Vol. 214, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 5, 1995 Pages 286-294

Functional identification of the transcription start site and the core promoter of the juvenile hormone esterase gene in *Trichoplusia ni*

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Received August 1, 1995

SUMMARY: The juvenile hormone (JH) esterase gene in *T. ni* encodes a protein that is responsible for the degradation of JH. The 5' structural characterization of this JH-sensitive gene was accomplished using reverse transcription PCR (RT-PCR) and northern analysis. The transcriptional start site of the JHE gene was biochemically determined by two methods: a 5' rapid amplification of cDNA ends (RACE) procedure which produced independent products with a sequence identical to the sequence of an exon encoded 5' to the putative first intron and by northern analysis with intronic and exonic probes. Both the transcription start site and the region containing the core promoter were also functionally identified by use of an *in vitro* transcription assay. The product of the *in vitro* transcription reaction, under the control of the putative core promoter region, initiated at the same base as identified by the RACE procedure, whether the reaction was driven by lepidopteran or by dipteran nuclear extracts. This result is the first functional identification of the core promoter region and transcription start site of any JH-sensitive gene.

The investigation of gene regulation by hormones is a vital area of study. Arthropods in general, and insects that metamorphose specifically, provide an excellent model systems to test the mechanisms used in hormonal gene regulation. This advantage in insects exists because hormonal gene regulation during the metamorphic change is dictated mainly by two hormones, juvenile hormone (JH) and ecdysone (1,2).

Juvenile hormone esterase (JHE) is a gene which plays a major role in the developmental decisions of *Trichoplusia ni* (3). The JHE gene in *T. ni* encodes a protein, synthesized in the fat body and distributed through the hemolymph, which is responsible for the degradation of JH to JH acid (4). While each molt is initiated by a pulse of ecdysone, it is the JH titer which determines the type of molt that will occur. If the JH titer is high, the result is a larval to larval molt. If the JH titer is low, the result is a larval to pupal molt.

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0006-291X/95 $12.00

Juvenile hormone esterase has been shown to be induced by two stimulants. Within the final larval stadium, JHE is induced on the second day by neurosecretions from the brain (5). Additionally, on the fourth day of the instar, JHE is induced directly in the fat body by JH (6). Furthermore, the induction by JH has been shown to be due to an increase in the abundance of the mRNA and an increase in the rate of transcription (7).

We report here the structural and functional identification of the transcriptional start site as well as the structural characterization of the first exon and first intron. The structural identification of the transcription start site was accomplished using two independent biochemical methods: RACE and northern analysis. *In vitro* transcription reaction, driven by lepidopteran or dipteran nuclear extracts, resulted in initiation at the same base as identified by the RACE procedure. This is the first structural and functional characterization of a core promoter of any JH-sensitive gene.

MATERIALS AND METHODS

Insect treatment. Larvae of *Trichoplusia ni* were reared and staged as described previously (5). The nomenclature used to identify the developmental stage is the number of the instar, followed by the day of the stadium (e.g. 5th instar, day 2 = L5D2).

RNA preparation. Total RNA was extracted from *T. ni* larvae as described (8). Poly (A+) RNA was purified by oligo (dT) cellulose chromatography as previously described (9).

Isolation and sequencing of the 5' region of the JHE gene. The genomic clone had been isolated as previously described (7). DNA sequencing was performed as previously described (10).

Northern blot analysis. Equal volumes of the same poly (A+) RNA sample from late L5D1 larvae were electrophoresed on a formaldehyde 1.2% agarose gel. After electrophoresis, the gel was rinsed in water 4 times for 15 minutes each. The gel was then stained with ethidium bromide, photographed, and the poly (A+) RNA then transferred onto nitrocellulose membrane. After ultraviolet irradiation with a Stratagene crosslinker, prehybridization and hybridization took place with the conditions and solutions as previously described (11). The filter was probed at 60°C with a ^{32}P-labelled oligonucleotide. The filter was then washed and exposed as described (11). Following are the sequences of the oligonucleotides used: Probe 1: 5'TTATCTAA GGACCTCCAGAGAGACCACATGTATGGAGAAGATACTAATACCACGCGCTCT3' (-250 to -196), probe 2: 5'GCGCCGTACCAAAATGTCATTCCTAGAAGAAGCACCGG CCATAATTTCCACAAG3' (-68 to-11), Probe 3: 5'GGGTAGGAGGGCGACGTCCGAA GGGCGTCATGTCTGGTCGGGGTCGCAC (+11 to +59), Probe 4: 5'ACTGAAAACGT ACCGACCTAGACCTCTTATCATAGTATCAGTGGACACAATTAG3' (intronic sequence).

Rapid amplification of cDNA ends. Minor modifications to procedure in the commercially available Gibco-BRL 5' rapid amplification of cDNA ends kit are noted as follows. Four µg of poly (A+) RNA from late L5D1 larvae were used as a template for the first strand cDNA synthesis. The following oligonucleotide was also used to prime the first strand synthesis: 5'GCCGGTACCGACAGACACGACGGCGCCTCCGCGT3' (+105 to +130). The first strand was tailed using dCTP. Due to the low JHE transcript abundance, it was necessary to include [α-^{32}P]dCTP to the PCR reaction for detection of the PCR product. One of the two oligonucleotides used in the PCR reaction was a 22 base oligo (dG) with an engineered Kpn I restriction site. The other oligonucleotide was: 5'CTTGCGGACGCTCGCCCTCCTACTC3'

(+36 to +60). The radioactive PCR product was electrophoresed on a 15% polyacrylamide gel and the product was removed from the gel, raised in 80µl TE (10mM Tris, 1mM EDTA) (pH 8.0), crushed and eluted at room temperature for three hours. Twenty µl of the elution was used as template for PCR. PCR was performed as according to the commercially available PCR reagent system of Gibco-BRL.

Reverse transcription-PCR (RT-PCR). Six ug of late L5D1 larvae poly (A+) RNA served as template for a reverse transcription reaction. The sequence of the oligonucleotide primer used in the reverse transcription reaction was: 5'GCCGGTACCGACAGACACGA CGGCGCCTCCGCGT3' (+105 to +130, Kpn I). This primer was also used in the subsequent PCR reaction. The other oligonucleotide PCR primer used was 5'CTAGTCTAGAAGACAT-GACGCCTTGCGGACG3' (+45 to +24, Xba I). The conditions of the PCR reaction are as described above. After 30 cycles, another 1µl of Taq polymerase was added, and the reaction continued for an additional 30 cycles.

Cloning of PCR products. Immediately after the PCR reaction, 1u Klenow fragment was added, and allowed to fill in the ends of the PCR products at room temperature for one hour. After phenol/chloroform extraction, and ethanol precipitation, the product was resuspended in water and digested with the restriction enzymes which corresponded to the engineered restriction sites. Upon completion of digestion, phenol/chloroform extraction was performed followed by ethanol precipitation. The product was electrophoresed on a 2% low melt agarose gel, excised, phenol/chloroform extracted, and then ethanol precipitated. The product was then ligated in Bluescript S/K+ vector, digested with Kpn I and Xba I. The cloned PCR product was verified by sequencing.

In vitro transcriptional analysis. *In vitro* transcription was performed using either *Drosophila* embryo nuclear extract (Promega) or prepared SF9 lepidopteran cell line nuclear extract. The *in vitro* transcription reaction was incubated at 30°C for one hour. The contents of the reaction with the *Drosophila* extract are as follows: 1X transcription buffer (Promega), 1.5mM rNTP, 4mM creatine phosphate, 5u *Drosophila* embryo nuclear extract (Promega). If α-amanatin was added, the final concentration was 0.1 µg/ml. The SF9 *in vitro* transcription reaction conditions were similar with the following modifications. The transcription buffer and *Drosophila* embryo nuclear extract were replaced with 15 µl of SF9 cell line nuclear extract. After completion of either reaction, phenol/chloroform extraction followed the incubation. The product was ethanol precipitated and resuspended in water. An antisense oligonucleotide corresponding to the coding sequence of the luciferase reporter transcript that was end-labelled with ^{32}P was hybridized to the transcripts in the presence of hybridization buffer (250mM Tris pH 8.3, 200mM KCl, 2.5mM EDTA pH 8.0). Hybridization proceeded at 58°C for 20 minutes, followed by incubation at room temperature for 10 minutes. The primer extension reaction was initiated by the addition of reverse transcriptase and incubated at 37°C for 30 minutes. The final components of the primer extension reaction were as follows: 0.5mM dNTP, 10mM $MgCl_2$, 1mM DTT, and 15u reverse transcriptase. Next, 80% formamide was added and after mixing incubated at 85°C for 2 minutes. The reaction was loaded onto a 6% polyacrylamide denaturing gel. After electrophoresis , the gel was transferred to 3M paper, and dried under a vacuum at 80°C for 2 hours. The gel was exposed as previously described (11).

Preparation of Lepidopteran cell line nuclear extract. Cells were harvested by centrifugation then washed once in PBS. After centrifugation and resuspension in 10mls T.E. (10mM Tris, 1mM EDTA) (pH 8.0), the cells were homogenized manually. Centrifugation yielded a pellet which was resuspended in 20 mls extraction buffer and homogenized again. After homogenization, one volume of saturated $(NH_4)_2SO_4$ was added and stored at 4°C for 30 minutes. Ultracentrifugation at 46000 rpm in a TY65Ti rotor for 3 hours yields a pellet. The supernatant is removed and 0.33 g/ml of $(NH_4)_2SO_4$ is added and incubated at 4°C for one hour. Centrifugation at 8000 rpm in a SS34 rotor for 20 minutes produced a pellet to which 1ml dialysis buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 2 mM DTT) was added. Dialysis was performed overnight in a volume of 100 mls dialysis

buffer. After ultracentrifugation at 40000 rpm in a TY65Ti rotor for one hour, the supernatant is removed and frozen immediately in liquid nitrogen.

JHE core promoter construction. PCR cloning was used to prepare the core promoter construct. Oligonucleotide primers placed at the boundary of the core promoter sequence contained engineered restriction sites, Kpn I and Bgl II, for the subsequent insertion of the PCR product into the PGL3 basic vector (Promega). A genomic clone containing the promoter sequence was used as template for the PCR reaction. The PCR conditions were identical to those described above.

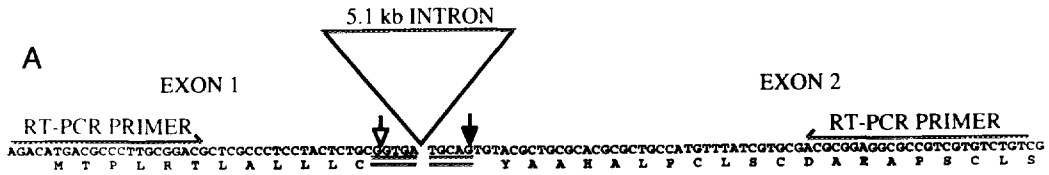
RESULTS AND DISCUSSION

Primer extension of the JHE transcript which had been performed provided no conclusive results. The extension product signal on autoradiograms was not sharp, possibly due to pausing of the enzyme arising from secondary structure of the transcript (data not shown).

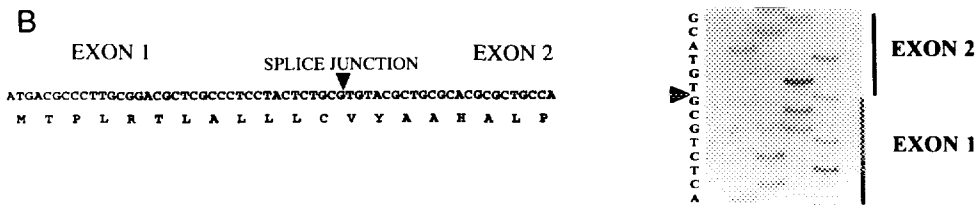
The genomic sequence 5' to the position of the primer extension stop was analyzed and an ATG codon was located that was followed by codons for primarily hydrophobic amino acids. An oligonucleotide made to this region was used with a reverse primer made against the coding region for the mature protein in an RT-PCR approach, using first strand cDNA to larval mRNA as template (Fig. 1). Subsequent sequencing of the cloned PCR product and comparison with the genomic sequence demonstrated that the open reading frame of apparent exon 1 had been joined without interruption to apparent exon 2, to yield an open reading frame encoding a signal peptide, which then continued into the amino terminus of the mature protein (Fig. 1).

With the identification of apparent exon 1, it was necessary to firmly identify its 5' boundary. Therefore 5' rapid amplification of cDNA ends (RACE) was performed. An oligonucleotide was used to prime a reverse transcription reaction and the cDNA was C tailed and subsequently used as template for PCR (Fig.2). After cloning of the PCR product, the sequence of the RACE product identified the transcriptional start site to be 27 nucleotides 5' to the inferred translation start codon. The sequencing of multiple RACE products did not provide any evidence of more than one transcription start site (Fig.2). The RACE product identified a CA motif to be present at the 5' end of transcripts. Such a motif is commonly present at eukaryotic transcription start sites, and the GCAGT motif located at the initiation site has been described as an arthropod transcription initiator element (13). TCAGT has been identified as the primary arthropod initiator sequence do to its overrepresentation in arthropod promoters. Its cognate, GCAGT, is less , although significantly, overrepresented as well. The arthropod initiator, as initiator sequences in general, is described to function as a possible "landing pad" for transcriptional machinery.

The boundaries of the first exon were verified through a second experimental technique. The 2.8 kb JHE transcript was probed in northern analysis with oligonucleotide primers which



SCHEMATIC OF RT-PCR STRATEGY



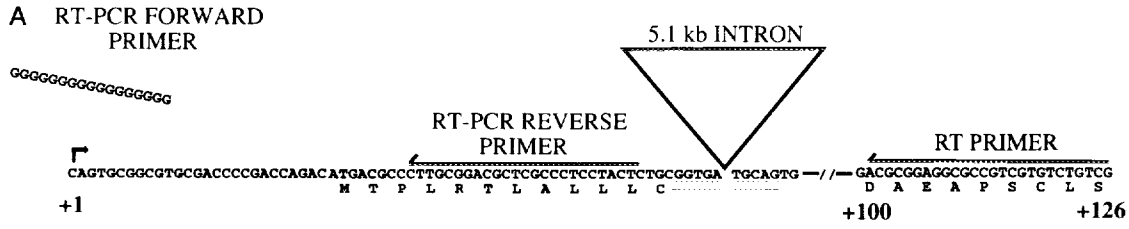
SEQUENCE OF RT-PCR PRODUCT FROM LARVAL mRNA

Figure 1. Reverse transcription PCR (RT-PCR) of larval mRNA to determine splice junction. (A) Diagram of the RT-PCR strategy used to verify the splice junction demarked by the double underlined consensus splice sites (12) bracketing the intron. Genomic sequences corresponding to the oligonucleotide primers are overlined, the consensus acceptor site is marked by the filled arrow, and the consensus donor site is marked by the hollow arrow. (B) Sequence of the RT-PCR product. The open reading frame is conserved from the first exon into the second exon. The splice junction is marked by a black arrow.

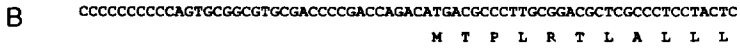
corresponded to different locations in the JHE gene (Fig. 3). Two oligonucleotide probes were located 5' of the RACE-identified 5' boundary of the first exon, i.e. 5' to the putative transcriptional start site. Both of these probes failed to hybridize to the JHE transcript (Fig. 3).

A probe designed against the first putative intron also failed to hybridize to JHE transcript. However, hybridization was demonstrated with an oligonucleotide probe located entirely within the defined boundaries of the putative first exon (Fig. 3). Thus independently confirming our placement of the short first exon 5' to the 5.1 kb first intron. With the report of a 4.3 kb intron in the 5' region of the *Heliothis virescens* JHE gene, this large first intron could possibly be an evolutionarily conserved character of the genomic organization of JHE genes (14), although a difference of 800 bases in the length of this intron between these two so closely related species is quite large. Further, we have located an intron in the *T. ni* genomic sequence starting at approximately base +250 that was not reported for the *Heliothis virescens* JHE gene.

While the transcription start site had been identified, and verified by a second experimental technique, it is important to demonstrate that the putative start site is transcriptionally func-



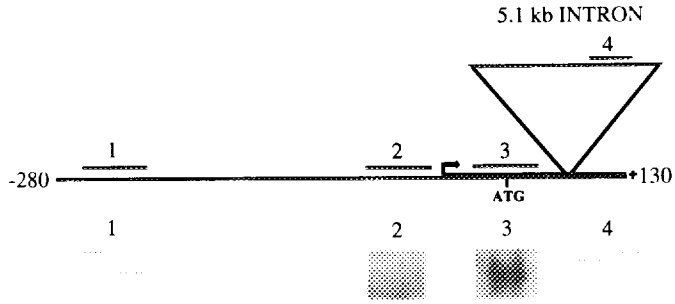
SCHEMATIC OF RACE STRATEGY



SEQUENCE OF RACE PRODUCT

Figure 2. 5' rapid amplification of cDNA ends (RACE) to identify the transcription start site. (A) Diagram of the experimental strategy. The genomic sequences corresponding to the reverse transcription primer and PCR primer are overlined, while the oligo (dG) forward primer is indicated above the inferred transcription start site. The consensus splice sites are double underlined and shown for orientation purposes. Nucleotide positions are marked underneath the nucleotide sequence in reference to the inferred transcription start site. (B) Sequence of the RACE product. The poly C tail is shown 5' of the transcription start site.

tional. A core promoter construct (-61 to +28) was prepared and used for *in vitro* transcriptional analysis. As seen in Figure 4, panel A, SF9 nuclear extract along with other components necessary for transcription allowed the JHE core promoter to directly function to actively transcribe the reporter luciferase coding sequence. It is also clear that the transcripts were produced by RNA polymerase II since the transcriptional activity were inhibited with α-amanatin (Fig. 4).



NORTHERN ANALYSIS OF THE 2.8kb LATE L5D1 JHE TRANSCRIPT USING 4 OLIGONUCLEOTIDE PROBES

Figure 3. Northern analysis of the 2.8 kb JHE transcript. Oligonucleotide primers corresponding to different genomic locations, numbered 1 to 4 and underlined were used to probe late L5D1 larval mRNA. The bent arrow represents the biochemically identified transcription start site (Fig. 2). The thick black line represents the sequence corresponding to the JHE transcript. The hatch mark above the ATG identifies the position of the presumed start codon. Hybridization is demonstrated by probe 3, while the other probes, located in regions not corresponding to transcript sequence, failed to hybridize.

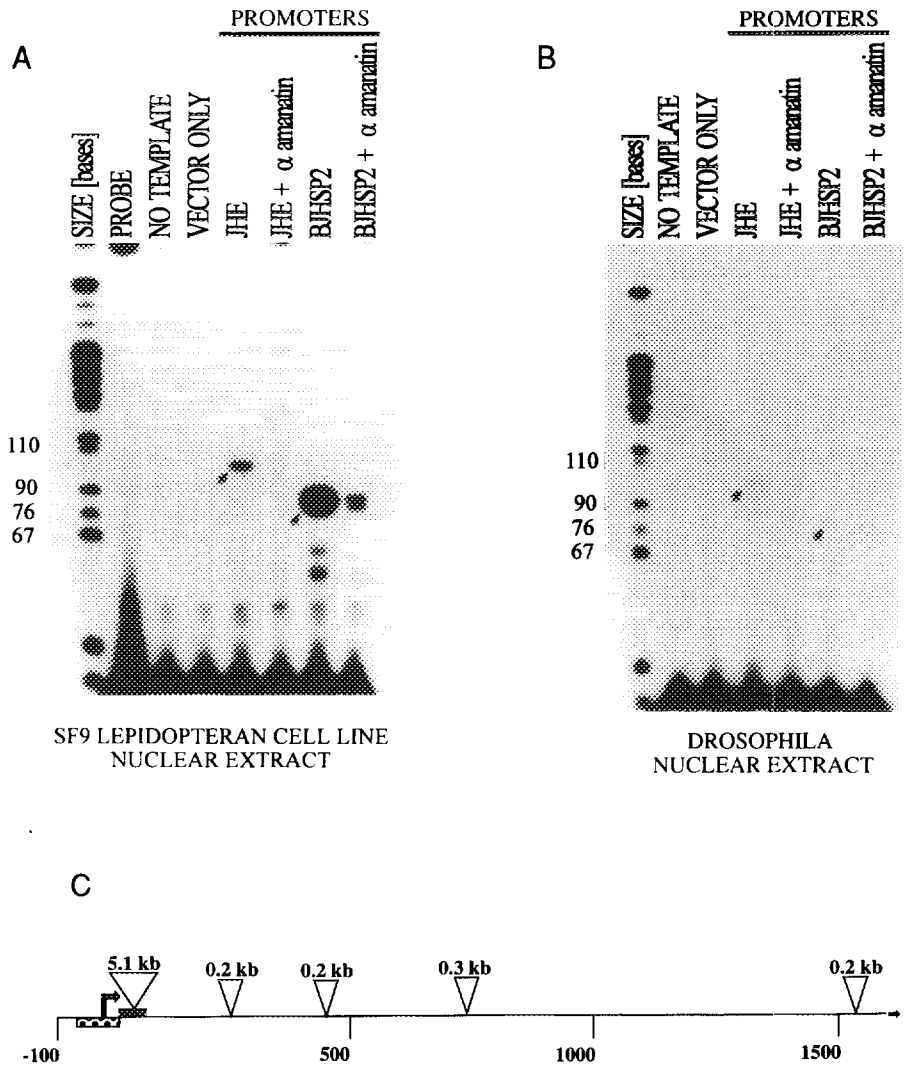


Figure 4. *In vitro* transcriptional assay to demonstrate the function of the JHE core promoter. Initiation and extension directed by the JHE core promoter and the BJHSP2 promoter (serving as a positive control) are marked as promoters. Arrows mark the primer extended transcriptional products, which are sensitive to α-amanatin. (A) Transcriptional analysis of the JHE core promoter driven by SF9 nuclear extract. (B) Transcriptional analysis driven by *Drosophila* embryonic nuclear extract. (C) A diagrammatic representation of the genomic organization of the 5' region of the JHE gene. The black box marks the presumed signal peptide. The stippled box represents the core promoter, and the bent arrow marks the transcription start site. Positions of introns are shown by triangles, with the approximate size of the intron indicated above each. The arrow denotes the continuation of the coding region.

The position of migration of the extension product for the *in vitro* produced JHE transcript corresponds to the size of a product which would have a transcription start site identical to that which we identified by RACE (Fig. 4). On a sequencing gel, the primary extension product

migrated to a position which corresponds to the C nucleotide of the motif GCAGT (not shown). A minor portion of the extension products migrated faster to a position corresponding to position #2 of the transcript.

The core promoter, functional in an essentially homologous system, was then tested with a heterologous nuclear extract from *Drosophila* embryos (Promega). As had been demonstrated in Fig. 4A, the core promoter drove transcription and the primer extended products of the heterologous system were of the same size as their homologous system counterparts (Fig. 4B). The transcriptional machinery provided by the *Drosophila* nuclear extract did not have as dramatic of transcriptional activity as seen in the homologous system. However, it did independently demonstrate that the JHE core promoter contains sequence capable of directing the binding of the minimal transcriptional apparatus.

Since the JHE gene is inducible by two possibly different transcriptional activators, one being neurosecretions from the brain prior to metamorphic commitment and the other being JH after metamorphic commitment, there may be other unidentified response elements present in either the core promoter, or other 5', 3', or intronic locations. Further experimentation is necessary to identify and locate the response element(s) which direct the JH-mediated transcriptional induction of the JHE gene. It remains clear that a gene which is transcriptionally induced by a hormone, such as JHE, must contain elements within its DNA sequence which allow a JH-sensitive transcription factor to bind that affects the rate of transcription of the basic transcription apparatus.

Acknowledgments. We thank Dr. Davy Jones for his timely and comprehensive review of this manuscript. This study was supported by grant DK39197.

REFERENCES

1. Nijhout, H. F. and Wheeler, D. E. (1982) *Q. Rev. Biol.* 57, 109-133.
2. Riddiford, L. M. (1994) *Adv. Insect Physiol.* 24, 213-274.
3. Jones, G. (1985) *Entomol. Exp. Appl.* 39, 170-176.
4. Venkatesh,K., Abdel-Aal,Y.A.I., Armstrong,F.B., Roe,R.M. (1990) *J. Biol. Chem.* 265, 21727-21732.
5. Jones G., Wing K. D., Jones D. and Hammock B. D. (1981) *J. Insect Physiol.* 27, 85-91.
6. Jones, G., Jones, D. and Hiremath, S. (1987) *Insect Biochem. Molec. Biol.* 17, 897-904.
7. Venkataraman V., O'Mahony P. J., Manzcak M. and Jones G. (1994) *Dev. Genet.* 15, 391-400.
8. LeMeur M., Glanville N., Mandel J. L., Gerlinger P., Palmiter R. and Chambon P. (1981) *Cell* 23, 561-571.
9. Desrosiers R., Friderici K. and Rottman F. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 3971-3975.

10. Sanger F., Nicklen S. and Coulson A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 80, 3963-3965.
11. Jones G., Brown N., Manczak M., Hiremath S. and Kafotos F. C. (1990) *J. Biol. Chem.* 265, 8596-8602.
12. Mount, S. M., Burks, C., Hertz G., Stormo, G. D., White, O. and Fields, C. (1992) *Nuc. Acids Res.*20, 4255-4262.
13. Cherbas, L. and Cherbas, P. (1993) *Insect Biochem. Molec. Biol.* 23, 81-90.
14. Harshman, L. G., Ward, V. K., Beetham, J. K., Grant, D. F., Grahan, L. J., Zraket, C. A., Heckel, D. G.and Hammock, B. D. (1994) *Insect Biochem. Molec. Biol.* 24, 671-676.