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Tenebrio molitor antifreeze protein gene identification and regulation

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Abstract

The yellow mealworm, *Tenebrio molitor*, is a freeze susceptible, stored product pest. Its winter survival is facilitated by the accumulation of antifreeze proteins (AFPs), encoded by a small gene family. We have now isolated 11 different AFP genomic clones from 3 genomic libraries. All the clones had a single coding sequence, with no evidence of intervening sequences. Three genomic clones were further characterized. All have putative TATA box sequences upstream of the coding regions and multiple potential poly(A) signal sequences downstream of the coding regions. A TmAFP regulatory region, B1037, conferred transcriptional activity when ligated to a luciferase reporter sequence and after transfection into an insect cell line. A 143 bp core promoter including a TATA box sequence was identified. Its promoter activity was increased 4.4 times by inserting an exotic 245 bp intron into the construct, similar to the enhancement of transgenic expression seen in several other systems. The addition of a duplication of the first 120 bp sequence from the 143 bp core promoter decreased promoter activity by half. Although putative hormonal response sequences were identified, none of the five hormones tested enhanced reporter activity. These studies on the mechanisms of AFP transcriptional control are important for the consideration of any transfer of freeze-resistance phenotypes to beneficial hosts.

Keywords: Gene family; Hormone regulation; Intron; Promoter identification; Response element

1. Introduction

The life cycle of yellow mealworm beetle, *Tenebrio molitor*, can encompass four months to several years, depending on the number and duration of the instars, and this, in turn, can be influenced by environmental conditions. In colder climates, larvae overwinter in a quiescent state, possibly in part regulated by juvenile hormone (JH), (Chippendale, 1984; Connat et al., 1984). *T. molitor* is freeze-susceptible and its survival is facilitated by the accumulation of antifreeze proteins (AFPs) (Ramsey, 1964; Tomchaney et al., 1982). After 30 years of effort by various groups, the hyperactive *T. molitor* AFP, TmAFP, was purified and its corresponding cDNA cloned and expressed in recombinant bacteria (Graham et al., 1997).

Mass spectral analysis of *T. molitor* hemolymph showed multiple AFP isoforms (Liou et al., 1999). Conceptual translation of 17 cDNA clones yielded 8 different TmAFP isoforms, all of which encoded 84, 96, or 120 amino acids, consisting of 5, 6 or 7 repeats respectively, of a 12-residue sequence, Thr–Cys–Thr–X–Ser–X–X–Cys–X–X–Ala–X (where X can be any residue). The first three residues of each amino acid repeat, Thr–Cys–Thr, are stacked and form a flat surface of the β-helix, which is complementary to the surface of ice (Liou et al., 2000). A related insect, the fire-colored beetle, *Dendroides canadensis*, has a family of similar antifreeze proteins that have 40–66% amino acid identity with TmAFP isoforms and share the first three residues of each repeat (Duman et al., 1998; Li et al., 1998).

TmAFP transcripts are found throughout larval development and peak in the final larval instar; levels decrease at pupation and further again in the adult stage (Graham et al., 2000). Larval-stage message levels could be experimentally increased by low temperature, desiccation or starvation, but not by photoperiod. Similarly, transcripts encoding the *T. molitor* desiccation stress protein 28 (dsp28) are also developmentally regulated and levels increased in response to low temperatures as well as desiccation (Graham et al., 1996a,b). Thus there is a

Abbreviations: AFP, antifreeze protein; JH, juvenile hormone; EcRE, ecdysone response element; JHRE, juvenile hormone response element.

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correlation between development, various stresses and low temperature survival.

The relationship between hormone titer and overwintering, however, is less clear. Overwintering beetle larvae, *T. molitor* and *Psacothea hilaris,* show high JH titers (Munyiri and Ishikawa, 2004; Xu et al., 1992). In *D. canadensis*, antifreeze activity increases by either low temperature or JH, but polyol synthesis, which is important for overwintering, is not induced by JH (Horwath and Duman, 1983; Andorfer and Duman, 2000). In the gall fly, *Eurosta solidaginis,* glycerol levels were not increased by JH analogue application (Hamilton et al., 1986). In contrast, cryoprotectant titers were stimulated by JH analogues in diapausing rice stem borers, *Chilo suppressalis* (Tsumuki and Kanehisa, 1981; Li et al., 2002).

It is not known how TmAFP genes are regulated, although their regulation may be complex, with developmental as well as environmental influences. In the moth, *Choristoneura fumiferana*, the only other insect, apart from the beetles, in which AFP sequences have been isolated, the AFP genes appear to be developmentally regulated and not directly controlled by environmental stresses such as photoperiod or temperature (Doucet et al., 2000). In the winter flounder fish, Type I AFP genes are regulated by photoperiod with longer days inducing the production of growth hormone that, in turn, inhibits AFP gene transcription in concert with a protein factor (Fourney et al., 1984; Fletcher et al., 1989). Because it is complex, an understanding of the transcriptional regulation of *Tenebrio* AFP can only be achieved by first isolating some of the corresponding genes. We report here this accomplishment as well as our initial experiments to understand AFP gene regulation.

2. Materials and methods

2.1. Insect rearing

T. molitor were maintained in our laboratory in the dark at 22±1 °C, and provided with wheat bran and moist papers, as previously described (Graham et al., 2000). Large, near-last stage larvae were collected from cages for DNA isolation.

2.2. Libraries, screening and genomic clones

Three *T. molitor* genomic libraries were used. One contained Mbo I-partially digested DNA in the BamH I site of EMBL 3 (Bouhin et al., 1993; kindly provided by Dr. H. Bouhin). Two other libraries were constructed from late larval DNA. A λZAP II genomic DNA library and a pBeloBAC11 BAC genomic DNA library were constructed using standard methods (Bio S & T Inc., Montreal, Canada). Libraries were hybridized with a ^{32}P-labelled TmAFP (2–14) cDNA (Liou et al., 1999) and positive clones were either in vivo excised to pBluescript (λZAP II vector manual, Stratagene, La Jolla, California), or subcloned into pBluescript SK II (BAC clones). All resulting clones were sequenced on both strands (3100 Genetic Analyzer, Applied Biosystems).

The first two λZAP II clones sequenced (designated BST1 and BST2) were used to design two sets of specific primers flanking the AFP coding region (BST1 forward primer with an EcoR I site, 4302PL1: 5′-CCG GAA TTC CCC AAA ATG AAA CCG GAA A; BST1 reverse primer with a Hind III site, 4302PR1: 5′-TGG ATA TAATACAGG TTA AGC TTA C ; BST2 forward primer with an EcoR I site, 3068PL1: 5′-CCGGAA TTC GGC AGT CGA TCG AAG TAA AC; BST2 reverse primer with a Hind III site, 3068PR1: 5′-TGG ATA GAA TAC AGG TTA AGC TTA C). A degenerate forward primer with an Xho I site spanning the initiation codon was also synthesized (5′AFPXhoI: 5′-CCG CTC GAG ATG GCA TTC AAI ACI TGT ICT TTT A). Several reactions using 100 ng of the *T. molitor* genomic DNA template and combinations of these primers (4302PL1/4302PR1 and 3068PL1/3068PR1) were done (95 °C for 5 min; 3 cycles of 94 °C for 40 s, 52 °C for 40 s, 72 °C for 1 min; 25 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 1min; 72 °C for 10 min) and the products were digested with EcoR I/Hind III and subcloned into pBluescript SK II. After amplification (with 5′AFPXhoI/4302PR1 and 5′ AFPXhoI/3068PR1 at 95 °C for 10 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min), the products were digested with Xho I/Hind III and subcloned into pBluescript SK II. All clones were sequenced twice.

Two EMBL3 specific primers (EMBL3left: 5′-CTT ATG CCC GAG AAG ATG TTG AGC AAA CTT A; EMBL3right: 5′-TCA TTA CTG AAC ACT CGT CCG AGA ATA ACG A) and two TmAFP degenerate primers (TmPR1: 5′-CAK YGG CAA TAA CAC TCG GTA CAC AA; TmPR2: 5′-TAC ACA ARC ACA TAA CTA TAA CTG CT; where K=G+T, R=A+G, Y=C+T), designed by alignment of the cDNA sequences of five isoforms 1–3, 2–14, 3–14, C-9, D-16 (Liou et al., 1999), were used together (EMBL3left/TmPR1 and EMBL3right/TmPR1) under the same PCR conditions (95 °C for 5 min; 30 cycles of 94 °C for 45 s, 61 °C for 45 s, 72 °C for 3.5 min; 72 °C for 10 min), and with the EMBL3 library as a template. The amplified products were then used as templates for nested PCRs (EMBL3left/TmPR2 or EMBL3right/TmPR2 at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2.5 min; 72 °C for 10 min). The nested PCR products were then directly ligated into a T/A cloning vector (PCR2.1; Invitrogen, Burlington, Canada), plasmid DNAs were isolated (Qiagen miniprep kit, Qiagen, Mississauga, Canada) and subsequently sequenced on both strands.

Sequences of all the *T. molitor* AFP genomic clones were aligned, conceptually translated and compared to previously isolated cDNA clones using web-based algorithms. One of these products, from the EMBL3 library and including ~1000 bp upstream of the TmAFP coding region was designated B1037 and used for 5′ region regulatory analysis.

2.3. 5′-upstream region analysis

B1037 in PCR2.1, obtained from the 5′ upstream region of a TmAFP genomic sequence, was used with a promoterless vector, pGL3-Basic (PGB, Promega), and an intron-containing PGB to make a series of 5′-sequence-containing constructs. In

order to add an exotic intron to PGB, a 245 bp sequence derived from the first intron in the hsp70 family member (GenBank accession number: AY178988) from the migratory locust, *Locusta migratoria*, was PCR-amplified using locust hsp70 specific primers (Lmhsp70Intron1HindIII: 5′-AAT TCA AGC TTT GTG CCT GCA ATA TAC GCT AAC and Lmhsp70-Intron1NcoI: 5′-CTT TTA CCC CAT GGT TCT GGA GAC AAA AAT TAC AAG AAA C). The locust hsp70 sequence in PCR2.1 was the template for PCR (94 °C for 5min; 3 cycles of 94 °C for 45 s, 57 °C for 40 s, 72 °C for 30 s; 25 cycles of 94 °C for 40 s, 68 °C for 40 s, 72 °C for 30 s; 72 °C for 10 min). The amplified products were Hind III/Nco I digested and cloned into pGB and designated GLBin. The full-length sequence of B1037 was cloned into the intron-containing vector, GLBin, by Hind III/Xho I digestion of DNA and vector, and the new construct was designated GLBinB4. Subsequently, this plasmid DNA was digested with Hind III/Nco I to remove the intron, and the plasmid was treated with T4 DNA polymerase to generate blunt ends and self-ligated, according to the manufacturer's instructions (Gibco, Burlington, Canada). The intronless plasmid was named GLBB46. A partial sequence of B1037 was cut out by Mlu I/Hind III and subcloned to PGB, and designated PGBMH1. Three smaller constructs were made using PCR. Reactions of PGBBMHA1 (BMluIA: 5′-ACG ACG CGT TGC CCT AAA GAG TAC AAT ATA AT and BHindIIIA: 5′-TCC CAA GCT TAA AGT ATG GCG AAA CCT CTA A), PGBBMHAC2 (BHindIIIA/BMluIC, BMluIC: 5′- CAC AAC GCG TGA AAG GTA GGC GGA TCT GG), PGBBMHC1 (BMluIC/BHindIIIC, BHindIIIC: 5′-GTG AAA GCT TTA ACT CAT TTG TGT ACC ATG CTA C) were performed using B1037 DNA template and conditions of 94 °C for 5 min, 94 °C for 25 s, 55 °C for 25 s, 72 °C for 25 s, 28 cycles; 72 °C for 10 min. The constructs were designated PGBBMHA1, PGBB-MHAC2, PGBBMHC1. Lastly, two more primers (BMluIB: 5′-GAG TAC GCG TTG TTG AGA GGT TTC GCC ATA C; BHindIIIB: 5′-GTG CAA GCT TGC GTC ATG TGA ATA AAA CGA TAA C) were synthesized and PCR accomplished using the GLBinB4 template at 94 °C for 5min, 94 °C for 25 s, 55 °C for 25 s, 72 °C for 25 s, 28 cycles; 72 °C for 10 min. This full-length amplification product with an internal Mlu I site was digested with Mlu I/Hind III, cloned into PGB, and designated PGBBT1. Partial digestion of PGBBT1 was done and the DNA was ligated again, with the result that a new construct with two extra Mlu I/Mlu I fragments tandemly added to the 5′ end of the BT1 insert, and designated PGBBMHB74. These constructs are diagramed in the Results section (Fig. 2).

Since no *Tenebrio* cell line was available, a hormonally responsive spruce budworm CF-203 cell line (Kethidi et al., 2004; Hu et al., 2004; kindly provided by G. Caputo and Q. Feng) was used for transfection. The cells were cultured in HyQ SFX-INSECT medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum and kept at 23 °C and seeded at 4×10^5 cells/well in 6-well tissue culture plates. The next day, 1.5 μg of each plasmid and 0.15 μg internal control plasmid, pRLA5 (consisting of a *C. fumiferana* AFP gene promoter sequence), were mixed and made up to 100 μl with serum-free medium. In a separate tube 10 μl of Lipofectin (Invitrogen) was mixed with 90 μl of serum-free medium. The two mixtures were left for 30 min at room temperature and then combined and incubated for another 10 min. Serum-free medium (800 μl) was pipetted into the transfection mixture, and then added to each well. With occasionally shaking, after 24 h, the transfection mixture was removed from the plate's wells and 2 ml of fresh growth medium supplemented with 10% fetal bovine serum, was added to each well. After 72 h, the cells were centrifuged at 12 000 ×*g* for 1 min and the supernantant discarded. Lysis buffer (250 μl) was added to cell pellet and gently shaken for 30 min at room temperature. After re-centrifuging (1 min at 12 000 ×*g*) and collecting supernatants, luciferase activities were determined with the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega).

For all the constructs, three replicates of each transfection were performed. One-way analysis of variance (ANOVA) was done using GraphPad software (San Diego, USA).

An internet-based computer program TRANSFAC at (www.biobase.de) was used to search for transcription factor binding sites.

2.4. Hormone-response tests

Two TmAFP gene 5′-upstream region constructs (GLBinB4 and GLBB46) and one TmAFP core promoter construct (PGBBT1) were selected for analysis. CF-203 cells were cultured and transfected as previously described with either 1.5 μg of these constructs (GLBinB4 or GLBB46 or PGBBT1) along with 0.15 μg pRLA5. After 24 h, with occasional shaking, the transfection mixture was removed from plate wells and 2 ml of fresh growth medium supplemented with 10% fetal bovine serum (containing hormones or hormone free controls) were added to each well. Hormone treatments with GLBB46 and GLBBinB4 were selected as previously suggested (Palli et al., 1995; Kethidi et al., 2004) and done at final concentrations of 25 μM 9-cis retinoic acid, 100 μM methoprene (a JH analogue), 1 μM JH I, 1 μM JH III, 1 μM 20-hydroxyecdysone. Hormone treatments with PGBBT1 and GLBB46 were done using serial dilutions of 20-hydroxyecdysone at 0, 0.1, 0.5, 1.0, 10, 50 μM. Cells were collected at 24 h after adding the hormone, centrifuged at 12000 ×*g* for 1 min and the supernantants discarded. Lysis buffer (250 μl) was added to the collected cells and gently shaken for 30 min at room temperature. After recentrifuging (1 min at 12000 ×*g*), supernatants from the cell lysate were assayed for luciferase activity as previously described. Three replicates of each transfection were performed. One-way analysis of variance (ANOVA) was done using GraphPad software (San Diego, USA).

3. Results

3.1. Genomic DNA library screening and sequence analysis

When the genomic libraries were hybridized with the ^{32}P-labelled TmAFP cDNA fragment, four genomic clones

were obtained (BST1, BST2, and BST3 from the λZAP II library and 7KSK4, representing two identical clones from the BAC library). All four clones, BST1 (4302 bp), BST2 (3068 bp), BST3 (4578 bp) and 7KSK4 (5682 bp), when conceptually translated contained a single coding sequence. When aligned with the known isoforms, they appeared to be well conserved with 6 or more 12-residue repeats (starting with Thr–Cys–Thr, with the first repeat sharing the Ala–Cys–Thr variation, that has been observed in various TmAFP cDNA clones). All of the λZAP II genomic clones had 6 repeats and the BAC clones had 7 repeats. These sequences have been deposited in GenBank (accession numbers DQ224365 to DQ224368), and are not shown here.

Polyadenylation signal sequences (AATAAT, AATATA or AATAAA) were identified at positions 19, 148, and 423 downstream of the stop codon, in all the λZAP II clones. As well, the λZAP II clones contained 5′ upstream regions (1351, 57, 2501 bp for BST1, BST2, BST3, respectively). BST1 and BST3 contained a TATA box consensus sequence (TATAAA) 563 and 500 bp upstream of the ATG codon, respectively, with a CAAT box consensus sequence (CCAAT) 247 bp further upstream of the putative TATA-boxes. The two identical BAC clones were larger with 4233 bp of putative 5′-upstream sequence, with a possible TATA-box, 226 bp upstream of ATG codon and a CAAT-box 306 bp further upstream. The 4 genomic clones are diagramed in Fig. 1.

In addition to these four different genomic sequences, 7 TmAFP 5′-genomic sequences with a portion of the coding sequence were obtained from the EMBL3 library using a nested primer PCR strategy. Sequence analysis of these 7 clones (GenBank Accessions number DQ176767 to DQ176773) showed that C951, D950, E827, and F951 had very well conserved 5′-upstream sequences; A1139, B1037 and G1267 had different 5′-upstream sequences. All except A1139, had a TATA box consensus sequence (2 each in B1037, C951, D950 and E827; 1 in F951; 4 in G1267), and B1037 and G1267 also had a CAAT box consensus sequence. It should be noted that if TATATA is an acceptible alternative to TATAAA in *T. molitor*, then TATA boxes for some of these TmAFPs might be slightly closer to the coding region than indicated, because TATATA sequences were identified between the TATAAA and the transcriptional start sites. Since B1037, appeared to encode a studied isoform, 2–14, it was selected for promoter analysis.

Comparison of the 11 genomic sequences with known hormonally regulated genes revealed no sequence elements closely similar to either the *Drosophila melanogaster* hsp27 gene ecdysone response element (Dmhsp27 EcRE, Grad et al., 2002), the JH response element (JHRE, Zhou et al., 2002) of the *L. migratoria* JH inducible gene, *jhp21*, or the JHRE of *C. fumiferana*, JH esterase gene (Kethidi et al., 2004). However, sequences similar to the putative JHRE of *dsp28* from *Tenebrio* (Graham et al., 1996b) were noted (Table 1) as well as some similarity to a short sequence upstream of a cuticle protein gene (Rondot et al., 1998).

3.2. TmAFP gene promoter analysis

When TmAFP 5′-upstream sequences ligated to a luciferase reporter were transfected to CF-203 cells, luciferase activities directed by the *Tenebrio* 5′-sequences, were compared to those from a promoterless vector, PGB (Fig. 2). GLBB46 and GLBinB4 (F and H, respectively in Fig. 2) constructs both contained the entire 5′-sequence (B1037) from the genomic

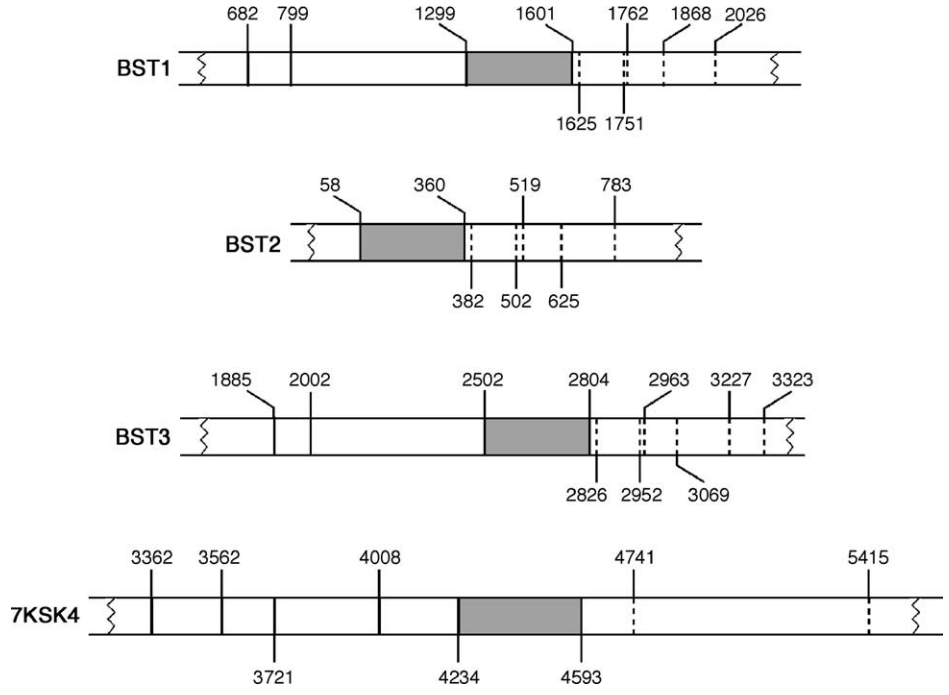

Fig. 1. Gene organization of BST1, BST2, BST3, and 7KSK4 four genomic clones (not drawn to scale). Grey boxes represent coding sequences, solid lines represent TATA box consensus sequences, dotted lines represent poly(A) signal sequence. Numbers are nucleotide locations in the respective clones.

Table 1
Sequences found in the 5′-region, upstream to TmAFP coding regions, which are similar to the putative JHREa and JHREb reported from *dsp28*

DSP28	Putative JHREa	Putative JHREb
	AAAGTTGCGTAGCTTT -501	AAGAGATAGCAATATAA -305
TmAFP		
A1139	AAAGGTTGAGAAACGCT -628	
B1037		AGGTGTTGTGCAATATA -637
C951	TTGAGTTACATACCTTT -691	
D950	TTGAGTTACATACCTTT -690	
E827	TTGAGTTACGTACCTTT -690	
F951	TTGAGTTACATACCTTT -710	
G1267	AAAAACTGATAGCTTT - 882	
BST1		ATGACTTAGCATCATAA -819
BST3		ATAAGGTACCTATATAG -88
7KSK4		ATGTAATAACAATATTA -741

The underlined bases are dyad symmetrical ones. The numbers on the right of sequences represent base pairs upstream of ATG.

Table 2
Effect of hormones on luciferase reporter activity directed by 5′-upstream region sequences (with or without an exotic intron) of the TmAFP gene

Hormone treatment	GLBB46 activity ratio	GLBBinB4 (with Lmhsp70 intron) activity ratio
No hormone	1	1
25 μM 9-cis retinoic acid	0.97±0.02	0.43±0.04
100 μM methoprene	0.94±0.02	0.75±0.05
1 μM juvenile hormone I	0.96±0.07	0.66±0.07
1 μM juvenile hormone III	0.98±0.06	0.35±0.04
1 μM hydroxyecdysone	1.02±0.07*	0.43±0.07
No promoter, no hormone	0.71±0.07	0.16±0.03

* Data from a separate experiment.

sequence, cloned into PGB. However, in GLBinB4, an hsp70 intronic sequence from *L. migratoria* was also included, and it showed a 6.1-fold increase in luciferase activity over the promoterless construct (construct A, Fig. 2, $P<0.001$). Whereas a 1.4-fold increase in luciferase activity was seen when the intron not included. Deletion constructs consisting of only 5′ or 3′ portions of the promoter region (constructs B–D and E) showed no increase in luciferase compared to promoterless plasmids. However, PGBBT1 (construct I, Fig. 2) that contained the mid portion of the promoter and was the shortest among the 8 constructs, showed a 9.6-fold increase in reporter activity over the controls ($P<0.001$). When truncated additional copies of the Mlu I gene fragment were included in the construct, activity was decreased by half (compare constructs PGBBT1 and PGBBMHB74, I and G in Fig. 2, $P<0.001$). Taken together, these results suggest that the *T. molitor* sequence used to derive the plasmid that was the most active in the transfection assays represents 143 bp of an AFP core promoter. This sequence contains a TATA box (717 to 722 in 1037 bp sequence).

3.3. Hormone regulation

There was no significant change in luciferase reporter activity when cells transfected with GLBB46 (F in Fig. 2) were treated with one of 5 different hormones ($P>0.62$; Table 2). Curiously, luciferase activities decreased significantly when cells transfected with GLBBinB4 (H in Fig. 2) were treated with any of the hormones ($P<0.001$, Table 2). When cells were transfected with the intronless plasmids, PGBBT1 and GLBB46 (I and F in Fig. 2), after exposure to different juvenile hormone (not shown) and ecdysone concentrations,

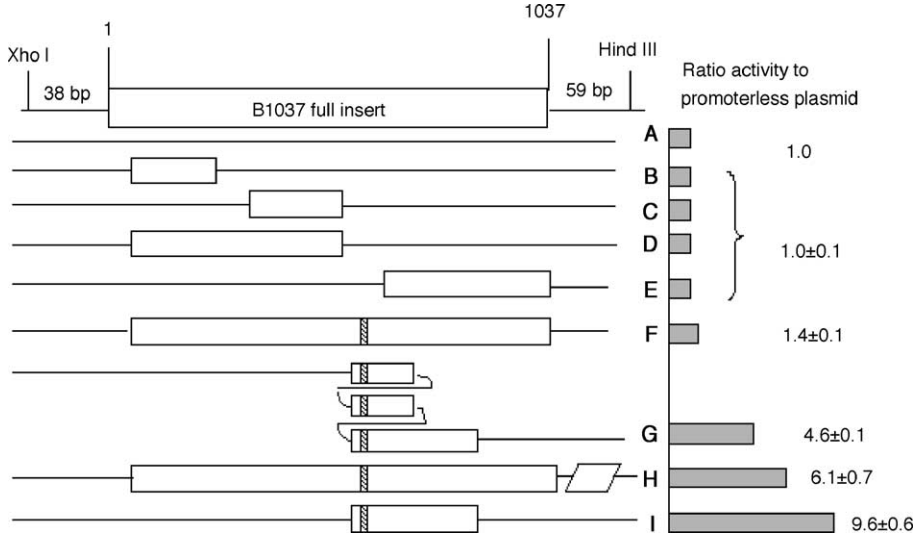

Fig. 2. Constructs (not drawn to scale) containing the 5′ upstream region of the B1037 genomic clone, ligated to a luciferase reporter sequence and transfected to insect cell lines, were used to identify the TmAFP core promoter. Blank rectangles represent promoter region sequences, the rhombus represents the Lmhsp70 first intron sequence, the lines represent vector sequence, and the striped boxes represent the position of the TATA box consensus sequence, TATAAA. A is the promoterless vector PGB, B is PGB with the 73–291 bp sequence of B1037, C is PGB with the 404–657 bp sequence, D is PGB with the 73–657 bp sequence, E is PGB with the 771 to the end, F (GLBB46) is PGB with the entire B1037 insert, G is PGB with the double tandem linked 657–776 bp sequence to 657 to 799 bp of B1037, H (GLBinB4) is PGB with entire B1037 sequence as well as a locust hsp70 gene family first intron, and I is PGB with the 657–799 bp sequence.

Table 3
The effect of various concentrations of hydroxyecdysone on luciferase reporter activity directed by a GBBB46 and a core promoter from B1037 (BT1)

Hydroxyecdysone concentration (μM)	PGBBT1 activity ratio	GLBB46 activity ratio
0	1	1
0.1	0.92±0.11	1.03±0.23
0.5	0.85±0.15	1.08±0.12
1.0	1.11±0.08	0.94±0.23
10	0.89±0.11	0.95±0.24
50	1.03±0.12	1.09±0.13

the luciferase reporter activities did not change significantly ($P>0.1$, Table 3).

4. Discussion

Genomic sequences corresponding to TmAFP isoforms containing 6 or more 12-residue repeats have now been identified. Genomic clones were isolated from three different libraries. The lambda (BST1, BST2 and BST3) and EMBL3 (A1139, B1037, C951, D950, E827, F951, G1267) clones contained relatively small inserts, but not surprisingly, the two identical clones isolated from the BAC library had large inserts. When the ~68 kb BAC clones (7 K and 13 M) were subjected to Southern analysis, only a single AFP coding region could be detected (data not shown). Thus, unlike the fish Type I AFPs that are tandemly arrayed (Scott et al., 1985), and the *C. fumiferana* AFP genes, *CfAFP2.7a* and *CfAFP2.7* (Doucet et al., 2002), the members of the TmAFP gene family examined here appear not closely linked.

When the 11 genomic clones, including the four lambda, one BAC and seven EMBL3 clones were conceptually translated, they showed approximately 46–91% identity with previously isolated TmAFP cDNAs. None of the TmAFP genomic sequences had intronic sequences. In contrast to these beetle genes, fish AFPs have introns of ~0.5 to 1.2 kb (Chan et al., 1997; Wang et al., 1995) and CfAFP genes have introns of ~3.0 to 3.6 kb. The fact that the TmAFP genes are intronless may simply reflect a selective pressure to retain the rather compact genome of *T. molitor*. Indeed, several genes from this insect have few or very small (49–55 bp) introns (Graham et al., 1996a; Feng and Happ, 1996; Mathelin et al., 1998; Rondot et al., 199; Lee et al., 1996), even though intronic sequences can be useful for transcriptional control (Lemoine et al., 2004).

After inspection of the sequences upstream the coding regions in the AFP genomic clones, putative CAAT boxes and TATA boxes (or short AT-rich regions downstream of the tentatively identified CAAT boxes) were noted. The upstream regions from all the clones analyzed were similar in that they did not appear to contain internal repeats nor show any significant sequence identity to repetitive elements archived in Genbank (September, 2005). As well, no sequences corresponding to well-known ecdysone or JH receptor elements were found.

One of the EMBL3 clones, B1037, contained a 952 bp sequence upstream of the first AUG of the coding region and had a coding region that appeared to correspond to the known isoform 2–14. By itself, the entire sequence ligated to a luciferase coding region enhanced the reporter activity 1.4-fold. Such modest, but consistent enhancement, suggested weak promoter activity or the presence of a sequence that partially supressed transcriptional activity in addition to a core promoter. When a 143 bp fragment was used alone (PGBBMH74), luciferase activity increased ~10-fold, demonstrating not only that this region contained the core promoter, but again suggesting that regions proximal or distal to this 143 bp sequence may contain negative regulatory element (s). Curiously, when the first 120 bp of this fragment (containing the putative TATA sequence) was added in duplicate to the PGBBT1 construct (Fig. 2), luciferase activity decreased by half. Perhaps tandemly linked, triplicate copies of the TATA box interfere with the binding of the transcription machinery. A similar report showed that when a fish myoglobin gene construct containing a TATA box duplication was transfected into cell lines, no reporter activity was detected (Small et al., 2003). These authors suggested that the additional TATA sequence could result in silencing of gene expression, perhaps through the assembly of an inhibitory complex.

The addition of an intron to the B1037 sequence enhanced luciferase activity 6.1-fold over controls and 4.4-fold over the same sequence without the intron (Fig. 2). This enhancement is similar to the average increase in expression (5-fold) when an intron, derived from either the host or the transgene, was introduced into a fish AFP gene construct and transferred to *Drosophila melanogaster* (Duncker et al., 1997). Similarly, intronic sequences have been found to increase mRNA levels following transient expression in cell lines (Huang and Gorman, 1990). As well, intron-containing promoter constructs of the human angiotensin II type 2 receptor (hAP2) gene enhanced luciferase activity 6.7-fold over intronless, control constructs (Warnecke et al., 1999), and this has been attributed to an enhancement of posttranscriptional processing including polyadenylation and capping (Lou et al., 1996; Ohno et al., 1987). Since the addition of an intron enhanced luciferase activity, it is curious, even given the possible pressure to maintain a small genome, that none of the TmAFP genomic coding regions isolated here, contains intervening sequences.

Previous studies showed that although weeks of low temperatures and desiccating conditions increased larval TmAFP transcript levels, developmental regulation was crucial since no cold-induced AFP increases were detected in pupae (Graham et al., 2000). As well, Horwath and Duman (1983) reported that juvenile hormone induced AFP activity in the related beetle, *D. canadensis*. The cell line, CF-203, used to investigate regulation by developmental hormones, was developed from the midgut of *C. fumiferana* (Sohi et al., 1993) and these cells are invaluable in that they respond to the molting hormone, 20-hydroxyecdysone, and the larval maintenance hormone, JH (Hu et al., 2004; Kethidi et al.,

2004). Since inspection of the sequences adjacent to the core promoter showed high similarity to putative JHREs identified upstream of *dsp28* (Table 1), CF-203 cells transfected with the TmAFP DNA were treated with JH III, the JH used in *Tenebrio*, and 4 other hormones or hormone analogues. It is an intriguing observation that all of the constructs containing the exogenous intron from a locust hsp70 gene family member, showed a decrease in reporter activity after hormone exposure. We consider it unlikely that the intron contained suppressor sequences recognized by these several hormones. Rather, the various hormone treatments may have stressed the cells and resulted in an overall decrease in mature AFP transcript levels. It is known that heat shock and other stresses can interfere with splicing activity (Yost and Lindquist, 1986; Simpson and Filipowicz, 1996), and inhibition of splicing can affect mRNA transport (Brodsky and Silver, 2000). Interestingly, it has been suggested that most Hsp genes do not have introns due to the inhibition of splicing activity by the very conditions that induce their transcription. If the CF-203 cells are indeed exquisitely sensitive to hormone treatments, future inquiry should avoid the use of intervening sequences in reporter constructs with this cell line. When we repeated our experiments using DNA without exotic introns to assess the influence of hormones on reporter activity, there were no differences observed, even in constructs that did or did not include the sequence resembling the putative *Tenebrio* JHRE (Table 2).

Thus, in vitro studies suggest that steroid hormones or hormones thought to have nuclear receptors similar to steroid hormones do not regulate TmAFP gene expression. Nevertheless, AFP levels increase in quiescent, overwintering larvae, suggestive of endocrine regulation, and a neuroendocrine-regulated pathway for AFP gene expression has been reported for fish AFPs (Fourney et al., 1984). Although hormone regulation of quiesence has not been studied in vivo in *T. molitor*, diapause regulation by hormones has been widely studied in other insects. These studies include those on the cotton bollworm, *Helicoverpa armigera*, and the silkworm, *Bombyx mori*, whose diapause-related hormone encoding genes have been cloned and characterized (Gade et al., 1997; Zhang et al., 2004a,b, 2005; Xu et al., 1995). A peptide hormone, hypertrehalosemic hormone, induced the cryoprotectant trehalose in the cockroach, *Periplaneta americana* (Sun et al., 2002), and a 41-amino acid peptide hormone is involved in the diapause of leaf beetle, *Gastrophysa atrocyanea* (Tanaka et al., 2003). These reports suggest that it would be useful to examine the role of peptide hormones in the regulation of *T. molitor* diapause and the control of TmAFP gene expression during the overwintering stage.

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