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Transcription of antifreeze protein genes in *Choristoneura fumiferana*

W. Qin, D. Doucet, M. G. Tyshenko and V. K. Walker*

Department of Biology, Queen's University, Kingston, Ontario, Canada

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Abstract

Antifreeze proteins (AFPs) are encoded by approximately 17 genes in the spruce budworm, *Choristoneura fumiferana*. Northern analysis using 6 different cDNA probes showed isoform-specific patterns that varied during development. Transcripts for the majority of isoforms were most abundant in the second instar overwintering stage, but some were also detected in first instar and even in egg stages. *In situ* hybridization using riboprobes corresponding to two 9 kDa protein isoforms showed differential AFP expression even in second instars; CfAFP10 RNA was detected in all tissues, but CfAFP337 RNA distribution was more limited. Two genomic regions encoding three AFP genes have been isolated. Presumptive regulatory regions conferred transcriptional activity when placed upstream of a luciferase reporter sequence and transfected into a *C. fumiferana* cell line. The CfAFP2.26 core promoter is an 87 bp sequence containing a TATA box, whereas the CfAFP2.7 core promoter is a 76 bp sequence with both a TATA box and CAAT box, which directed higher reporter activities when tested *in vitro*. Reporter activity was not enhanced with five different hormones, although lower activities were observed with all intron-containing constructs. AFP message half-life, as assessed using reporter assays, was not appreciably influenced by isoform-specific-3′UTRs. These studies successfully demonstrate the temporal and spatial diversity of AFP expression encoded by this small gene family, and underscore the complexity of their regulation.

Received 10 November 2006; accepted after revision 16 February 2007. Correspondence: Dr Virginia K. Walker, Department of Biology, Queen's University, Kingston, Ontario, K7L 3 N6, Canada. Tel.: +1 613 533 6123; fax: +1 613 533 6617; e-mail: walkervk@biology.queensu.ca

Abbreviations: AFP: Antifreeze protein; JH: Juvenile hormone; UTR: untranslated region

Introduction

Choristoneura fumiferana, the spruce budworm, has a freeze-avoidance strategy for overwintering. Such insects can produce low molecular weight cryoprotectants to lower the freezing point of their haemolymph, avoid the initiation of ice growth by sealing themselves in cocoons, eliminate materials which could act as nucleators, increase production of energy sources, and even produce heat shock proteins (Hsps) and other stress proteins for protection against cold shock and injury (Denlinger *et al*., 1991; Lee, 1991; Danks *et al*., 1994; Joanisse & Storey, 1996). In addition, *C. fumiferana* synthesizes antifreeze proteins (AFPs) (Tyshenko *et al*., 1997; Walker *et al*., 2001). AFPs depress the freezing point of the haemolymph by adsorbing to seed ice crystals and inhibiting their growth. Although AFPs have been most widely studied in fish (Davies & Sykes, 1997; Ewart, 2002), insect AFPs are hyperactive, compared to the majority of fish proteins, presumably due to the more extreme winter conditions experienced by terrestrial organisms.

Similar to fish AFPs that are encoded by hundreds of gene copies (Hew *et al*., 1988), *C. fumiferana* AFPs (CfAFPs) also are multicopy, with ~17 copies. These are divided into three classes: 'long and short-3′UTR genes' encoding 9 kDa CfAFPs and 'intermediate length 3′UTR genes' encoding 12 kDa CfAFPs (Doucet *et al*., 2002). Photoperiod regulates the transcription of AFPs in winter flounder, with longer days inducing the production of growth hormone that inhibits the transcription of the fish Type I AFP in the liver (Fourney *et al*., 1984). Seasonal variation in AFP levels in the fire-coloured beetle, *Dendroides canadensis*, also are influenced by day length (Andorfer & Duman, 2000), but in the yellow meal worm beetle, *Tenebrio molitor*, low temperature increased AFP mRNA abundance (Graham *et al*., 2000).

The details of AFP gene regulation in *C. fumiferana* have not been examined. It is known that after hatching in the summer, first instar spruce budworm larvae move away from the egg masses, spin communal silk hibernacula and molt to second instar larvae (Fig. 1). During winter diapause, the production

of AFPs (Tyshenko *et al*., 1997; Walker *et al*., 2001) and glycerol (Han & Bauce, 1993) allow the larvae to survive temperatures of –30 °C or lower. In the following spring, they leave the hibernacula and complete the life cycle through third to sixth instar larvae and adult moths (Han & Bauce 1993). The cloning of seven cDNAs and two genomic clones (Doucet *et al*., 2000; Doucet *et al*., 2002) has now provided tools for the analysis of AFP gene regulation in early instar spruce budworm larvae. Specifically, we have investigated if members of this gene family are differentially regulated during the crucial overwintering stage, and if the identification of core promoters or transcript properties can provide insight into their expression.

Results

Sequence of CfAFP2.26 and CfAFP2.7

Two *C. fumiferana* genomic clones, CfAFP2.26 and CfAFP2.7, were completely sequenced (not shown) and have been deposited as GenBank accession numbers AF325857 to AF325859. CfAFP2.7 (9428 bp) contains a complete coding region as well as upstream and downstream sequence corresponding to a member of the long-3′UTR class of AFP isoforms, and named *AFP-Lu1* (Doucet *et al.* 2000). A single phase 1 intron (3597 bp, starting at nt 4163) was identified by comparison with the cDNA sequence and interrupts the signal sequence. *AFP-Lu1* encodes a 9 kDa protein and 4 putative polyadenylation signals at nts 8404, 8462, 9316–9319 and 9393 were identified. Sequences corresponding to a TATA box and a CAAT box were found upstream of the first exon at –84 and –116 nts. Further upstream of *AFP-Lu1*, and on the same strand, lies a second AFP gene, designated 2.7a, with 7 potential polyadenylation signal sites at 1296, 1553, 1754, 2207, 2261, 2737 and 2866 nts.

CfAFP2.26 (6797 bp), encodes *AFP-lu1*, a seemingly typical intermediate 3′UTR class of gene encoding a 12 kDa AFP. A single phase 1 intron (2995 bp) and a putative TATA box at –129 bp upstream of the first exon were sequenced, but no CAAT box was identified. Poly(A) signal sequences (Tyshenko *et al*., 1997) were found at 5684, 6106, 6602, 6617, 6665 and 6668 nts.

Promoter identification and hormone response

Promoter sequences identified by sequence analysis were further investigated by ligating the 5′ upstream sequences from AFP-Lu1 and AFP-lu1 to a luciferase reporter and transfecting into CF-203 cells. Luciferase activities driven by the CfAFP2.26 5′ region alone or the 5′ region plus the AFP coding and intron sequence were similar, but 7-fold greater than a control plasmid without a promoter (Fig. 2).

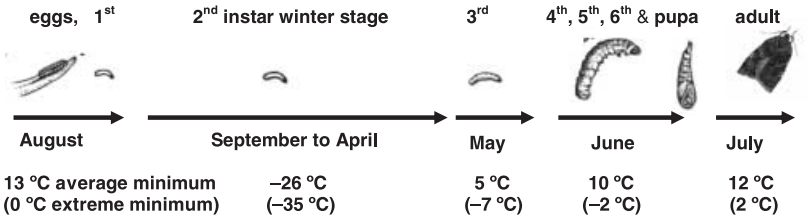

Figure 1. Life cycle of *Choristoneura fumiferana*. The time line indicates when a particular life stage of spruce budworm is normally found in the Northern boreal forest. Since the insects were originally collected just outside Sault Ste. Marie (46°31′N, 84°19′-W), the average minimum temperatures (and extreme minimum temperatures; Environment Canada, http://www.climate.weatheroffice.ec.gc.ca) are given for this region, corresponding to the conditions that each stage must survive. The sizes are representative rather than true dimensions, but typically eggs are 1 mm, 2nd instars are 2–4 mm, mature larvae are 20–25 mm, pupae are 15 mm and adults are 13 mm. The sketches are modified from those from Forest Health, Government of Alberta.

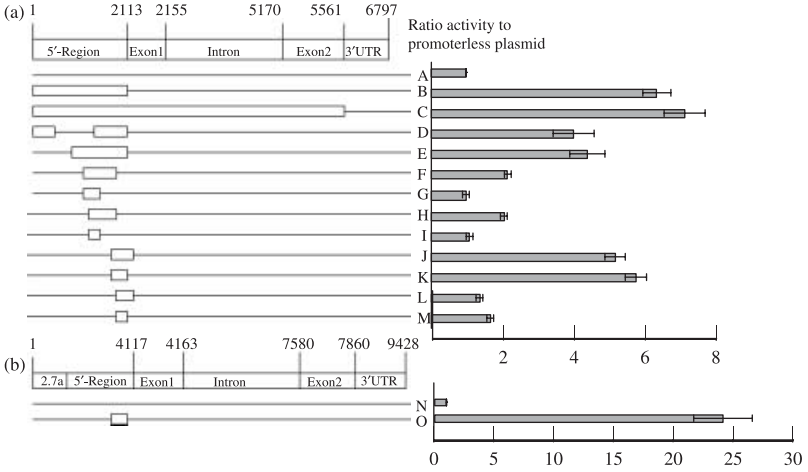

Figure 2. Promoter analysis of CfAFP genes, diagrammed to show constructs and luciferase reporter activity. (a) CfAFP2.26 gene and promoter constructs: A is the promoterless plasmid PGB, B is PGB2.26A5, C is PGB2.26B20, D is PGB2.26A5AA6, E is PGB2.26A5KK8, F is P1C1, G is P1D2, H is P2C2, I is P2D3, J is P3A3, K is P3E2, Lis P4A5, M is P4E1, and K is 87 bp and includes a putative TATA box. (b) CfAFP2.7 gene and promoter constructs: N is the promoterless plasmid, O is 76 bp and includes a putative TATA box and CAAT box. Other constructs are not shown.

Table 1. The effect of hormones on luciferase reporter activity directed by the CfAFP flanking region, intron and coding sequences

Hormone Treatment (μM)	PGB2.26B20 (with a 3 kb intron) activity ratio
No hormone	1
9-*cis* retinoic acid (25 μM)	0.41 ± 0.02
Methoprene (100 μM)	0.47 ± 0.05
Juvenile hormone I (1 μM)	0.26 ± 0.06
Juvenile hormone III (1 μM)	0.46 ± 0.07
Hydroxyecdysone (1 μM)	0.34 ± 0.04
No promoter, no hormone	0.17 ± 0.01

Table 2. The effect of hydroxyecdysone and juvenile hormone I on luciferase reporter activity directed by AFP 5′-flanking region sequences

	PGB2.26A5 transcript ratio	
Hormone concentration (μM)	hydroxyecdysone	juvenile hormone I
0	1	1
0.1	0.98 ± 0.12	1.00 ± 0.05
0.5	1.00 ± 0.08	0.88 ± 0.13
1.0	0.92 ± 0.11	1.12 ± 0.12
10	1.05 ± 0.07	0.92 ± 0.09
50	0.78 ± 0.15	1.00 ± 0.06

Thus, a core promoter resides in the 5′ region. When plasmids containing certain deletions of this region (D and E, Fig. 2) were used, reporter activities remained higher than promoterless controls ($P < 0.001$). Cells transfected with two of the constructs (J and K) had luciferase activities that were similar (5-fold) to the full-length plasmids ($P < 0.001$). The other 6 constructs (F-I and L-M) had low activities. Taken together, it is likely that the core promoter is located in the 87 bp region found in P3E2 (K, Fig. 2) and it includes the TATA box identified by sequencing at –129 bp.

Similarly, the construction of plasmids containing smaller fragments derived from the CfAFP2.7 genomic clone showed that the core promoter for this gene was likely contained in a 76 bp fragment of PGB2.7MH3. This region directed significantly more luciferase reporter activity than controls (O, Fig. 2; $P < 0.001$) and includes a CAAT box and TATA box that was identified by sequence analysis.

Since *C. fumiferana* second instars have the highest AFP activity of any life stage (Tyshenko *et al*., 1997), we reasoned that the genes might be developmentally regulated and as a consequence, be influenced by hormones. However, there was no enhancement of luciferase reporter activity when cells were transfected with PGB2.26B20 (C, Fig. 2) and reporter activities decreased with all hormone treatments (Tables 1, $P < 0.001$). To investigate if reporter activity could be suppressed after treatment, the dose–response of two different hormones showing this response was examined. However, when cells transfected with a construct containing the 5′ sequence (PGB2.26A5, B in Fig. 2) were treated with different concentrations of hydroxyecdysone and JH I, luciferase reporter activities did not change significantly ($P > 0.1$; Table 2).

Developmental and tissue regulation of AFP transcripts and proteins

When Northern blots of *C. fumiferana* eggs and early instar larvae were hybridized with cDNAs representing the short, intermediate and long-3′UTR gene classes, overall, transcripts were most abundant in the second instar stage (Figs 3 and 4), concordant with previous observations of high AFP activity at this developmental stage. The long-3′UTR probe, CfAFP337, hybridized to two major transcripts (1.1 and 1.5 kb, Fig. 3), which could also be detected in first instars and very faintly in eggs (Fig. 4). Transfer of second instars to diapausing conditions did not appear to result in a change in transcript levels (Fig. 3, compare L2 lanes). Similar results were observed when the short-3′UTR probe, CfAFP339, was used to detect 0.67 and 1.2 kb transcripts in second instars, with hybridization intensity independent of light and temperature conditions (Fig. 4).

Both short and long-3′UTR transcripts disappeared almost completely within the first 48 h after larvae broke diapause following transfer to ‘summer’ rearing conditions, with messages with long-3′UTRs persisting a few hours longer (Fig. 4, L2 48 h). Other probes designed to detect transcripts that encode 9 kDa AFPs revealed similar patterns (not shown). The accumulation of intermediate length 3′UTR transcripts, however, was less predictable and varied depending upon the probe used. Transcript abundance for CfAFP501 (1.0, 1.4 and 2.9 kb) dropped to low levels after transfer to 24 °C, similar to the short and long-3′UTR isoforms (Fig. 4, and not shown). Some other messages with intermediate 3′UTRs such as the 1.0 kb CfAFP10, 18 and 104 transcripts persisted for 4–5 days after transfer (Fig. 4). For CfAFP104 and CfAFP18, low transcript levels were still present even after the insects had molted to third instar larvae (Fig. 4 and not shown). In contrast, transcripts (1.0 and 1.4 kb) from a putative intermediate 3′UTR gene, 2.7a, peaked in abundance during the first instar and disappeared with 48 h after the change in rearing conditions (Fig. 4). Real time PCR was used to assess CfAFP transcript levels in second instar larvae of a selected, ‘non-diapause’ strain derived from the parent population. CfAFP transcripts could be readily detected, but the abundance of mRNA was reduced (2-fold difference in the cycle threshold values) when compared to the wild-type strain (both at 4 °C; 0 l:24D).

In situ hybridization of the 2–4 mm second instar larvae showed that an antisense hybridization probe, corresponding to the long protein (CfAFP10) appeared to detect AFP mRNA in every tissue. In contrast, an antisense probe for the short protein (CfAFP337) detected AFP transcripts in

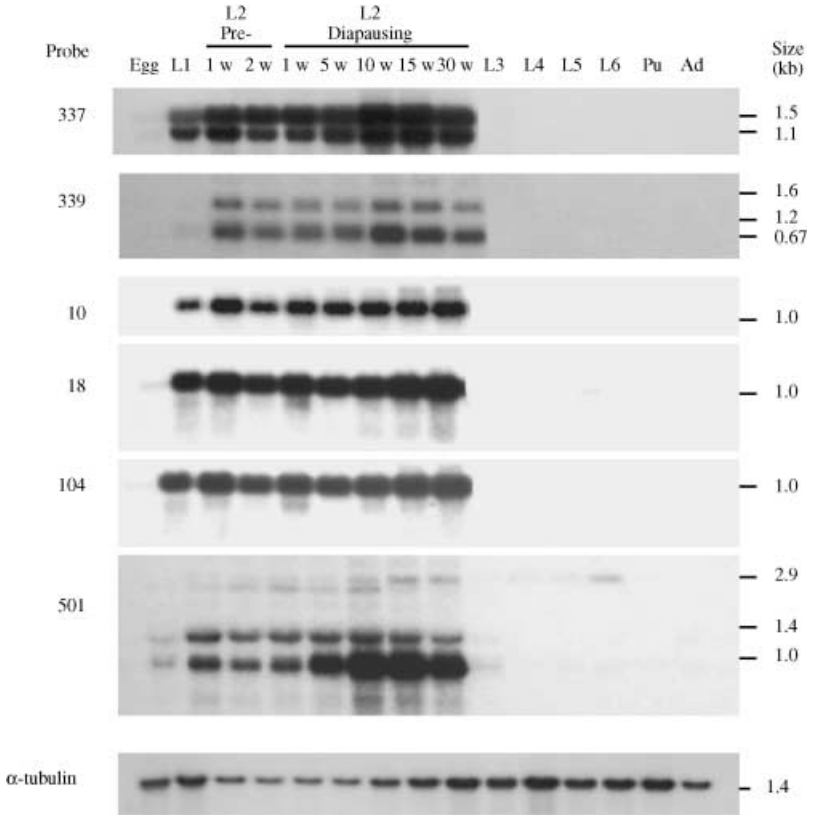

Figure 3. Northern blots showing transcript accumulation of several CfAFP isoforms during different developmental stages. Eggs, all six larval instars (indicated with L and a number), pupal (Pu) and adult (Ad) stages of the insects were used. Size of the AFP mRNAs (in kb) is indicated at the right. Second instars 1 and 2 weeks (w) at 23 °C before transfer to 2–4 °C (L2 Pre-) were assayed as well as second instars 1, 5, 10, 15 and 30 weeks after transfer to cold storage (L2 diapausing). The probes used were short-3′UTR (339) and long-3′UTR (337) both of which encode a 9 kDa AFP and intermediate length 3′UTR cDNAs (10, 18, 104, 501), which encode 12 kDa AFPs. The blots were reprobed with *Drosophila* α-tubulin for estimation of RNA loading, and representative blots are shown.

fewer tissues, but was particularly prominent in the midgut (Fig. 5). No mRNA was detected with either one of the two sense probes used as controls.

To determine if AFP accumulation reflected message levels shown by Northern blots and *in situ* hybridization, Western blot analysis was undertaken. AFP appeared in first instars, 9 h after hatching from the egg (Fig. 6a,b) and persisted at high levels in second instars prior to transfer to 2–4 °C (Fig. 6c). One week after breaking diapause at the end of the second instar, AFP levels declined and cross-reacting material was not detected in one-day-old third instar larvae (Fig. 6c,d).

AFP transcript half-life and structural analysis

Since different isoform transcripts showed a surprising range of temporal and spatial expression we considered the possibility that promoter elements might not be as important as the variability that could be contributed by the ~800 nt difference in 3′UTR length. The half-life of a short-3′UTR isoform (CfAFP339) and a long-3′UTR isoform (CfAFP337), with similar 9 kDa coding sequences but bearing-3′UTRs of dissimilar lengths (211 and 996 nts, respectively), were determined by the transfection of constructs (339UTR and 337UTR) into CF-203 cells. New transcript synthesis was arrested with actinomycin D. With both constructs, the rate of mRNA degradation was linear over the period tested (short-3′UTR: $R^2 = 0.97$; long-3′UTR: $R^2 = 0.95$), allowing transcript half-lives to be determined. Transcripts with long and short-3′UTRs, had rather similar half-lives of 2.9 and 2.4 h, respectively ($P < 0.005$, Fig. 7).

When the short and long-3′UTRs from CfAFP339 and 337, respectively, were examined using the 'RNA analyser' algorithm (see Methods), both sequences appear to potentially contain secondary structures. The short and long-3′UTRs contained 4 and ~10 stem structures, respectively, with some 'stem-loop' motifs that appeared similar. The significance of these structures is unknown, and hence these figures are not included, but are available from the authors.

Discussion

Similar to all AFPs from metazoans that have been characterized to date, AFPs in *C. fumiferana* are encoded by a multicopy gene family. For example, there are 30–40 copies of both liver and skin-type AFP genes in winter flounder (Scott *et al.*, 1985; Gong *et al.*, 1996). In ocean pout the 40 AFP gene copies found in Southern latitudes apparently amplified to as many as 150 copies in colder Northern waters, suggesting that selective pressure can increase the gene copy number (Hew *et al.*, 1988). Spruce budworm has to endure much harsher winter environments than

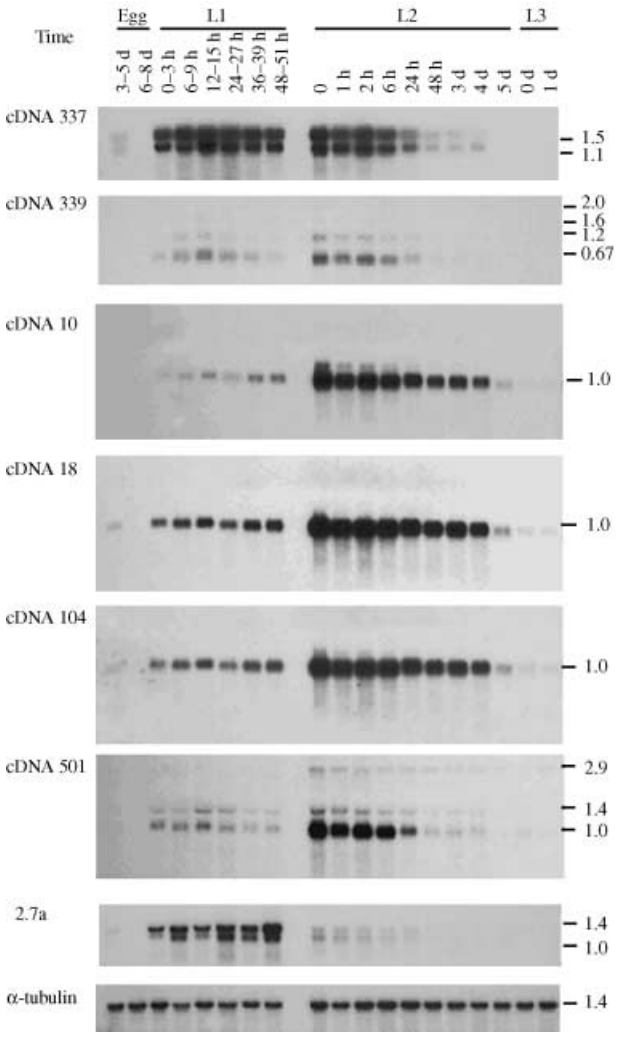

Figure 4. Detailed Northern blot analysis of CfAFP isoforms during early instars of *C. fumiferana* development. Samples were taken at different times after egg laying (lanes 1 and 2), during first instar (L1) from 0 h to 51 h after hatching (lanes 3–8), during second instar (L2) kept at 2 °C (0 l: 24D) for 30 weeks and transferred to normal rearing conditions (23 °C, 16 l: 8D). After 30 weeks (lane 10) second instars exited their hibernacula in the first 24 h of normal rearing conditions and were sampled after several hours (h) or days (d) at normal conditions (lanes 11–18). They were also sampled during third instar (L3) (lanes 19–20). Fresh diet was supplied after 24 h in L2 (lanes 15–20). Lane 9 contains no RNA. The probes used are described in Figure 3 with the exception of 2.7a [10]. Probing of the blots with a *Drosophila* α-tubulin fragment was done to assess loading in each lane; representative examples of the blots are shown.

ocean fish, but CfAFP is hyperactive compared to the Type I, II and III fish AFPs, possibly explaining the lower copy number of ~17 AFP loci in these insects (Doucet *et al.*, 2000; Doucet *et al.*, 2002). Although the global expression of the winter flounder AFP appears to be negatively regulated by growth hormone (Fourney *et al.*, 1984), the regulation of individual AFP genes has not been monitored. In budworm, however, the lower gene copy number, the availability of several genomic clones and the divergence of these genes during the last three million years (Tyshenko *et al.*, 2005), now allows an investigation into the regulation of a limited number of AFP genes, which would be more difficult in fish.

Analysis of the complete sequence of two genomic clones (deposited to GenBank, see Results) containing three AFP coding regions, facilitated the identification of two putative promoter regions. Constructs containing the 5′ region with intron and exon sequences, or simply the 5′ region, were used to drive a luciferase reporter in spruce budworm cell lines to identify the location of the core promoters. For one of the genomic clones, CfAFP2.7, the entire 5′ upstream sequence likely resides in the intergenic region between the two coding regions. Indeed, the core promoter was identified in a 76 bp fragment that contained sequences that matched a consensus CAAT box (at –116 bp) and TATA box (at –84 bp). Similarly, the core promoter in CfAFP2.6 was identified in an 87 bp fragment containing a TATA box (at –129 bp).

Since spruce budworm overwinters as diapausing, second instar larvae, it was initially surmised that similar to sequences encoding the fish AFPs, CfAFP genes would be expressed at a time when the temperature is low. Although transcripts encoding the majority of the isoforms (e.g. CfAFP339, 10 and 501) were most abundant at this stage, transcripts for long-3′UTR isoforms such as 337 could also be detected in first instars and even in eggs, periods in the summer when freeze resistance would presumably not be required (Fig. 1). Even more striking, some transcripts appeared to peak in abundance in first instars and mRNA levels either did not change (CfAFP337) or decreased (CfAFP2.7a) during the overwintering stage when temperatures can drop as low as –30 °C. An intermediate 3′UTR transcript (CfAFP104) persisted to the third instar, a spring stage (Figs 1 and 4), and low levels of AFP mRNA were also detected in the summer, fifth instar stage (Qin *et al.*, 2006). In addition to this seemingly complex regulation, more than one transcript was often detected with the different probes, suggesting that different polyadenylation sites seen in the genomic clones are used in many of the different transcripts. As well, some of the fainter bands seen on Northern blots could represent more distantly related isoform transcripts. Thus the spruce budworm seems similar to *Dendroides canadensis* since they also differ in their seasonal occurrence of AFP mRNAs. *Dendroides* DAFP-1 transcripts were found in insects collected from autumn to early summer, whereas DAFP-7 transcripts accumulated in insects collected in autumn and early winter (Andorfer & Duman, 2000).

We postulated that the observed differences in transcript abundance could reflect the relative stability of individual transcripts, especially when one class of CfAFP genes has 3′UTRs which are almost 5-fold longer than the short-3′UTR class. Message stability in other organisms has been

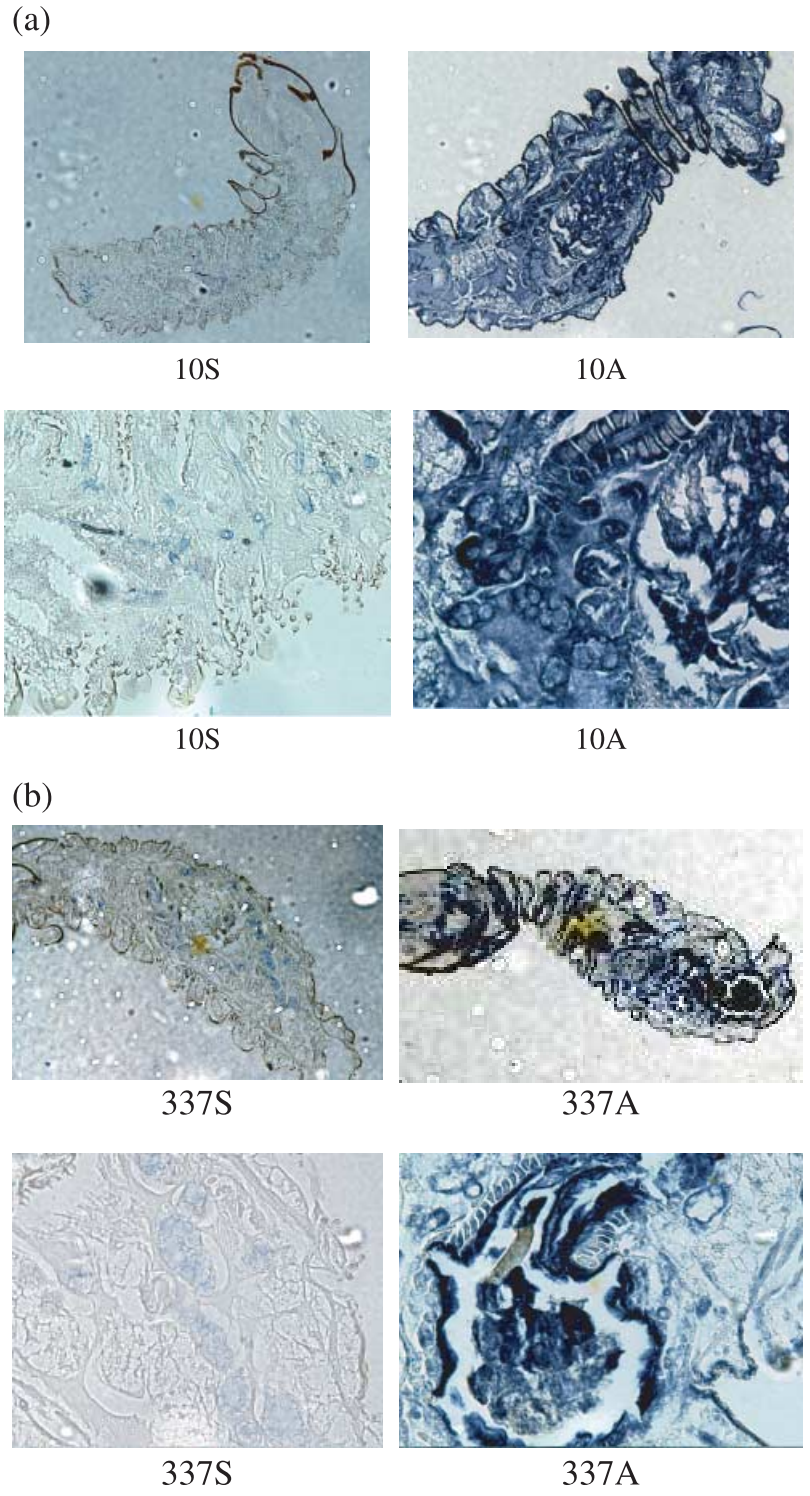

Figure 5. *In situ* hybridization of CfAFP mRNAs. Sections of second instar larvae (2–4 mm) were hybridized with (a) CfAFP10 (10; encoding a long protein isoform with an intermediate length 3′UTR) and (b) CfAFP337 (337; encoding a short protein isoform with a long-3′UTR) riboprobes. The sections were hybridized with sense (S) or antisense (A) probes and incubated with digoxingenin-AP antibody followed by staining (see methods). Representative slides were photographed at 10x (top images) and at 40x (lower image). All tissues in a sagital section of (a) and probed with 10 A are stained. Midgut cells and cells associated with the peritrophic membrane stain darkly, but fat body is more lightly stained when probed with 337 A (b).

attributed to such sequences; the decay of human c-fos and GM-CSF transcripts are mediated by distinct AU-rich elements in their 3′UTRs (Chen *et al.*, 1995). However, when 3′UTRs from the long and short CfAFP gene classes were used in luciferase reporter assays to determine message stability, the two transcripts showed rather similar half-lives of 2.4 h and 2.9 h, respectively. This result is consistent with the rather rapid disappearance of the CfAFP337 and 339 transcripts, as well as AFP cross-reacting material, when larvae were returned to summer-like conditions after breaking diapause (Figs 4, 6). Analysis of the long and short-3′UTR sequences showed that neither had

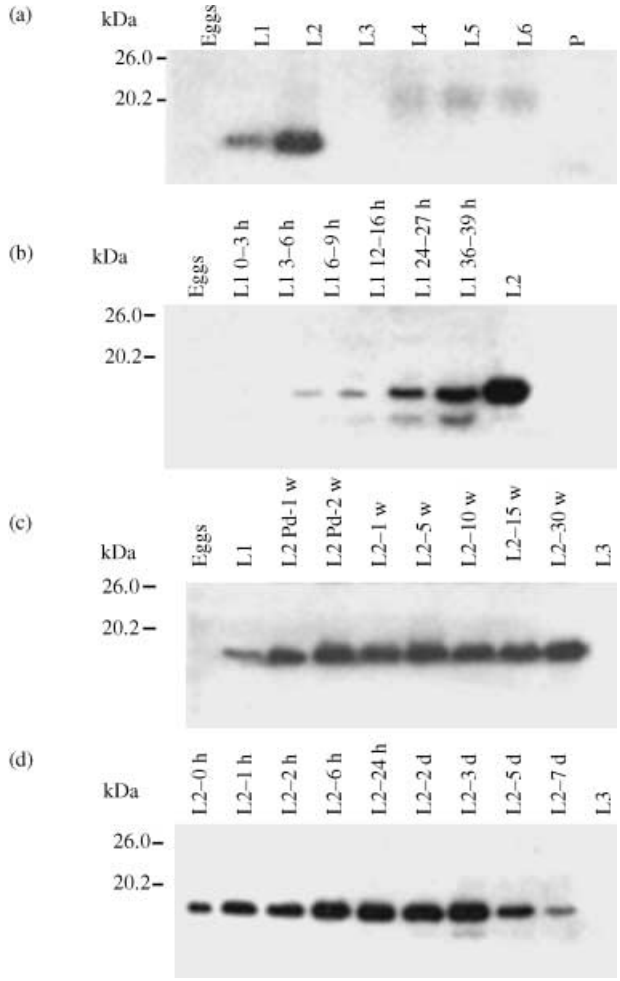

Figure 6. Western blots showing expression of CfAFP during development. (a) Eggs, all six larval instars (indicated with L and a number), and pupal (P) stages.(b) L1 to L2 larval instar developmental transition at various time points. First instars were timed from hatching until molting to second instars. (c) The effect of temperature during development. Eggs, L1, second instars 1 and 2 weeks (w) at 23 °C (L2 Pd-1 or 2), and second instars 1, 5, 10, 15 and 30 weeks after transfer to 2–4 °C (L2–1w to L2–30w). The L3 stage is shown within 24 h after molting. (d) L2 to L3 larval instar transition after the return of second instars to 23 °C after 30 weeks at 2–4 °C. Times: 0–24 h and day 2–7 for second instars and day 1 after molt to the third instar are shown. Marker proteins are shown on the left. For all Western blots, polyclonal anti-CfAFP, that recognized both short and long isoforms [23], was used at a 1 : 5000 dilution and chemiluminescence exposure was for 10–30 s.

classic AU-rich RNA destabilizing elements (Zubiaga *et al.*, 1995; Peng *et al.*, 1996), but both could be folded *in silico* to secondary structures with multiple stem-loops, reminiscent of the folded structure of bicoid 3′UTR, important for transcript localization in *Drosophila* development (MacDonald, 1990; MacDonald & Kerr, 1997) or as substrates for dsRNA ribonuclease, determinants of mRNA stability in plants (Yang & Stern, 1997). Unlike the case for the Type I AFP, where there is a 40% increase in levels of tRNA alanine isoacceptors in winter-caught fish (Pickett *et al.*, 1983), there is no evidence of translational control of CfAFP expression; Western blots indicated that AFP was present in first instars, most abundant in second instars (independent of temperature) and declined after a week post diapause (Fig. 6). Together, the Western and Northern analysis as well as the half-life determinations, show that the expression of CfAFP genes is not limited to the overwintering stage and that there is variation from gene to gene that cannot be attributed to 3′UTR-determined destabilizing elements.

Since AFPs accumulate in insect haemolymph, it is not surprising that AFP genes are transcribed in the fat body (analogous to the production of AFPs in fish liver). However, AFP transcripts are also found in beetle midgut and epidermal tissues (Duman *et al.*, 2002; Olsen & Duman, 1997). Similarly, AFPs are found in the skin and intestinal fluid of the winter flounder (Gong *et al.*, 1996), where it is speculated that these proteins may serve as a barrier to external ice crystal growth. CfAFP mRNAs are likewise found in multiple tissues, as demonstrated by *in situ* hybridization (Fig. 5). It is not known if the midgut and epidermis secrete AFPs to the haemolymph, or if, analogous to the fish, they serve a separate function in the epidermis as protection against ice growth that could penetrate the hibernaculum. In the midgut it has been suggested that AFPs could protect against residual nucleators that may not be completely cleared prior to overwintering (Olsen & Duman, 1997). The different distribution of the long and short isoforms even in a single larval stage certainly underscores the challenge to be faced in understanding the roles of the various CfAFP isoforms.

The observation that these AFP mRNAs are also present in eggs and first instar larvae indicates that they are not strictly diapause-specific. As well, AFP mRNA was detected using RT-PCR in second instars in a strain selected to bypass diapause, although at slightly lower levels. Increased mRNA accumulation and AFP cross-reacting material were not seen when spruce budworm larvae were transferred from standard light and temperature conditions to diapause conditions (2–4 °C, 0 l: 24D). Together, these results suggest that, unlike beetles where AFPs are at least

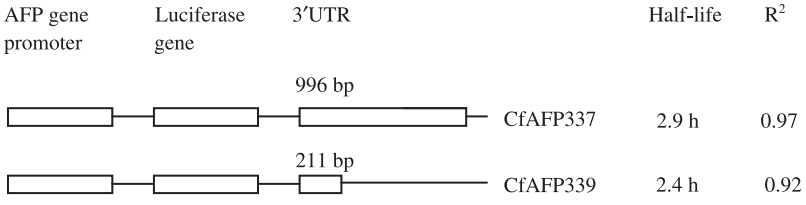

Figure 7. Half-life studies of plasmids bearing a long or short-3′UTR. Constructs (depicted as 3 boxes) containing a luciferase reporter sequence and a long or short CfAFP 3′UTR, 996 bp (CfAFP337) and 211 bp (CfAFP339), respectively. Similar half-life estimates ($P < 0.005$) and results of regression analysis are shown to the right of the diagram.

partially regulated by the reduction in day length and low temperature (Andorfer & Duman, 2000; Horwath & Duman, 1983; Horwath & Duman, 1982), or elevated by starvation and low temperature (Graham *et al.*, 2000), CfAFP isoform accumulation appears to be relatively independent of environmental conditions.

Glycerol is used as a cryoprotectant in concert with AFPs during overwintering in spruce budworm larvae. Glycerol accumulation in *C. fumiferana* is confined to the second instar, and it again differs from CfAFPs in that there is an absolute requirement for low temperature as a prerequisite for production, with elevated glycerol levels achieved by exposure to lower temperatures (Han & Bauce, 2000).

In the seeming absence of simple light level or low temperature regulation of these genes, but a clear influence by development, neuroendocrine regulation is possible. Transcripts encoding the ecdysone receptor are present throughout spruce budworm development including the second instar stage (Kothapalli *et al.*, 1995; Perera *et al.*, 1998) and the high titres of JH esterase present at the first day of every instar, excepting the second (Kethidi *et al.*, 2004), suggests that JH may initiate diapause. However, despite the reported presence of both hormones at the peak of CfAFP mRNA accumulation, we were unable to identify any sequences corresponding to known ecdysone or putative JH receptor elements in 2112 and 3306 bp upstream of the two isolated CfAFP genes. Initially, JH I treatment appeared to decrease the level of reporter activity in transfections, but subsequent experiments showed that this result was due to the presence of the intron in the constructs. When the intron was removed, there was no repressive effect on reporter activity at any JH I concentration used. Further work should be done to examine a possible negative regulation of CfAFP gene expression mediated by elements in the intervening sequence, and to ensure that the cell line used has appropriate hormone receptor levels. However, in the absence of these experiments, we suggest that hormone treatment stressed the CF-203 cells resulting in a non-specific decrease in reporter activities. Stress is known to reduce expression in other systems, possibly due to splicing or mRNA transport inefficiencies (Yost & Lindquist, 1986; Simpson & Filipowicz, 1996; Denegri *et al.*, 2001; Shomron *et al.*, 2005).

Other neuroendocrine signals could be important in the developmental regulation of these genes. A neuroendocrine-regulated pathway for AFP gene expression has been reported for fish AFPs (Fourney *et al.*, 1984). In diapausing silk moth (*Bombyx mori*) embryos, it is speculated that diapause hormone controls the accumulation of glycogen that is subsequently converted to sorbitol, a cryoprotectant (Chino, 1957; Yamashita & Hasegawa, 1985). However, it must be noted that, to date, this peptide hormone has only been shown to be a regulator of *B. mori* diapause (Denlinger, 2002). With the cloning of these CfAFP genes and cDNAs, the identification of the promoter elements, and the investigation of the transcriptional activity of various isoforms, the way is now clear for similar investigations with *C. fumiferana* CfAFPs.

Experimental procedures

Insect rearing and sampling

Choristoneura fumiferana Clemens (Cf; Family: Torticidae) were originally collected from Sault Ste. Marie, Ontario and used to establish a population maintained by Canadian Forest Services. Diapausing second instars were kept at 2–4 °C in the dark (0 l:24D) for 12–30 weeks. All other instars were kept at 23 °C ± 2 °C under a photoperiod of 16 l: 8D as described previously (Doucet *et al.*, 2002). Samples were obtained 3–5 days and 6–8 days after egg laying and from 0 to 51 h after hatching into first instars.

Diapausing second instars kept for 30 weeks in the dark at 2–4 °C were sampled at 1, 2, 6, 24 and 48 h. Second instars were also sampled 3, 4 and 5 days after transfer to standard rearing conditions (23 °C ± 2 °C, 16 l: 8D). These larvae exited their hibernacula = 24 h after transfer to standard conditions and were also sampled after molting to third instar larvae. A second strain of *C. fumiferana* was also obtained that had been selected from wild populations to bypass the almost obligate diapause period ('non-diapause' strain). Although the supply of these insects were severely limited, larvae were sampled 5 days after hatching and kept under standard conditions or transferred to 2–4 °C; 0 l:24D until required.

Genomic DNA analysis

A *C. fumiferana* genomic library was constructed in λDASH II vector (Stratagene, La Jolla, CA) and screened using an isolated cDNA (CfAFP337) as previously described (Doucet *et al.*, 2000; Doucet *et al.*, 2002). Two CfAFP genomic clones (CfAFP2.26 and CfAFP2.7) were obtained and partially sequenced as reported (Doucet *et al.*, 2002). The complete sequences of these two genomic clones were obtained by designing primers to determine previously unsequenced regions as well as correcting ambiguous regions. Genomic DNA was sequenced on both strands, in both directions, and analysed using internet-based computer programs (Expasy tools).

RNA isolation, Northern analysis and real time PCR

Total RNA from eggs and larvae instars was isolated using Trizol reagent (Gibco, Burlington, Canada) and treated with DNase I (Sigma, Oakville, Ontario, Canada). For Northern analysis, the RNA was fractionated by gel electrophoresis and transferred to Hybond N nylon membrane (Amersham-Pharmacia, Baie d'Urfe, Quebec, Canada). Each time point represented a pool of RNA isolated from 10 individuals. A probe specific for each isoform was synthesized by PCR, using consensus AFP primers (SPF and SPR; 12). Northern blots were hybridized with ^{32}P-dCTP-labelled probes (= 1×10^8 cpm/μg) at 42 °C in 50% formamide/5x SSC/5xDenhardt's solution/0.5% SDS and 100–150 μg of sheared salmon sperm DNA (Gibco) for a minimum 16 h (Sambrook *et al.*, 1989). The CfAFP10, 337, 18, 104 and 501 isoform cDNAs were previously isolated from a second instar cDNA library (Tyshenko

et al., 1997) and the blots were reprobed with an α-tubulin fragment as previously described (Doucet *et al*., 2000). Three washes (0.1XSSC/0.5%SDS at 65 °C for 20 min) were done prior to film (Biomax MS, Eastman Kodak, Rochester, New York, USA) exposure with intensifying screens at –80 °C for four days.

Since material was so limited for the analysis of the selected, 'non-diapause' *C. fumiferana* strain, real time RT-PCR was used. RNAs were isolated as described above, repurified, and used with degenerate AFP primers (AFPm1: 5′-ATA ASG WCT TCA ACA WCT ACY RRT WC and AFPm2: 5′-TYA RTY TMR GAC GAC TTT CAT GSC CT where S = *G* + C, W = A = T, Y = C + T, R = A + G, M = A + C) with or without QuantiTect RT mix (Qiagen, Mississauga, Canada). Actin primers (CfActin1 5′-CTT CTA CAA TGA GCT GCG TGT GGC and CfActin2: 5′-CTC GAA CAT GAT CTG TGT CAT CTT C) were used to amplify the control sequence. Serial dilutions of the templates were made and 5, 10, 25, 50, 100 or 250 ng were used in each 25 μl reaction with each set of primers at 50 °C for 30 min; 95 °C for 15 min, 94 °C 15 s, 72 °C 30 s, 79 °C 15 s, 45 cycles. Amplification was monitored and transcript quantification was done according to the manufacturer's protocol (Cepheid, Sunnyvale, CA).

In situ *hybridization*

Diapausing second instar larvae, kept at 4 °C (0 l: 24D) for 30 weeks, were fixed in formalin prior to being embedded in paraffin and 10 μm tissue sections were then made. Hybridization probes were made to specifically detect the CfAFP10 and 337 isoforms by selecting sequences with the lowest percentage of identity to each other for the design of PCR primers. Cf10NotI and Cf10SalI (5′-ATA AGA ATG CGG CCG CGT GAC GTC ACA CCG TGA G; 5′-ACG CGT CGA CAG AGT ATC AGA AAA TAT TGC AC) were used to amplify a 148 bp fragment from CfAFP10 cDNA. Analogously, Cf337NotI and Cf337SalI (5′-TAT TCA TTG CGG CCG CTT ATG GGT CAG TAA GCC GAG; 5′-ACG CGT CGA CCG GTC GAT TCG CCG AGT TTG) were used to amplify a 452 bp fragment from CfAFP337 cDNA. Both reactions were performed at 94 °C for 5 min; 94 °C for 1 min, 55 °C 40 s, 72 °C 45 s, 30 cycles; 72 °C for 10 min and both amplification products were *Not*I/*Sal*I digested and cloned into pBluescript SK II vector, and designated Cf10NS and Cf337NS, respectively. Sense riboprobes were transcribed from *Sal*I-linearized Cf10NS and Cf337NS plasmid DNAs using T3 RNA ploymerase and antisense probes were transcribed from *Not*I-linearized Cf10NS and Cf337NS using T7 RNA polymerase, following the protocol of DIG-RNA labelling kit (Roche, Laval, Canada).

Tissue sections were hybridized with the four probes, following the manufacturer's protocol. Briefly, after treating the slides with xylene, 4% paraformaldehyde–PBS, followed by equilibration in 5x SSC, the slides were prehybridized (2 h) at 58 °C in hybridization buffer (50% formamide, 5x SSC, 40 μg salmon sperm DNA) and hybridized (overnight) at 58 °C in hybridization buffer with one of the four riboprobes at 2 ng/ml. After hybridization they were washed for 30 min in 2x SSC at room temperature, 60 min in 2x SSC, and at increasingly high stringency to 60 min in 0.1 × SSC, 65 °C. After blocking, antidigoxingenin-AP, Fab fragment antibody was diluted to 250-fold in 1 × blocking buffer (Roche) and incubated with the slides for 2 h at room temperature. Subsequently, they were washed and stained with 200 μl 50x diluted NBT/BCIP stock solution overnight and then washed according to standard protocols and mounted in AquaPerm Mounting medium (Immunon™ Thermoshandon, Pittsburgh, USA). The slides were examined at 10X or 40× magnification and pictures were taken using an Olympus BX 51 camera.

Western blot analysis

Eggs and larvae (first to sixth instars) were homogenized in an equal volume of 1 × phosphate buffered saline (PBS), 2 mM phenylmethyl-sulphonyl fluoride and 1 mM phenylthiourea. After centrifugation at 13000 xg for 5 min, the samples were loaded on 18% SDS-PAGE gels (Sambrook *et al*., 1989). The gels were electroblotted on to Immobilon PVDF membrane (Millipore, Nepean, Canada) at 30 V for 3 h, membranes were blocked in 5% skim milk buffer for 8 h and incubated with rabbit anti-CfAFP antiserum (detects short and long isoforms) for 2.5 h, washed in 1 × PBS, 0.05% Tween-20 and incubated for 1 h with goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (Pierce Chemical, Rockford, IL), and subsequently washed to remove excess, unbound antibody. Detection was carried out by reacting the membrane for 2 min with enhanced chemiluminescence reagent (Amersham-Pharmacia, Baie d'Urfe, Quebec, Canada), blotting dry and exposing to Biomax MS film for 10–120 s.

AFP gene promoter identification

A 6797 bp fragment of the CfAFP2.26 genomic clone was used for promoter identification. The 5′-flanking regions as well as the intron and entire coding sequence (with the stop codon, TAG, changed to a non-stop codon, GAA), were cloned into a promoterless vector, pGL3-Basic (PGB), containing the firefly luciferase coding region (Promega, Madison, WI, USA) using PCR. Subsequently, CfAFP2.26 in pBluescript SK II vector (0.01 μg) was used as a PCR template with primer SKT3plus/2.26HindIIIA for the 5′ flanking region and SKT3plus/2.26HindIIIB for the longer product that included the transcribed region (SKT3plus: 5′-CCC TCA CTA AAG GGA ACA AAA GCT G; 2.26HindIIIA: 5′-CAT GAA GCT TGA TAA TTC AGA AAA ATG TAG ACA AC; 2.26HindIIIB: 5′-CAT GAA GCT TCA TTA GCT CTC AAT GTA CAT CCA GAA) at 94 °C for 5min; 94 °C 1 min, 57 °C 1 min, 72 °C 1 min, 3 cycles; 94 °C 40 s, 60 °C 40 s, 72 °C 6 min, 25 cycles; 72 °C for 20 min. The two amplified products were *Hin*dIII-digested and cloned into PGB and designated PGB2.26A5 and PGB2.26B20, respectively. The 5′ flanking region was also ligated to a reporter vector containing the *Renilla* luciferase coding region, pRL-CK (Promega), using *Mlu*I/*Hin*dIII digestion. This latter construct, designated pRL2.26A5, was used as an internal control. Each luciferase gene plasmid containing the AFP promoter region sequence (1.5 μg) and PGB vector (1.5 μg), or control, was co-transfected into a *C. fumiferana* midgut cell line (CF-203 cells; kindly provided by Drs Q. Feng and G. Caputo) with pRL2.26A5 (0.15 μg) using lipofectin reagent (Invitrogen, Burlington, Canada) following the manufacturer's instructions. After 72 h, the cells were collected by centrifugation at 5000 xg and lysed using passive lysis buffer (Promega). Luciferase activity was assayed using the Dual-Luciferase reporter assay system reagents prepared according to the manufacturer's protocol (Promega). As described by the manufacturer, after luminescence measurements, the ratio of firefly luminescence to *Renilla* luciferase activity was determined, and this ratio was then normalized to the control construct, which was arbitrarily assigned a value of 1. Routinely, three replicates of each transfection were performed and each extract was assayed three times for firefly and *Renilla* luciferase activities.

Further promoter analysis used PGB2.26A5. Two more constructs were made by removing the *Apa*I/*Apa*I and *Kpn*I/*Kpn*I fragments

using single restriction enzyme digestions, and the resulting plasmids were designated PGB2.26A5AA6 and PGB2.26A5KK8, respectively. A PCR cloning strategy was then used to make 8 serial smaller insert constructs using PGB2.26A5 as template DNA to generate P1C1 (2.26MluIp1: 5′-ATC GAC GCG TAC ATA CAT AAT TCT GCA AGT CTT TG; 2.26HindIIIC: 5′-AAC CCA AGC TTT TTC ATA AGA ATC AAC AGT GGA ATG), P1D2 (2.26MluIp1: 5′-ATC GAC GCG TAC ATA CAT AAT TCT GCA AGT CTT TG; 2.26HindIIID: 5′-AAC CCA AGC TTG TCA ATA TCG TAA CAT AAC CAG GTA G), P2C2 (2.26MluIp2: 5′-ATC GAC GCG TCA ATA CGC TGA ACT ATG CGA CGA TC; 2.26HindIIIC: 5′-AAC CCA AGC TTT TTC ATA AGA ATC AAC AGT GGA ATG), P2D3 (2.26MluIp2: 5′-ATC GAC GCG TCA ATA CGC TGA ACT ATG CGA CGA TC; 2.26HindIIID: 5′-AAC CCA AGC TTG TCA ATA TCG TAA CAT AAC CAG GTA G), P3A3 (2.26MluIp3: 5′-TCG ACG CGT TAT TAT CAG AGC AAA AAA ATT GC; 2.26HindIIIA: 5′-CAT GAA GCT TGA TAA TTC AGA AAA ATG TAG ACA AC), P3E2 (2.26MluIp3: 5′-TCG ACG CGT TAT TAT CAG AGC AAA AAA ATT GC; 2.26HindIIIE: 5′-TCC CAA GCT TAA CTC TTT TTC ATA AGA ATC AAC AGT G), P4A5 (2.26MluIp4: 5′-ACG ACG CGT AAT ACA AAT TAT AAC ATT CCA CTG; 2.26HindIIIA: 5′-CAT GAA GCT TGA TAA TTC AGA AAA ATG TAG ACA AC), P4E1 (2.26MluIp4: 5′-ACG ACG CGT AAT ACA AAT TAT AAC ATT CCA CTG; 2.26HindIIIE: 5′-TCC CAA GCT TAA CTC TTT TTC ATA AGA ATC AAC AGT G). All reactions were performed at 94 °C 5 min; 94 °C 30 s, 55 °C 30 s, 72 °C, 30 s, 3 cycles; 94 °C 30 s, 57 °C 30 s, 72 °C 30 s, 28 cycles, 72 °C 10 min

An analogous strategy was used for CfAFP2.7 clone promoter identification (not presented, but details are available from the authors). The core promoter construct, PGB2.7MH3, was made using the CfAFP2.7 5′ sequence in the PCR 2.1 vector as an amplification template (0.01 μg) with primers 2.7MluIp1/2.7HindIIIp1 (2.7MluIp1: 5′-ACT ACA CGC GTA TCA GGG CAA TTG CTA TTT ATA T; 2.7HindIIIp1: 5′-ACC TAA AGC TTT CTC ACT TGA AGA GCA GCT G; 94 °C for 5 min, 94 °C 30 s, 55 °C 30 s, 72 °C 30 s, 30 cycles; 72 °C for 10 min).

As indicated, at least three replicates of each transfection were performed as described above. One-way analysis of variance (ANOVA) was done using GraphPad (San Diego, USA).

Hormone response tests

The 5′-flanking region plus intron and coding sequence construct (PGB2.26B20) and a construct containing only the 5′-flanking region (PGB2.26A5) of CfAFP2.26, were selected for analysis. CF-203 cells were cultured and transfected as previously described with either 1.5 μg PGB2.26B20 or PGB2.26A5 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), was added to each well. Hormone treatments with PGB2.26B20 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, and 1 μM 20-hydroxyecdysone. Hormone treatments with PGB2.26A5 were carried out using serial dilutions of 20-hydroxyecdysone and JHI at 0, 0.1, 0.5, 1.0, 10, and 50 μM. Cells were collected 24 h after adding the hormone, centrifuged at 12000 xg for 1 min and the supernatants discarded. Lysis buffer (250 μl) was added to the cell pellet and gently shaken for 30 min at room temperature. After recentrifuging (1 min at 12000 xg), the supernatants of the cell lysates were assayed for luciferase activity as previously described. Three replicates of each transfection were performed. ANOVA was done as described above.

3′ UTR half life assays and analysis

CfAFP337 (AF263009), a cDNA with long-3′UTR, and CfAFP339 (AF286207), a cDNA with short-3′UTR, were selected for half-life analysis. The 3′UTR of CfAFP337 was PCR-amplified using a 337XbaIP primer (5′-GAG CTT CTA GAG CCA TGA AAG TCG TCC GAG ATT G) and SKFseIB primer (5′-TGT CAG AGG CCG GCC AGT GAG CGC GCG TAA TAC GA) with CfAFP337 cDNA in pBluescript SK II as the template. PCR was performed (94 °C 5 min, 94 °C 30 s, 62 °C 30 s, 72 °C 60 s, 30 cycles, 72 °C 10 min) and the products were digested with *Xba*I/*Fse*I, cloned into PGB2.26A5, and designated 337UTR. The 3′UTR of CfAFP339 was amplified using *Fse*I site primers (339P1: 5′-AGA GCT AGG CCG GCC ATG AAA GTC GTC TGA AAC T and 339P2: 5′-AGC TGC TGG CCG GCC TAT GGT AAA AAA ATG TAT CTC AAA ATT, 94 °C for 5 min; 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, 3 cycles; 94 °C 30 s, 65 °C 30 s, 72 °C 60 s, 25 cycles; 72 °C for 10 min). The amplification product was digested with *Fse*I, cloned into PGB2.26A5, and designated 339UTR. A control plasmid, with the *Mlu*I/*Hin*dIII region of pRL-TK (Promega) replaced with a *Mlu*I/*Hin*dIII fragment from PGB2.26A5, was designated pRLA5.

CF203 cells were co-transfected with (1) 10 μg 337UTR and 1 μg pRLA5 control plasmid and (2) 9 μg 339UTR and 1 μg pRLA5 plasmid (the concentration difference ensures that the numbers of plasmid molecules were standardized in the transfections), as previously described. Aliquots of co-transfected cells were first collected 20 h post-transfection (termed sample time 0) when actinomycin D (20 μg/ml; Sigma, Oakville, Canada) was added to the transfected cells. Sampling was done immediately and after 3, 6, 10, and 24 h. RNA was isolated as described for real time PCR analysis and quantitified using the RiboGreen RNA Quantification kit (Molecular Probes, Burlington, Ontario, Canada). A QuantiTect SYBR Green RT-PCR kit (Qiagen) was used to monitor the mRNA degradation rate with serial time points. To avoid the detection of endogenous mRNA, primers were designed based on the sequence of the transfected plasmid's luciferase gene (Forward primer, Flucp3: 5′-GTA CTT CGA AAT GTC CGT TCG GTT G and reverse primer, Flucp2: 5′-GCA ACT GCA ACT CCG ATA AAT AAC G), which amplified a fragment of 144 bp. Serial dilutions (0.2, 2.5, 25, 40, pg/ml) of plasmid 337UTR were used to generate a standard curve, with 1 μg of total RNA used in each assay. Real time RT-PCR was performed in 25 μl reactions and the following conditions: a 2-temperature cycle repeat (50 °C for 20 min and 94 °C for 15 min) followed by a 4-temperature cycle, repeated 45 times (95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s, 77.8 °C for 15 s) using the optics option. A minimum of three replications of each transfection experiment were performed followed by statistical analysis (ANOVA).

The sequences of the long and short-3′UTR were compared using RNA Analyser, http://wbbi001.biozentrum.uni-wuerzburg.de/server.html (Bengert & Dandekar, 2003).

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