulation of the complicated viral gene expression cascade. However, little is known about *ie1* transcriptional regulation in host cells except for limited information on the activation of the *ie1* promoter by viral regulatory proteins such as IE0 and IE1 (Kovacs et al., 1991), and IE2 (Yoo and Guarino, 1994).

Baculovirus infections start with the ingestion of polyhedra by insect larvae. Once swallowed, the polyhedra are lysed by the alkaline digestive juice within the midgut lumen and release viruses (ODVs: occlusion-derived viruses) which infect midgut cells. The viruses proliferate in the infected midgut cells, bud from the cell surface (BVs: budded viruses) to infect neighboring cells one after another, and finally kill their host insect with the production of a great number of polyhedra (Blissard and Rohrmann, 1990). Infections of baculovirus may occur throughout larval stages but viral multiplication seems to be affected by the developmental status of host insects which is regulated by the interplay of juvenile hormone (JH) and ecdysteroids (Riddiford, 1993). For example; i) it was previously reported that silkworm larvae infected with BmNPV were apt to show the symptoms of polyhedrosis disease mainly at the molting stage (Kobayashi et al., 1967), ii) many baculoviruses possess an ecdysteroid UDP-glucosyltransferase (*egt*) gene (Clarke et al., 1996), which prevents

molting by inactivating ecdysteroids during baculovirus replication (O'Reilly, 1995), and iii) ecdysteroid stimulates foreign protein production of recombinant AcMNPV in *Spodoptera frugiperda* cells (Sarvari et al., 1990). These observations suggest that ecdysteroid influences the multiplication of baculoviruses.

Ecdysteroid acts as the primary molting hormone in insects and plays important roles at the initiation of metamorphosis. The active form of ecdysteroid is 20-hydroxyecdysone (20E) which is synthesized as alpha-ecdysone (E) at the prothoracic gland or ring gland, secreted into hemolymph, and then enzymatically converted into 20E in the peripheral tissues (Sonobe and Yamada, 2004). 20E binds to a heterodimer consisting of the ecdysone receptor (EcR) and Ultraspiracle (USP), an ortholog of the vertebrate retinoid X receptor (RXR) forming a functional ecdysone receptor, and activates a number of ecdysone-regulated genes (Riddiford et al., 2000).

Recently, it was reported that insect hormones stimulated expression from a baculoviral essential regulatory gene *ie1* promoter, though the regulatory mechanism of the *ie1* promoter in the hormonal activation is obscure (Zhou et al., 2002). In this study, we investigated the *cis*-acting elements in the promoter sequence of BmNPV *ie1* as a first step to elucidating the host cellular factors involved in the possible regulation of the baculovirus gene expression cascade.

2. Materials and methods

2.1. Cells and virus

BmN cells were maintained in TC-100 (Nosan corporation) supplemented with 10% fetal bovine serum. The BmNPV (family *Baculoviridae*, genus *Nucleopolyhedrovirus*, species, *Bombyx mori NPV*) T3 strain and derivative recombinants were propagated in BmN cells. Viral titers were determined by plaque assay (Isobe et al., 2004).

2.2. Construction of reporter plasmids

A series of reporter plasmids which expresses the luciferase gene under the control of BmNPV *ie1* promoters, were constructed. Detail of construction of these reporter plasmids are given in Fig. 1.

2.3. Transfection and reporter assay

BmN cells were transfected with plasmid DNA as described elsewhere (Kojima et al., 2001). After the transfection, the cells were maintained for 48 hrs in TC-100 medium containing alpha-ecdysone (Sigma) or 20-hydroxyecdysone (Sigma) at an implicit concentration as indicated in the figure legends. Then, luciferase activity in the cells was

measured according to a method described elsewhere (Kojima et al., 2001). Ecdysteroids were dissolved in ethanol, then 0.1 mg/ml stock solutions were made in dH₂O, and serially diluted with TC-100 culture medium to make the culture medium containing ecdysteroids at the implicit concentration shown in the figures.

2.4. RT-PCR

Total RNA was extracted using ISOGEN (Nippon gene) from BmN cells maintained without E or with 0.1 µg/ml of E for 48 hrs. The total RNA was treated with DNaseI (TaKaRa) followed by two rounds of extraction with acid phenol. One microgram of total RNA was reverse-transcribed by using an Omniscript RT kit (QIAGEN) with a dT20 primer, and then PCR was performed using Ex-taq (TaKaRa) with specific primers for detecting mRNAs from the actin-3 gene and the ecdysteroid-inducible regulatory protein genes. The PCR (96°C for 10sec, then 30 cycles of 96°C for 5sec, 60°C for 5sec, and 72°C for 15sec, and finally, 72°C for 2min) was performed using the primers listed in Table 1. As a control, PCR amplification using non-reverse-transcribed total RNA was performed in the same conditions.

2.5. Recombinant BmNPVs

Recombinant BmNPVs (rBmNPV-Bie, -d03, and -n08) were constructed as follows. First, transfer plasmids were produced by inserting a *Sac* I - *Kpn* I fragment from PGV-Bie, -d03, and -n08 into the multi-cloning site (between *Sac* I - *Kpn* I sites) of pBK-blue (Nosan corporation). Then, these plasmids were transfected with the BmNPV genomic DNA into BmN cells and the rBmNPVs were obtained according to a method described elsewhere (Maeda et al., 1985). These rBmNPVs were plaque purified at least three times. For the reporter assay, BmN cells (1×10^6) were infected with rBmNPVs at a multiplicity of infection (M. O. I.) of 1, and maintained in TC-100 medium supplemented with E (at an implicit concentration of 0, 0.001, 0.01, 0.1, 1.0, and 5.0 µg/ml) for 6 hrs. The luciferase activity in the cells was then analyzed as described above.

3. Results and discussion

3.1. Ecdysone responsive elements in the *ie1* promoter

A computer-assisted analysis using a computer program, TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCHJ.html>) (Heinemeyer et al., 1998), identified tentative target sequences for insect transcriptional regulators such as FTZ-F1 and Broad-Complex Z4 (BR-C Z4) in the *ie1* promoter sequence (GenBank accession no. **L33180**, base 116395 - 116954) (Fig. 3a). A nonameric sequence (5'-GGACCTTGT-3')

homologous (67% identity) to the binding sequence for FTZ-F1 (5'-YCAAGGYAR-3') (Ueda et al., 1992) was located in the 5' terminal one-third of the *ie1* promoter. Another nonameric sequence (5'-TCAAGGACG-3') showing 78% identity to the FTZ-F1-binding sequence was observed in the center of the *ie1* promoter sequence. In addition, three tridecameric sequences (5'-TATGTAAATAAAT-3', 5'-TGAATAAATAAAT-3', and 5'-ATAATAAAAA AAA-3') homologous to the binding sequence (5'-WWWRKAAASA WAW-3') for another ecdysone inducible transactivator of *Drosophila*, Broad-Complex Z4 (BR-C Z4) (von Kalm et al., 1994), were located in the 3' terminal half of the *ie1* promoter sequence (Fig. 3a). FTZ-F1 is known as an ecdysone-pulse-inducible transactivator (Sun et al., 1994) and BR-C is known as an ecdysone-inducible transactivator (Karim et al., 1993). Thus, these were tentative target sequences for ecdysone inducible transcriptional regulators. FTZ-F1 was first reported in *Drosophila melanogaster* as an ecdysone-inducible regulator of transcription which activates transcription from the pair-rule segmentation gene *fushi-tarazu* (*ftz*) in a sequence-specific manner (Ueda et al., 1990). The *Bombyx* homologue of *Drosophila* FTZ-F1 (BmFTZ-F1) is a member of the nuclear hormone receptor superfamily known to be expressed just before molting and pupation (Yamada et al., 2000) and has been demonstrated to transactivate the transcription from *ftz* (Ueda and Hirose, 1990). BR-C is a key regulator gene in the morphogenesis of *Drosophila*, controlling ecdysone-responsive gene expression (Karim et al., 1993). These results suggest that ecdysone is involved in the regulation of transcription from the *ie1* promoter through interaction between the regulatory proteins and these tentative target sequences. Since *ie1* is located at the top of the NPV gene expression cascade, ecdysone could affect viral gene expression and replication through its involvement in the regulation of *ie1* expression. This idea was consistent with the previous observations that the transcriptional activity of *ie1* promoter was increased to a certain extent by ecdysteroid-treatment in uninfected insect cells or fifth instar silkworm larvae transfected with a plasmid containing a luciferase gene driven by the *ie1* promoter (Zhou et al., 2002) and that the expression of a foreign protein from recombinant BmNPV was enhanced in the presence of ecdysone (Chan et al., 2002) though further experiments are required to prove the biological significance of these tentative *cis*-acting sequences.

3.2. Response to ecdysone of the *ie1* promoter and responsive element

We examined the responsiveness of the *ie1* promoter in BmN cells using a reporter plasmid, PGV-Bie, which expresses luciferase under the control of the BmNPV *ie1* promoter (-549 to +11 of transcription start site), and observed an increase of *ie1* promoter activity in response to both E and 20E at an implicit concentration of between 0.001 and 5.0 $\mu\text{g/ml}$ with maximum activation at between 0.1 and 1.0 $\mu\text{g/ml}$ for both hormonal compounds (Fig. 2). Since the ecdysone concentration in hemolymph under physiological conditions in *Bombyx*

mori larvae at the fourth molting and at pupation has been reported to reach 0.4 µg/ml and 1.2 µg/ml, respectively (Kiuchi, 1992), our results suggested that the *ie1* promoter was responsive to ecdysone in BmN cells in the range of physiological concentrations observed in the silkworm during metamorphosis. On the other hand, other promoters such as the *Drosophila hsp70* promoter, *Bombyx lysozyme* promoter, and BmNPV *p35*, *egt*, and *pe38* promoters showed no response to E at 0.1 µg/ml in this study (data not shown), suggesting that the *ie1* promoter responded to E in a sequence-specific manner. We also found that the *ie1* promoter was up regulated not only with 20E but also with E. Since the active form of ecdysteroid is 20E, BmN cells possibly have the ability to convert E into 20E just like cells of silkworm larvae. This possibility is supported by the observation that 20E-inducible small heat shock proteins were expressed following E-treatment in an insect cell line (Schneider's S3 cells) (Haass and Kloetzel, 1990).

To identify the ecdysone-responsive sequence in the *ie1* promoter, we constructed a series of truncated *ie1* promoters (Fig. 3a). BmN cells were transfected with reporter plasmids possessing a promoter construct, cultured in the presence (at an implicit concentration of 0.1 µg/ml) or absence of E, and assayed for luciferase activity. Each experiment was duplicated with good reproducibility. Values were averaged, and the means were graphed. Responsiveness in the presence of E was essentially unaltered by deletion of the 5' half of the *ie1* promoter (Fig. 3b line chart for PGV-n02, -d01, -n05, -d02, -d03, and -n07), but was drastically reduced with PGV-n08 which has an *ie1* promoter construct (n08; -214 to +11 of transcription start site) lacking the 5' terminal 6 nucleotides (nts) of the d03 promoter (-220 to +11) and was completely lost on deletion of an additional 15 nts (PGV-d04) (d04 promoter; -199 to +11), while the basal promoter activities of these plasmids were almost identical (Fig. 3b bar chart for PGV-n07, -d03, -n08, and -d04). These results suggested that the 21 nucleotides between -220 and -200 (5'-GTGTTATCGA CCTGAGATTA A-3'), especially the hexameric sequence between -220 and -215, played an important role in the response of the *ie1* promoter to E. We found that the 5' terminal tridecameric nucleotides (5'-GTGTTATCGA CCT-3') of the region showed 77% identity to the ecdysone receptor response element reported for *Drosophila* (*DmEcRE*: 5'-RG(GT)TCANTGA(CA)CY-3') (Cherbas et al., 1991) and designated as *ie1EcRE*.

In these reporter plasmids, the sequence immediately upstream of the inserted promoter sequence is "5'-GGTACCGAGC TC-3'" and did not generate any artificial EcRE-like structure due to cloning. The structure of *ie1EcRE* was characterized by an imperfect palindrome similar to some EcREs such as *hsp27* and *Fbp1* of *Drosophila* (Antoniewski et al., 1993), and *Sgs-4* of *Drosophila* (Lehmann and Korge, 1995). These observations supported that *ie1EcRE* is the only ecdysone-responsive element of the *ie1* promoter in BmN cells. The nucleotide sequence of *ie1EcRE* differed from that of *DmEcRE* at positions -5, -2,

and +1 (Fig. 3a small italic letters). Previous studies showed that the mutation of *Drosophila melanogaster hsp27* EcRE at these positions reduces its affinity for EcR (Antoniewski et al., 1993; Ozyhar and Pongs, 1993), suggesting a difference in nucleotide sequence preference between *ie1*EcRE and *Dm*EcRE or the involvement of some unexpected ligand. Direct testing of these hypotheses awaits further experiments.

3.3. RT-PCR-based detection of ecdysone-inducible transactivator gene transcripts in BmN cells

The *ie1*EcRE, a tentative BmEcR-binding sequence, was thus the only ecdysone-responsive element of the *ie1* promoter in BmN cells, though there were several sequences homologous to the binding sequence for the other ecdysone-inducible transactivators, BmFTZ-F1 (Crossgrove et al., 1996) and BR-C (Li et al., 1994). We investigated the expression of the six genes which are components of EcR (BmEcR (Swevers et al., 1995), BmUSP (BmCF1; (Swevers et al., 1996; Tzertzinis et al., 1994))), BmFTZ-F1 (Ueda and Hirose, 1990), BmMBF1 and BmMBF2 (Li et al., 1994), and BR-C (Ijiro et al., 2004). As shown in Fig. 4, an RT-PCR demonstrated that all genes tested were constitutively expressed in BmN cells and showed no increase of mRNA as a ratio against that of *actin-3* in the presence of E. The results were verified by the observation that there was no amplification when total RNA was amplified without reverse transcription (Fig. 4 RT-). Among these gene products, only the “EcR-USP heterodimer” is known as a ligand (ecdysone)-specific activator which can activate the expression of target genes with EcRE in the presence of ecdysone (Koelle et al., 1991; Swevers et al., 1996), which might explain why only *ie1*EcRE is functional in BmN cells where the expression of transactivators was not increased by the treatment with E. However, the expression of FTZ-F1 (Sun et al., 1994) and BR-C (Reza et al., 2004) is strictly regulated in silkworms, where the tentative responsive elements for BmFTZ-F1 and BR-C may act as ecdysone-responsive elements *in vivo*.

Autographa californica NPV (AcNPV) is closely related with BmNPV (Gomi et al., 1999). The *ie1* promoter of AcNPV shows 96.6 % identity with that of BmNPV (19 mismatches in 560 nucleotides). In 6 tentative ecdysone responsive elements, the sequence of *ie1*EcRE and BmFTZ-F1 was conserved completely while the 4 remaining sequences have one mutation each (Fig. 3a lower-case letters), which is consistent with speculation that *ie1*EcRE has an important role in viral replication and predicts the functional importance of the BmFTZ-F1 element.

3.4. Responsiveness of the *ie1* promoter in the viral genome context

To examine whether *ie1*EcRE could be responsive to ecdysone in the context of the viral genome, we constructed three recombinant BmNPVs (rBmNPV-Bie, -d03, and -n08) which

contain a luciferase gene driven by the Bie (full length) promoter or a truncated (-d03 and -n08 promoter constructs) *ie1* promoter at the polyhedrin gene locus. BmN cells were infected with each recombinant BmNPV, cultured with various concentrations of E for 6 hrs, and assayed for luciferase activity.

As shown in Fig. 5, rBmNPV-Bie and -d03 were responsive to E while rBmNPV-n08 was not, indicating that the response to E of the *ie1* promoter in the viral genome is also dependent on *ie1*EcRE in BmN cells. These results suggested that *ie1*EcRE also acts as an ecdysone-responsive element for the *ie1* promoter during viral replication. However, our results don't exclude the significance of other tentative ecdysone-responsive elements in different cells and organs in which the expression of transactivators other than EcR could be responsive to E.

4. Conclusion

Our results indicated that *ie1*EcRE is the ecdysone responsive-element of the *ie1* promoter in BmN cells and responds equally to E and 20E (Fig. 2). Judging from the finding that 20E has 1000-fold greater affinity for BmEcR/BmUSP than does E (Makka et al., 2002), BmN cells have the ability to convert E into 20E, resulting in activation of the *ie1* promoter through the binding of 20E to the BmEcR/BmUSP heterodimer via *ie1*EcRE.

Viruses are obligate parasites whose replication is highly dependent on the host cell machinery and whose function is tightly controlled by the physiological conditions of the individual. This means that viral replication could be strongly affected by the physiological condition of the host. Insect life cycles are characterized by drastic changes in morphology and physiology, what is called metamorphosis, which occur with the programmed disruption and/or differentiation of internal structures (Swevers et al., 1996). With such intensive physiological change, insects seem not to be a profitable host for replicating viruses. In fact, it was reported that in *Heliothis virescens* larvae infected with AcMNPV, the occlusion body yield from insects whose development is completely arrested is more than fourfold greater than that from insects who have initiated prepupal development (O'Reilly et al., 1998). This led us to speculate that insect viruses have developed strategies to achieve maximal multiplication dealing with such marked changes in physiological conditions. This is the first report to identify an ecdysone-responsive element in a baculoviral gene promoter.

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Figure Legends

Fig. 1 Construction of reporter plasmids with truncated *ie1* promoters

Reporter plasmids were constructed as follows. First of all, the *ie1* promoter sequence with about 550 nucleotides was obtained from pDIEp-Luc (Kojima et al., 2001) by digestion with *Eco* RI, blunt-ended, and treated with *Sac* I. The DNA fragments containing the *ie1* promoter sequence were cloned between the *Hin* dIII / blunt and *Sac* I sites in PGV-p (Toyo Ink MFG. Co., Ltd) and the resultant plasmid was designated as PGV-Bie. The reporter plasmids possessing the 5'-terminal truncated *ie1* promoters were then constructed. In brief, each truncated *ie1* promoter sequence (n02, n05, n07, n08, n09, n11, d01, d02, d03, d04, d05, d06, t01, t02, and t03) (Fig. 3a) was amplified by PCR using PGV-Bie as a template and specific primers and the *ie1* promoter in PGV-Bie was then replaced using the restriction sites *Eco* RI and *Sac* I (PGV-n02, -n05, -n07, -n08, -n09, -n11, -d01, -d02, -d03, -d04, -d05, -d06, -t01, -t02, and -t03). Thick arrows and thin arrows show the luciferase ORF and the PCR-primers, respectively. Open boxes and gray boxes show the *ie1* promoters and the poly(A) signal, respectively. Abbreviations; E: *Eco* RI, H: *Hin* dIII, S: *Sac* I, Blunt: blunt-ended with T4 polymerase.

Fig. 2 Response of the *ie1* promoter to ecdysone

BmN cells were transfected with the reporter plasmid PGV-Bie and luciferase activity was analyzed. The activity of the *ie1* promoter at an implicit concentration of E (a) or 20E (b) presented in the figures is shown as relative luciferase units (luciferase activity in the cells at each concentration / at a concentration of 0). Data represent the mean \pm SD from triplicate experiments.

Fig. 3 Analysis of the ecdysone-responsive element

a) Tentative regulatory protein-binding sequences in the *ie1* promoter. The -549 to +11 region of the BmNPV *ie1* promoter sequence used in this study is presented. The binding motif for the BmFTZ-F1 (open box) and Broad-complex Z4 (BR-C Z4, hatched box), TATA-box (underlined), and CAGT motif (underlined) are indicated. The 5'-terminus of the Bie promoter and truncated *ie1* promoter constructs used in (b) are indicated by arrows. *DmEcRE* (*ie1EcRE*) is indicated by a thick underline. Lower-case letters indicate nucleotides not conserved between BmNPV and AcMNPV. Small italic letters indicate nucleotides not conserved between *ie1EcRE* and *DmEcRE*.

b) Responsiveness of truncated *ie1* promoter constructs to ecdysone. The bar chart shows the activity of *ie1* promoter constructs in the absence of E. Data are represented as relative luciferase units against the full-length *ie1* promoter (PGV-Bie). A line chart shows the

activation of each promoter construct by E as relative luciferase units (luciferase activity in the presence of E / in the absent of E).

Fig. 4 RT-PCR-based detection of ecdysone inducible transactivator gene expression
Total RNA from normal (E-) or E-treated (at an implicit concentration of 0.1 $\mu\text{g/ml}$, 48 hrs) (E+) BmN cells was used for RT-PCR after reverse transcription (RT+) or without reverse transcription (RT-). All genes tested were amplified and shown to be expressed even in normal conditions. Abbreviations; A3: cytoplasmic *actinA3*, EcR: BmEcR, CF1: BmCF1, FTZ: BmFTZ-F1, MBF1: BmMBF1, MBF2: BmMBF2, BR-C: broad-complex.

Fig. 5 Ecdysone-evoked response of the *ie1* promoter in recombinant BmNPV
The activity of *ie1* promoter constructs in the recombinant virus at the implicit concentration of E presented in the figure is given as relative luciferase units (luciferase activity in the cells at each concentration / in concentration of 0). Data represent the mean \pm SD from triplicate experiments.

Table 1

Table 1 Primers used for RT-PCR

Target gene	Genbank ID	length	sequences
Actin A3	<u>U49854</u>	416 bp	5'-cattcgcatc tacgtacttg-3' 5'-gggtcatctt ctctctgttg-3'
BmEcR	<u>D43943</u>	220 bp	5'-gatgacgac ttaacgggcc agttg-3' 5'-ggccatgccc gccttgccgt agttg-3'
BmCF1	<u>U06073</u>	206 bp	5'-gccaggeatg acgctgcacc gcaac-3' 5'-tctttctcg aagaacgtcc acttc-3'
BmFTZ-F1	<u>D10953</u>	206 bp	5'-ggtaggggta tcgtaaact gaag-3' 5'-gtctgggtag gtcctggcc tgcac-3'
BmMBF1	<u>AB001078</u>	217 bp	5'-aaagacctg ccactaaaat ttgtg-3' 5'-gcaataaatg aatcggagta aagtcttc-3'
BmMBF2	<u>D70818</u>	205 bp	5'-actgtgtac aagaccaatg caacc-3' 5'-cctgtcgtc tcatgcgta tcttg-3'
BmBR-C	<u>AB113088</u>	200 bp	5'-tcagcttga gccctattt cagag-3' 5'-gtgtgagacc ggagacacgg agcac-3'

Fig. 1

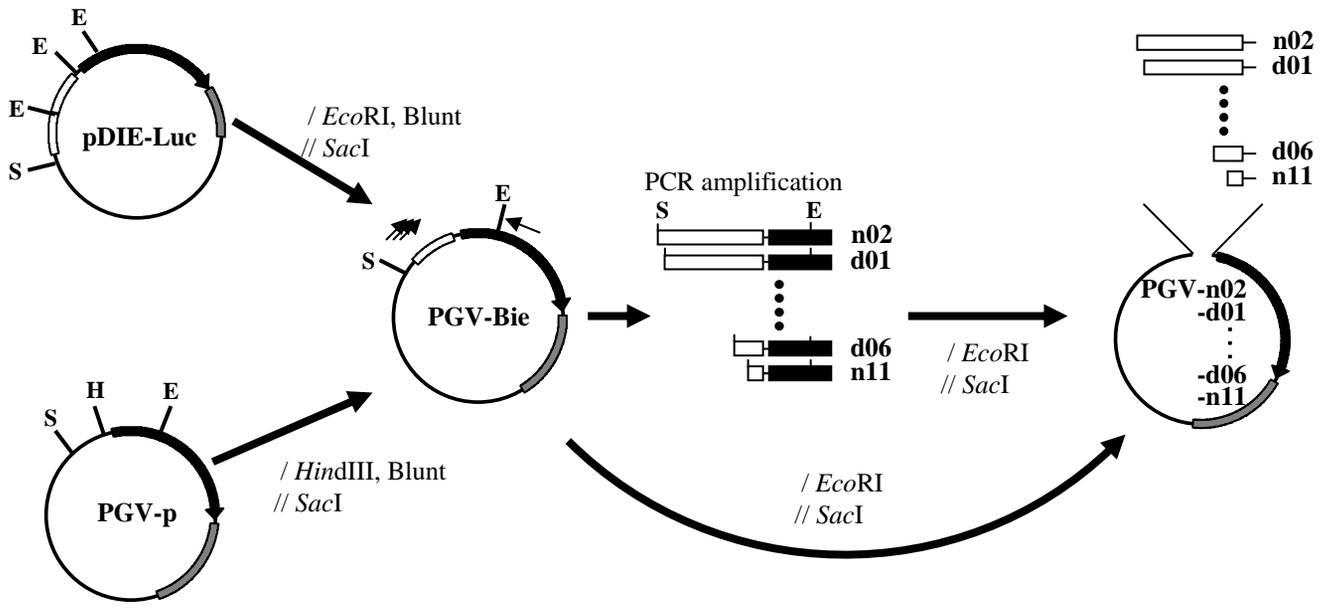
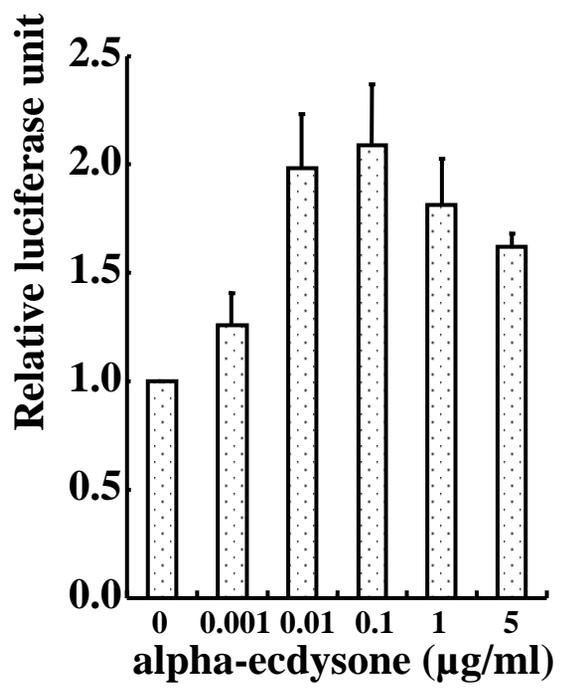


Fig. 2

a)



b)

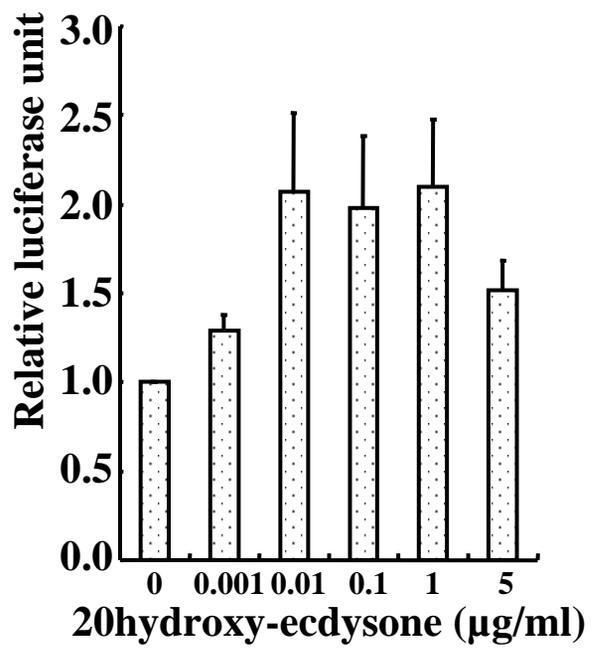


Fig. 3

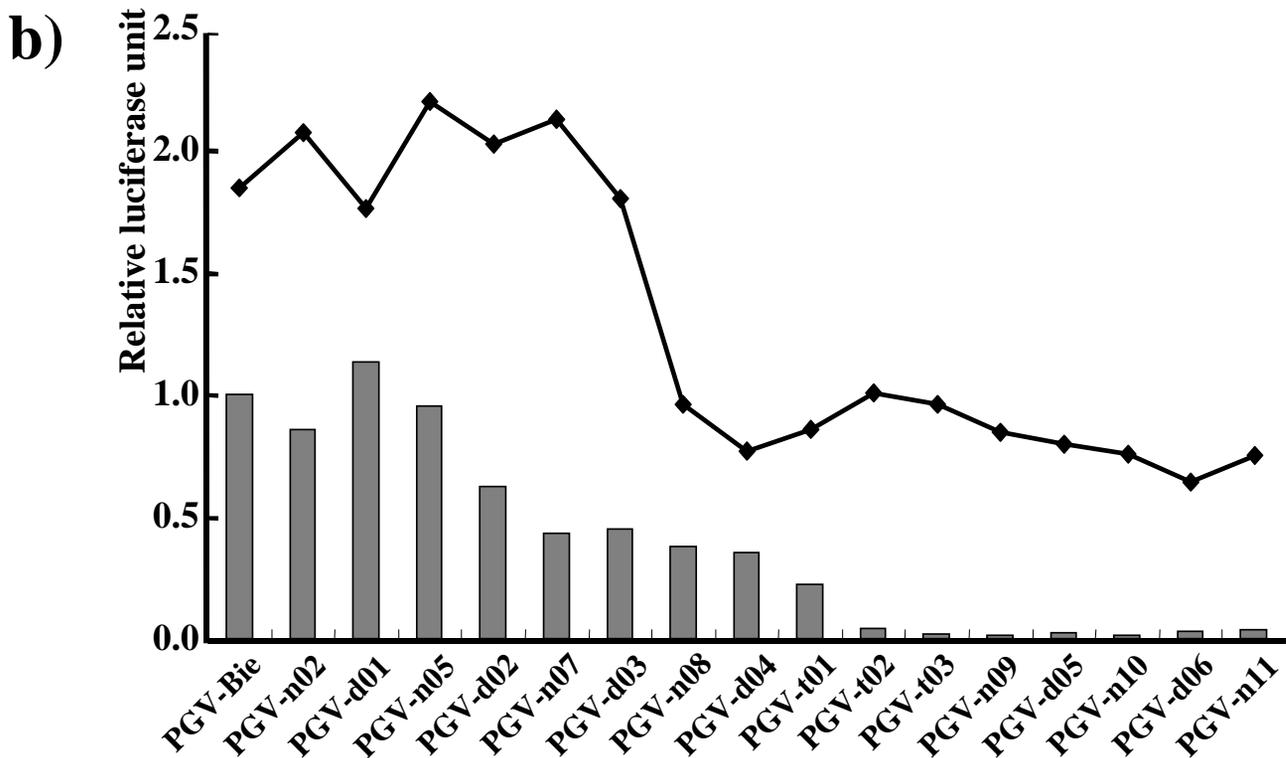
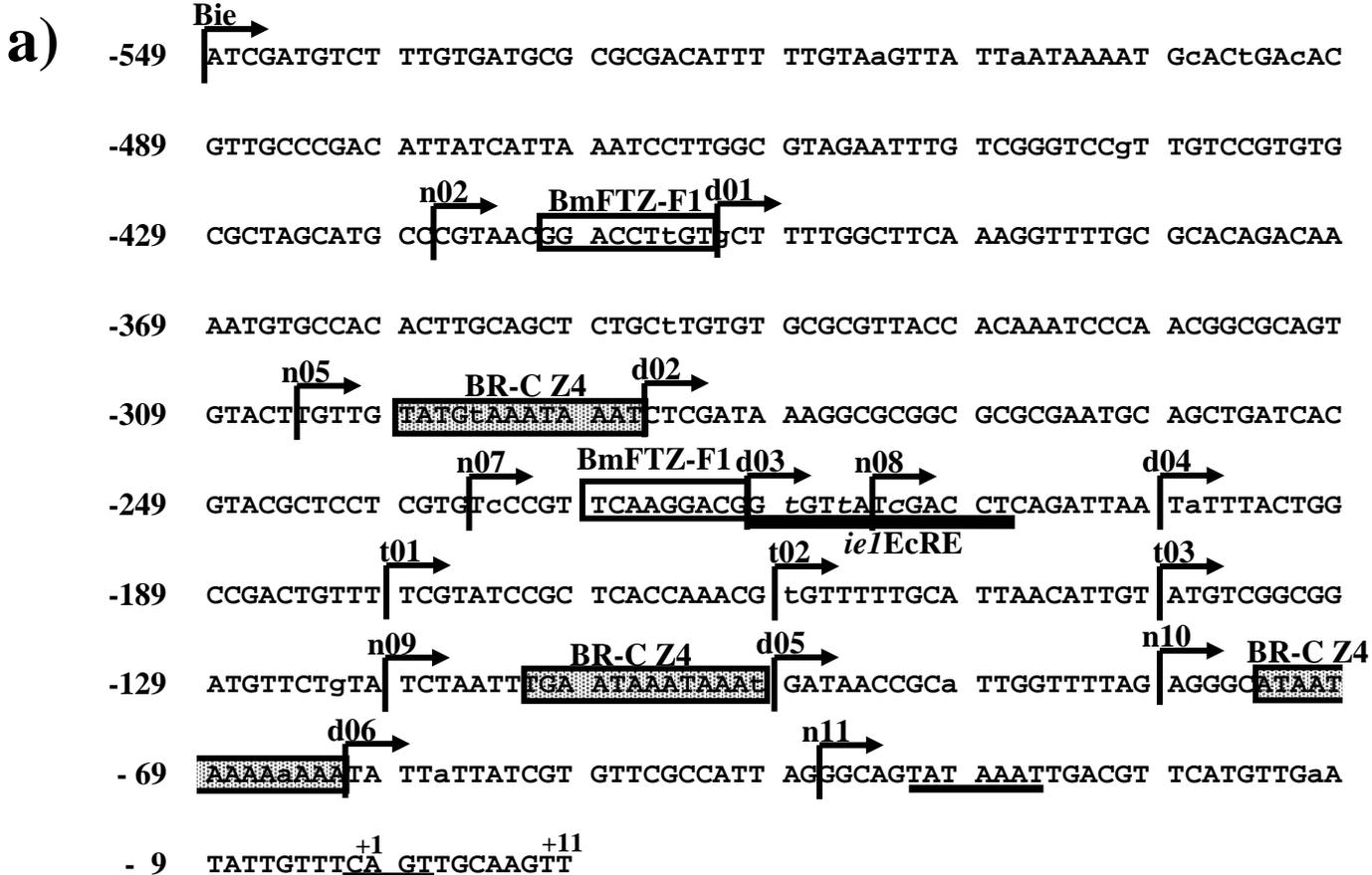


Fig. 4

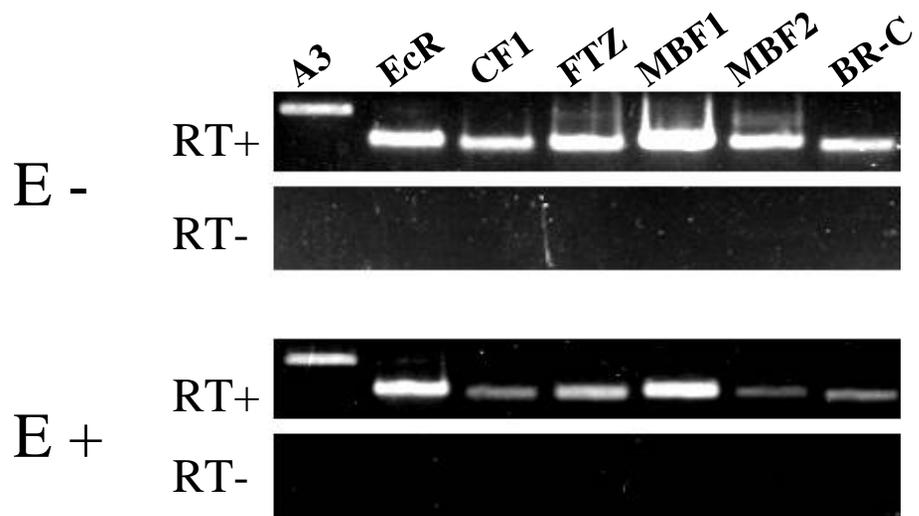


Fig. 5

