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Developing a Gene Specific Probe for Phosphoenolpyruvate Carboxykinase mRNA

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Developing a Gene Specific Probe for Phosphoenolpyruvate Carboxykinase mRNA


**by
Stephen M. Kuntz**

**Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biology from the
Department of Biology of Seton Hall University
May, 99**

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Finally, this thesis has given me a new-found respect for people who dedicate their lives to scientific research, a field that often times provides more questions than answers. Although I have left my scientific roots behind to entertain a management career in manufacturing, I will always remember the failures and successes of the laboratory.

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Abstract

Phosphoenolpyruvate carboxykinase (pepck) is the structural gene for the enzyme phosphoenolpyruvate carboxykinase (PEPCK). This enzyme plays an integral role in carbohydrate metabolism. The focus of this research project was to develop a gene specific probe for PEPCK mRNA in *Chironomus riparius* with the overall goal of using the probe as a biomarker for exposure to environmental contaminants. Both *Drosophila* (Oregon strain) and *Chironomus riparius*, an EPA approved organism for sediment toxicity testing, were investigated in this study. *Drosophila* DNA and mRNA served as controls, since pepck has been sequenced in this organism and not in *Chironomus*. DNA from both insects was isolated and purified. Primers specific for pepck were employed in the polymerase chain reaction (PCR) to verify the gene sequence in *Drosophila* and amplify it in *Chironomus*. Several PCR fragments from *Chironomus* were generated and sequenced but none showed significant homology to pepck. Subsequently, *Drosophila* larvae were exposed to 0.1mM and 1.0 mM concentrations of dexamethasone, a glucocorticoid known to induce pepck transcription. The purpose of this approach was to find a concentration of dexamethasone that might induce pepck in *Chironomus* leading to the identification of the gene through reverse PCR. Dexamethasone exposure times were one, three and six hours after which the organisms' RNA was isolated and

purified. The concentration of mRNA was then determined using a P³² - end labeled *Drosophila* pepck probe in solution hybridization. Solution hybridization results for the three time trials showed that dexamethasone had an acutely toxic effect on the *Drosophila* larvae.

Introduction

The scope of this research focused on identifying a gene specific probe for pepck mRNA in the non biting midge *Chironomus riparius*. The overall goal of the project was to utilize the probe as a non-specific biomarker for exposure to environmental contaminants.

The aquatic larvae of *Chironomus riparius* (Figure 1) are indigenous to freshwater ecosystems. Chironomid larvae reside within surficial sediments (upper 10 cm) of freshwater communities (Bervoets *et al.*, 1996). The larvae burrow into the upper sediment layer where they feed on particulate matter. The sediment layer acts as a reservoir for heavy metals and other pollutants. The chironomids' close association with the sediment makes them particularly susceptible to anthropogenic pollutants such as heavy metals. Their susceptibility to pollutants and broad ecological range makes them ideal candidates for use as environmental indicators (Dickman and Rygiel, 1996). In this capacity they serve as a tool for detecting and assessing the effects of environmental contaminants (Figure 1).

Experimental studies have been conducted to evaluate the effects of heavy metals and other pollutants on *Chironomus sp.* and other insects. Dickman and Rygiel, 1996, adopted a holistic approach using larval deformity

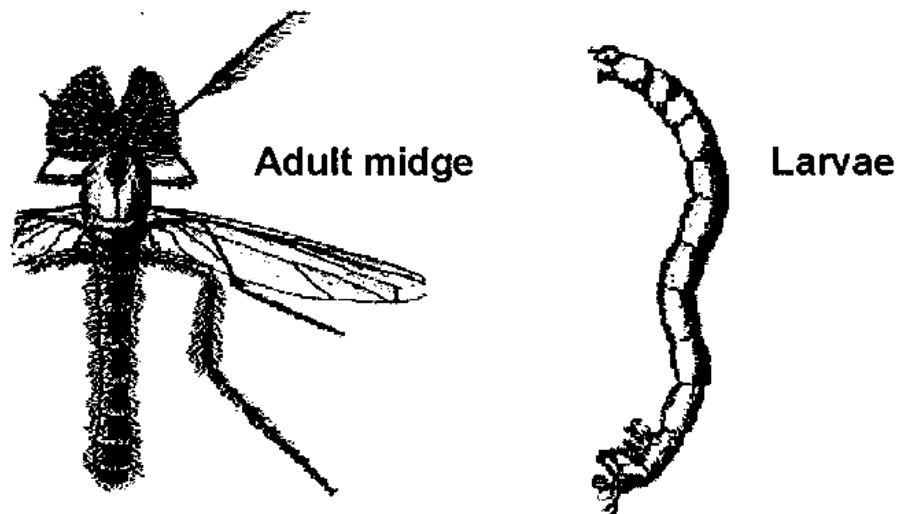


Figure 1. Depiction of *Chironomus riparius* (Borror and White, 1970).

and mortality as observable effects for exposure to heavy metals. Maroni *et al.*, 1986, used an alternative approach to study the effects of heavy metals on *Drosophila* larvae. Instead of focusing on the whole organism, Maroni *et al.*, 1986 concentrated specifically on a metallothionein cDNA to examine the organism's response to heavy metals. Metallothionein plays a protective role; the enzyme is responsible for binding toxic heavy metals such as cadmium. Additionally, environmental pollutants have been shown to induce a stress protein gene in the freshwater sponge *Ephydatia fluviatilis* (Müller *et al.*, 1995). Studying the effects of pollutants on specific genes and enzymes may eventually lead to the discovery of useful biological indicators. This project focused on using a pepck probe as a potential biomarker.

Pepck plays an integral role in carbohydrate metabolism. The enzyme is the rate-limiting enzyme in gluconeogenesis (Nebes and Morris, 1987). PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (PEP), an essential precursor for the anabolic reactions that yield glucose via gluconeogenesis (Figure 2). Insects utilize PEPCK in conjunction with pyruvate carboxylase to catalyze the conversion of pyruvate to PEP (Chippendale, 1978). In addition to the enzyme's role in gluconeogenesis, it is also capable of catalyzing the reaction in reverse, converting PEP to oxaloacetate. This anaplerotic reaction fuels the citric acid cycle, ultimately

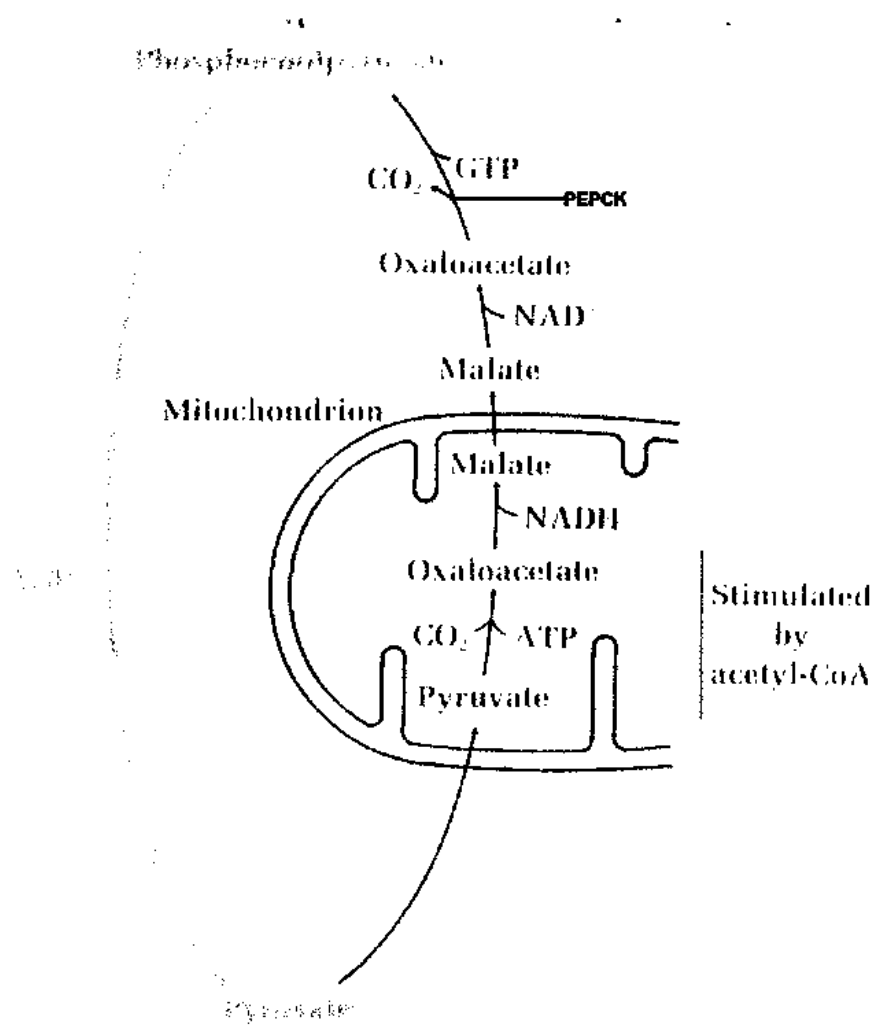


Figure 2.

PEPCK converts oxaloacetate to phosphoenolpyruvate. PEPCK is responsible for the catalytic conversion of oxaloacetate into phosphoenolpyruvate. (Lehninger, 1982).

generating energy (Lehninger, 1982). Pepck is a plausible candidate for use as a biological indicator because of its intimate involvement in carbohydrate metabolism. Heavy metals alter carbohydrate levels and exert undue environmental stress on organisms (Ortel, 1995). Organisms inhabiting polluted environments utilize carbohydrate metabolism to generate the energy necessary to stave off toxic insults and maintain homeostasis. Pepck expression is influenced by carbohydrate metabolism and pepck induction may be indicative of an organism's exposure to toxic levels of heavy metals.

In the vertebrate model, expression of pepck is primarily under hormonal regulation. Glucagon enhances pepck transcription, while insulin has an inhibitory effect. These hormones act on the gene via transcriptional factors that bind to regulatory elements (Figure 3) located on the pepck promoter (Wolf, 1994). Additionally, glucocorticoids bind to glucocorticoid receptors, located within the pepck promoter, to induce the gene (Wolf, 1994). Insulin has been identified in *Drosophila melanogaster* (LeRoith *et al.*, 1981) and has been implicated as an inducer of embryonic nervous system development in *Drosophila* (Pimental *et al.*, 1996). It is hypothesized that invertebrate insulin and invertebrate glucocorticoids would have regulatory effects on pepck similar to those observed in the vertebrate model.

Dexamethasone is a synthetic glucocorticoid that has anti-inflammatory and immunosuppressive effects. Dexamethasone is lipid soluble and diffuses

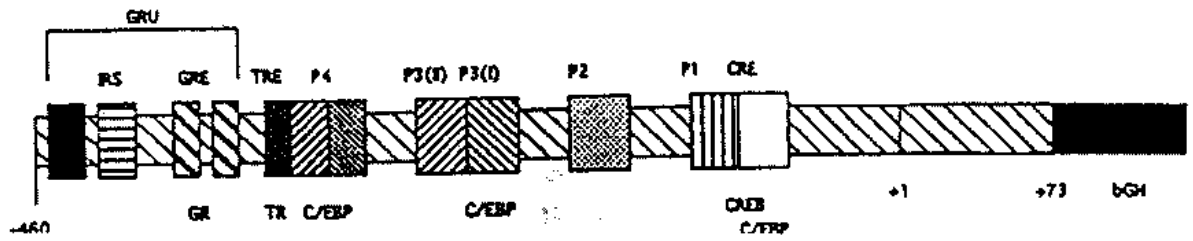


Figure 3.

Various protein-binding sites are located on the pepck promoter. Each box represents a regulatory element. This version of the pepck promoter is the murine model (Wolf, 1994).

through cellular membranes eventually binding to cytoplasmic glucocorticoid receptors. These receptor complexes bind to glucose response elements in various genes, including pepck, and induce gene expression (Goldfien, 1992). Dexamethasone has been used in numerous studies to induce pepck (Nebes and Morris, 1987; Sutherland *et al.*, 1995; Watford and Mapes, 1990). Dexamethasone will be employed in this study to induce pepck expression and assess the effectiveness of a primer designed specifically for pepck mRNA.

Materials and Methods

Organism Maintenance

Chironomus riparius larvae were a gift from Dr. Alan McIntosh of the University of Vermont. They were maintained at 20-22°C in the laboratory with a light cycle of 12 hours light and 12 hours dark. Their water was particle and carbon filtered at a pH of 7.1 and a hardness of 130 mg/L. Their substrate consisted of acid-washed sand and Cerophyll (Ward's Natural Science, Rochester, NY). Food consisted of crushed Tetra DoroGreen (TetraWerke, Germany) and was administered biweekly.

Drosophila strain, Oregon, were a gift from Dr. Elliott Krause of Seton Hall University. They were maintained in an incubator at 23°C on FORMULA 4-24 instant drosophila medium BLUE (Carolina Biological Supply Company, Burlington, NC).

DNA Isolation

DNA was isolated by modifying the protocol outlined in the SIGMA TRI-REAGENT™ Technical Bulletin MB-205, For Product No. T-1924, June 1995. Prior to the addition of Tri-Reagent (Sigma BioScience, MO), organisms were homogenized in 1 ml proteinase K buffer [5M NaCl, 25% SDS, 1M Tris (pH 7.4), 0.5 M EDTA pH 8.0] and centrifuged for 1 minute at 2000 rpm. A 100 µl volume of Proteinase K stock (2 mg of proteinase K enzyme in 1 ml of proteinase K buffer) was added to the supernatant and the samples were incubated at 50°C for 3 hours. Samples were divided into 250 µl aliquots and 750 µl volumes of Tri-Reagent (Sigma BioScience, MO) were added to each sample and processed according to the technical bulletin. The DNA pellet was then precipitated and exposed to a series of equal volume extractions (500 µl) using TE (10 mM Tris/HCL, 1 mM EDTA pH 7.4), CI (24:1 Chloroform to iso amyl alcohol) and PCI (1:1 buffered phenol to CI). Following extraction pellets were dissolved in dd H₂O and combined. DNA concentrations were determined using a spectrophotometer (Pharmacia LKB-Ultrospec III).

Primer Generation

Primer development was conducted utilizing the programs and databases available in the PCGene software. Aligned pepck sequences from various

invertebrates including, *Drosophila melanogaster*, *Ascaris suum* and *Haemonchus contortus* were used to search for sequence homology. Aligning the sequences from these organisms (Figure 4) identified consensus sequences. These sequences were utilized to develop four primers, an inside and outside primer set. The primers were synthesized by Oligos Etc. (Oligos Etc., NJ). The outside primers were designated KP25 and KM44 (no figure) and the inside primers were designated KP9 and KM37 (Figure 4). See the appendix section for primer data.

Polymerase Chain Reaction

Polymerase chain reactions were conducted following standard laboratory techniques using a GIBCOBRL PCR Reagent System (GIBCO-BRL, Gaithersburg, MD). Reactions were run on a Perkin Elmer Gene Amp PCR System 2400, thermal cycler (Perkin Elmer, CA). *Drosophila* genomic DNA (.158 µg/µl) and primer sets (20 pmol/µl) were run under the following cycle conditions: (1) Heat @ 94°C for 4 min (2) 40 cycles of 94°C @ 30 sec, 58°C @ 2 min (3) 72°C @ 3 min (4) 4°C until retrieved. Bands resulting from the genomic PCR were purified from gels (below), diluted 1:1000 using dd H₂O, and reamplified as follows: (1) Heat @ 94°C for 4 min (2) 35 cycles of 94°C @ 30 sec, 58°C @ 30 sec, (3) 72°C @ 3 min (4) 4°C until retrieved. Genomic *Chironomus riparius* DNA

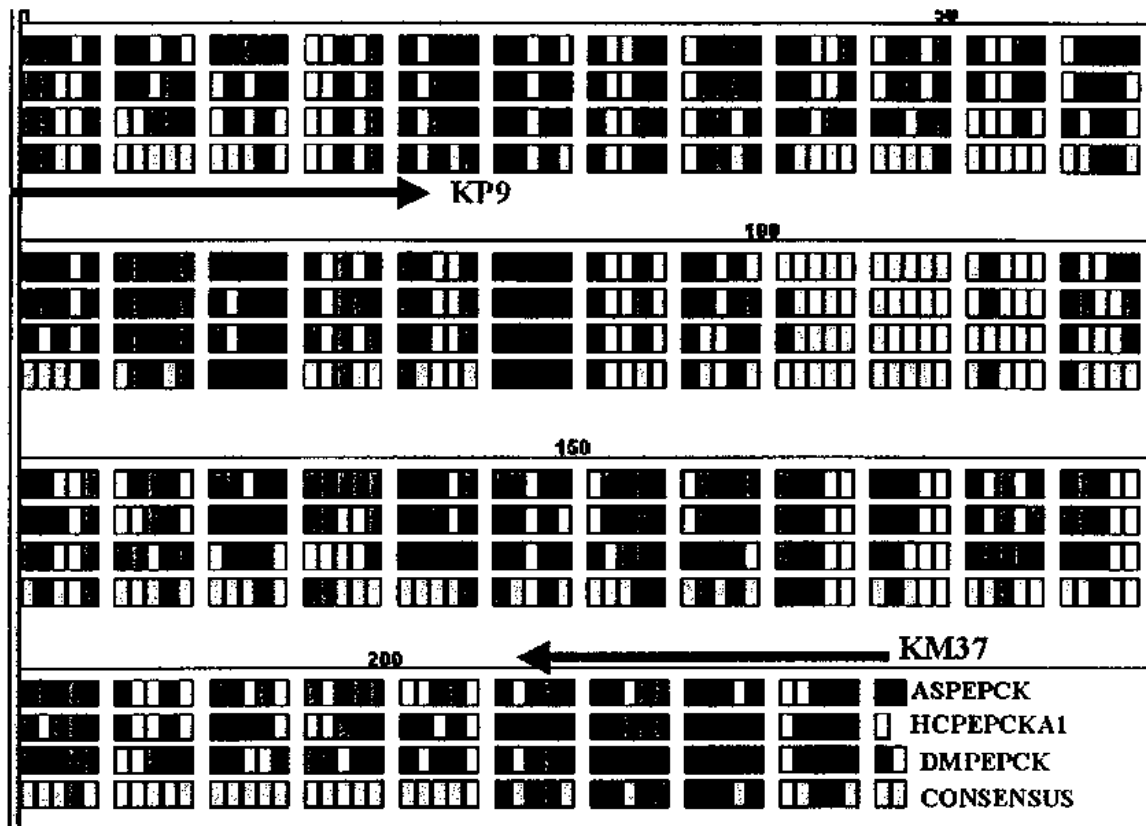


Figure 4. Pepck Sequence Alignments

Pepck sequence alignments of three invertebrate species were used to design PCR primers. Each colored square represents a DNA nucleotide. The bottom row represents the consensus between the three invertebrate species. Gray shaded squares are indicative of a lack of homology between the three species. KP9, the positive inside primer, is represented by the purple arrow at top and KM37, the minus inside primer, is represented by the purple arrow at bottom.

(.089 $\mu\text{g}/\mu\text{l}$) and primer sets (20 $\text{pmol}/\mu\text{l}$) were run under the following cycle conditions: (1) Heat @ 94°C for 4 min (2) 40 cycles of 94°C @ 30 sec, 50°C @ 2 min, (3) 72°C @ 3 min (4) 4°C until retrieved. In addition, a modified PCR protocol was developed to enhance specificity. The conditions of the modified PCR are as follows: (1) Heat @ 94°C for 5 min (2) 10 cycles of 94°C @ 1 min, 55°C @ 2 min (3) 72°C @ 1 min followed by (4) 30 cycles of 94°C @ 30 sec, 62°C @ 30 sec (5) 72°C @ 30 sec (6) 4°C until retrieved. Bands resulting from the genomic PCR were purified from gels (below), diluted 1:500 using dd H₂O, and reamplified as follows: (1) Heat @ 94°C for 4 min (2) 35 cycles of 94°C @ 30 sec, 65°C @ 30 sec, (3) 72°C @ 3 min (4) 4°C until retrieved.

Isolation and Purification of DNA Bands

PCR samples were routinely run on 1% agarose gels, however, 2-3% agarose gels were run if the bands required better separation. Bands were excised and cleaned following the protocol outlined in the Sigma Biosciences Nucleiclean kit (Sigma Biosciences, MO).

DNA Sequencing

An AmpliCycle™ Sequencing kit (Perkin Elmer, CA) was utilized for DNA sequencing. Samples were electrophoresed on a BIO RAD Sequi-Gen Sequencing Cell, powered by a BIO RAD POWER PAC 3000 (BioRad, CA). [³²P] α dATP (BioRad, CA) was incorporated into the DNA fragments for

detection. Gel exposure was between 24-48 hours on 14"x17" Wolf SCIENTIFIC Autoradiography film (Wolf Scientific, NY).

Dexamethasone Exposure Experiment

Drosophila (strain Oregon), third instar larvae were harvested after 4-5 days. The animals were divided into 18 test groups and 3 control groups, each group consisted of 15 animals. Test groups were exposed to either 0.1mM or 1.0 mM dexamethasone (Sigma Chemical, MO) at exposure times of 1, 3 and 6 hours. Dexamethasone concentrations and exposure times used for the experiment were chosen by extrapolating values from previously conducted dexamethasone exposure experiments involving cell culture (Watford and Mapes, 1990). All exposure times were performed in triplicate. The test animals were placed onto filter pads saturated with dexamethasone and the control animals were placed onto filter pads saturated with filtered water. The animals were removed following their designated exposure times.

RNA Isolation

RNA isolation was conducted following the protocol outlined in the TRI-REAGENT™ Technical Bulletin MB-205, For Product No. T-1924, June 1995 (Sigma Biosciences, MO).

Probe Selection

The minus primer, KM 37, was chosen as a foundation for the probe. Additional bases were added to the minus primer to make it a viable probe. The probe was prepared by OLIGOS ETC INC of South Orange, NJ and designated DPK 37. See appendix for the probe.

End Labeling of Probe

Probe DPK 37 was end labeled by first adding 8 μ l of γ [32 P] ATP (3,000 ci/mmol, BioRad,CA) to 25 pmol of dried probe followed by the addition of DEPCD treated water. T4 Polynucleotide Kinase forward reaction buffer, 5 μ l, (GIBCO-BRL, MD) was then added to the probe followed by the addition of 1 μ l T4 Polynucleotide Kinase (GIBCO-BRL, MD). This was incubated for 30 min at 37°C then an additional 1 μ l of T4 Polynucleotide Kinase was added to the reaction. The reaction was incubated for another 30 min at 37°C. Degradation of T4 kinase was conducted by incubating the reaction for 10 min at 65°C. Unincorporated γ [32 P] ATP was removed using a Micro Spin G-25 column. The column was prepared following the instructions outlined in the Pharmacia Biotech Certificate of Analysis (Probe Quant G-25 microcolumns, Pharmacia Biotech). This involved a series of three equal volume TES (10mM Tris-HCl, 1mM EDTA, 0.1% SDS) column rinses conducted prior to the addition of the sample. The sample was diluted with 25 μ l volumes of TES and run through the column.

Labeling efficiency was determined by diluting a 1 μ l of the labeling reaction into 5 ml scintillation buffer (Sigma-Fluor LSC Cocktail, Sigma Chemical, MO) and counted for 1 min on a BECKMAN LS 1801 beta counter (Beckman Instruments, PA) to determine the specific activity of the probe. See appendix section for result.

Solution Hybridization

Previously prepared *Drosophila* RNA (10 mg) was added to sample tubes followed by the addition of P³² end labeled probe DPK 37 and 2X solution hybridization buffer (1.5 M NaCl, 0.4% SDS, 8 mM EDTA, 40 mM Tris, pH 7.5). Samples were incubated overnight at 30°C. Samples were then diluted in 300 μ l S1 nuclease buffer (0.75 M NaCl, 2.8 mM Zn acetate, 70 mM Na acetate, pH 7.5) and digested with 1 μ l S1 nuclease for 1 hour at 37°C to degrade unbound RNA. RNA-DNA hybrids were precipitated with 100 μ l 50% TCA (trichloroacetic acid) at 4°C for 1 hour. The samples were filtered using Schleicher and Schuell # 30 Glass size 0.7 cm filters and a Schleicher and Schuell MINIFOLD® --DOT BLOT (Schleicher and Schuell, NH) to collect precipitated RNA-DNA duplexes and remove unhybridized probe (Figure 5). Filter disks were removed from the dot blotter, placed in 5 ml scintillation fluid, and counted using a BECKMAN LS 1801 beta counter (Beckman, PA).

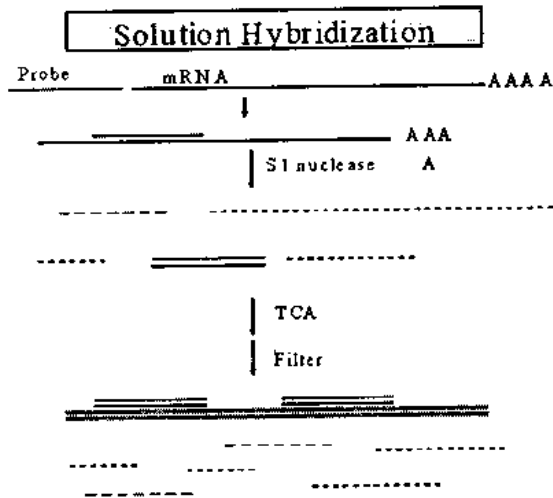


Figure 5.

The schematic outlines the steps involved in solution hybridization. S1 nuclease digests unbound DNA and TCA precipitates duplexes that are captured on the filter paper.

In addition to the test samples, control samples were run to evaluate the success of the solution hybridization. The controls consisted of an S1 Nuclease sample and a Total Precipitated Counts (TPC) sample and involved probe DPK 37 and a mouse liver control probe (Multi-NPA Kit, TX). RNA and S1 nuclease were absent from the TPC sample. This sample represented the total counts per minute (cpm). The S1 Nuclease sample contained no RNA and was prepared to assess the enzyme's digestion efficiency. This sample represented the background value.

Data Analysis

Larvae were sacrificed following their designated exposure times and their RNA was isolated and purified. The purified RNA was then hybridized with the P^{32} - end labeled probe. The solution hybridization results, expressed in counts per minute (cpm), for each triplicate exposure experiment were run in duplicate. Background value was determined by adding S1 nuclease to ^{32}P – end labeled probe, incubated for 1 hour at 37°C to allow for probe degradation, filtered, and counted. The average background value was 1610 cpm. Each duplicate was averaged and the background was subtracted from it. The resulting values were then averaged for each exposure time. These values were then subject to statistical analysis using two-way analysis of variance (ANOVA).

Results

Assessing PCR Primer Pair Viability

Inside and outside primer sets were run in the PCR using *Drosophila* genomic DNA. The purpose of this was to determine if the primers were capable of recognizing and amplifying an mRNA coding segment of pepck. The inside primers yielded a myriad of fragments while the outside primers generated one band that was the target size. This band was excised and purified. An additional PCR was run utilizing the purified band diluted 1:1000 to assess the specificity of the primers. To augment the experiment the inside primers were run using the same purified band as a template. Both the inside and outside primer sets generated crisp bands that were the appropriate size (Figure 6).

Chironomus riparius genomic DNA was run utilizing the same primer pairs under the same PCR conditions and the reaction failed to generate any bands. The annealing temperature was lowered to 56°C and still no bands were generated. A final PCR was run using a less stringent annealing temperature of 50°C and the inside primer and the outside primer pairs generated multiple fragments (Figure 7). All of the bands were excised and purified. A series of experiments was run to optimize the PCR and increase specificity. A modified PCR protocol, outlined in the methods section, ultimately generated two bands that met the designated size criteria (Figure 8). The modified protocol enabled

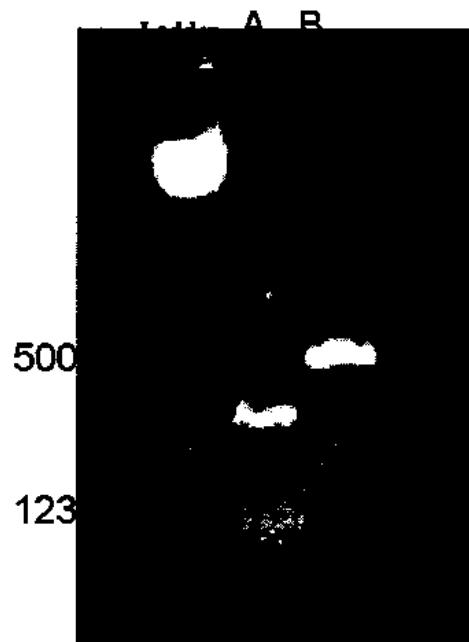


Figure 6.

PCR amplification using the inside and outside primer pairs. The first lane is a DNA molecular weight marker (123 bp ladder). The band in lane A was generated by the inside primer pair using the band from lane B as a template. The band in lane B was generated by the outside primer pair using *Drosophila* genomic DNA.

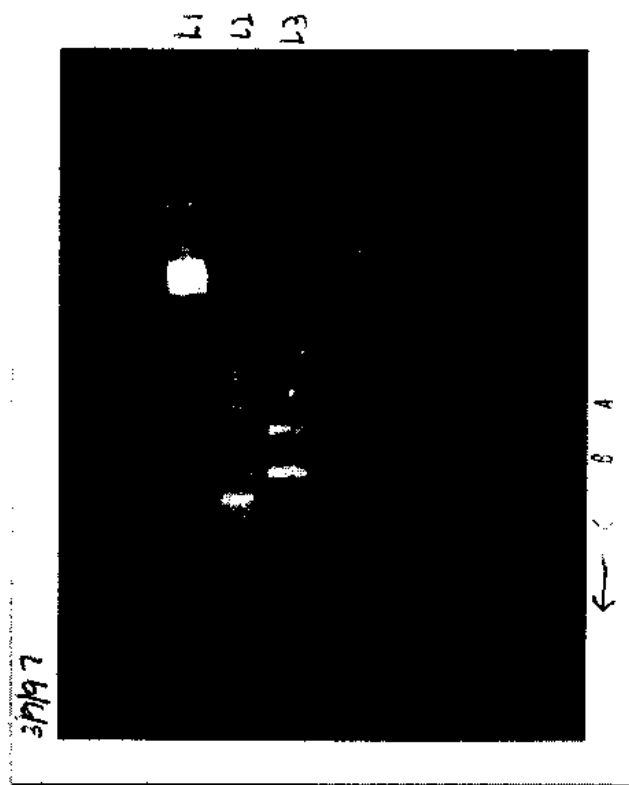


Figure 7.

A PCR experiment using *Chironomus riparius* genomic DNA at an annealing temperature of 50°C. The first lane is the DNA molecular weight marker. The bands in the second lane were generated using the inside primer pair

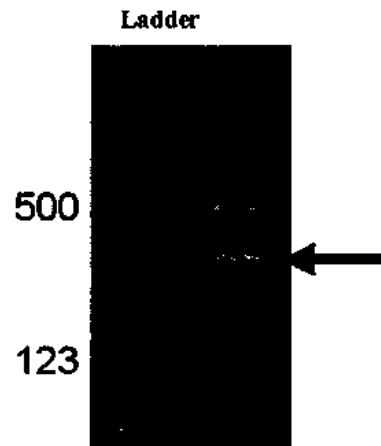


Figure 8.

Modified PCR experiment run on *Chironomus riparius* genomic DNA designed to enhance specificity. The bands generated in the right lane were produced using the outside primer pair. The band marked by the arrow was excised, removed, purified, and used as a template for the inside primer pair.

the generation of non-specific bands at a low annealing temperature and enhanced the specified binding of primers and generation of specific bands at the higher annealing temperature. The modified band generated two plausible target bands. The band that was slightly smaller than 500 bp was excised, purified and used as a template for the inside primers. The PCR was run at an annealing temperature of 65°C and generated a solitary band. The *Chironomus* band generated by the inside primer pair was run on a gel simultaneously with the *Drosophila* band proving the bands were virtually identical in size (Figure 9).

Identification of DNA Fragments Using Gel Sequencing

The *Drosophila* band generated by the inside primer pair was sequenced and compared to the known *Drosophila* pepck sequence obtained from the PCGene database (Gundelfinger *et al.*, 1987). The homology was 92% indicating that the inside primer pair had correctly amplified a sequence from pepck (Figure 10). This sequenced band served as the control. The *Chironomus* band derived from the same primer pair was also sequenced. When compared to the pepck sequence it had 47% homology, too low to conclusively identify it as pepck (Figure 11). There were some portions of the sequence that showed homology to the *Drosophila* sequence; however, the majority of the bases were not homologous.

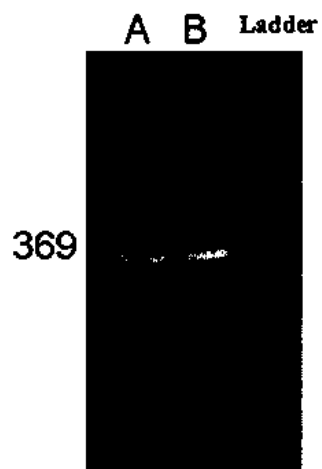


Figure 9

The band in Lane B is a pepck-coding segment from *Drosophila*. The band in Lane A was generated using the same inside primer pair and the smaller of the two bands from Figure 7 as a template. The comparison shows the striking similarity between the two bands.

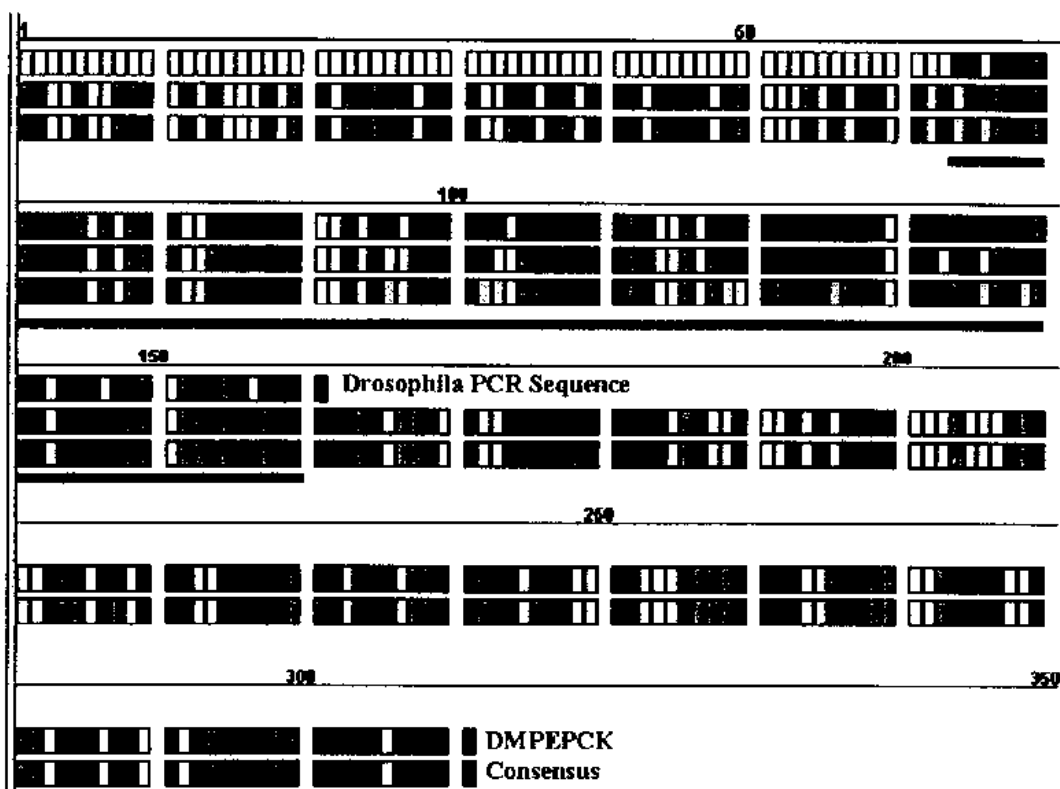


Figure 10. *Drosophila* Sequence versus *Drosophila* PEPCK

The *Drosophila* band generated by the inside primer pair compared to the known *drosophila* sequence from the PCGene database (underlined in purple). The consensus is represented by the bottom row. The gray shaded squares represent bases that were not homologous.

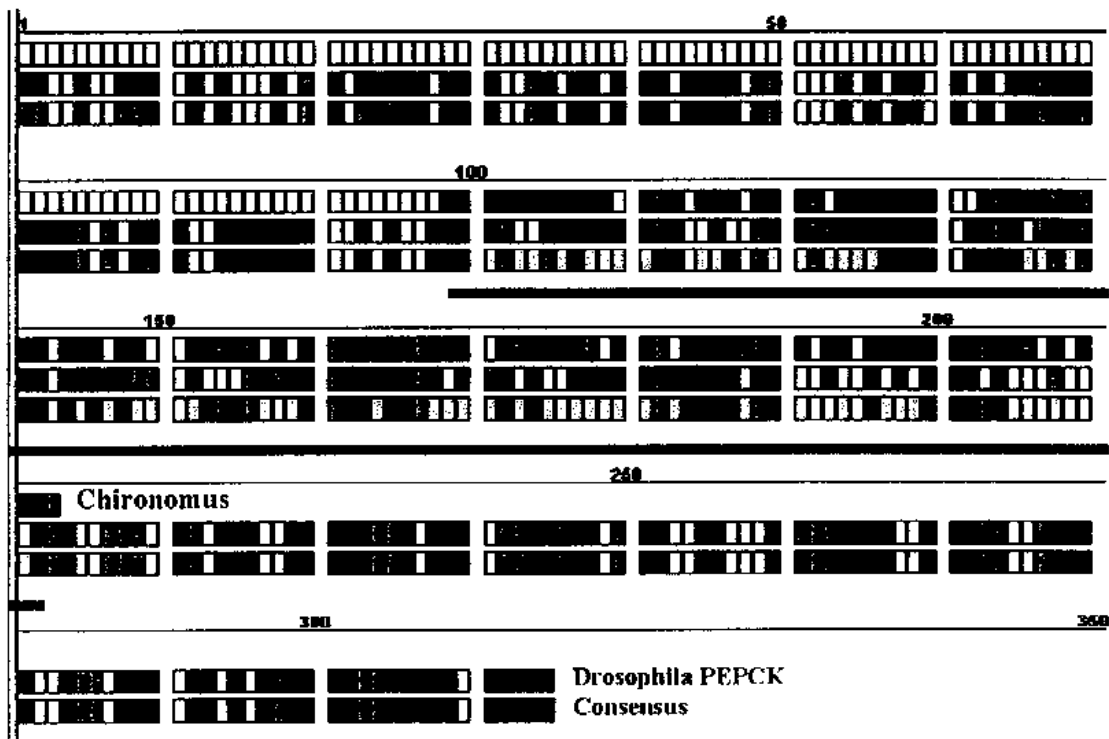


Figure 11. *Chironomus* Sequence versus *Drosophila* PEPCK

The *Chironomus* band generated by the inside primer pair compared to the known *Drosophila* sequence from the PCGene database (underlined in purple). The consensus is represented by the bottom row. The gray shaded squares represent bases that were not homologous.

Solution Hybridization

Drosophila larvae were homogenized following exposure to dexamethasone and their RNA was isolated and purified. Solution hybridization was conducted utilizing the purified RNA from the eighteen exposure groups and the three control groups. Solution hybridization experimental controls were implemented to ensure the test system functioned properly. The experimental controls included total precipitated counts (TPC) and S₁ nuclease. A mouse probe was utilized in addition to probe DPK 37 to supplement the experimental controls. The experimental controls were run in conjunction with the exposure samples. The S₁ nuclease control served as a background indicator while the TPC control was indicative of the total radioactivity precipitated by TCA. The average TPC for the mouse probe was 17,892 counts per minute (cpm) and the average count for probe DPK 37 was, 3,991 cpm. The TPC count disparity between the mouse probe and probe DPK 37 was probably attributed to probe quality. The S₁ nuclease control average for the mouse probe was 4,166 cpm and the average for probe DPK 37 was 1610 cpm. See appendix for actual readings.

Sample results were averaged for the three test groups comprising each exposure time and the S₁ nuclease value (1610 cpm), ie. the background, was subtracted out (Figure 12). The data from the 1.0 mM exposure groups were as

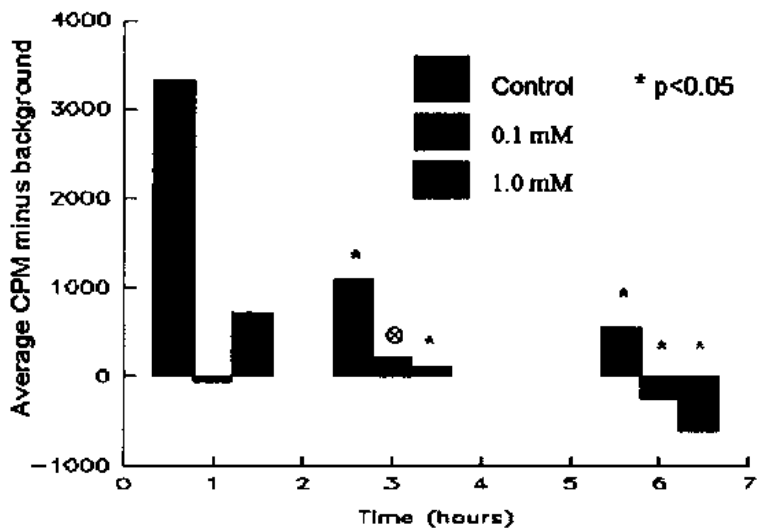


Figure 12

The graph represents the results for the control group and the two exposure groups. The three and six-hour control group are statistically different from the one-hour control group. Both the three and six-hour treatment groups are statistically different from the one-hour treatment groups.

follows: at one hour, 737 cpm, at three hours, 126 cpm, and at six hours the value drops below background (see appendix for actual readings). The data from the 0.1 mM exposure groups were as follows: at one hour, below background, at three hours 233 cpm, at six hours below background. Counts per minute for the control group decreased as exposure time increased. The results for the one, three and six hour control groups were 3330 cpm, 1096 cpm, and 557 cpm respectively (see asterisks on the table located in the appendix section for actual readings).

Two-way analysis of variance revealed that the three hour and six hour control groups were statistically different from the one hour control group and that the three hour control group was statistically different from the six hour control group. In addition, the three-hour and six hour 1.0 mM treatment groups were statistically different from the one hour 1.0 mM treatment group. Finally, the three-hour and six hour 0.1 mM treatment groups were statistically different from the one hour 0.1 mM treatment group.

Discussion

Biological assays are important research tools. They enable the researcher to evaluate various treatment effects and elucidate an organism's response to a particular treatment. In the field of environmental toxicology, biological assays are used to assess whether or not an organism has been exposed to an environmental pollutant and to determine the biological effects of pollutants. *Chironomus sp.* is often employed for use as a biological indicator because it is an EPA approved organism for sediment toxicity testing. Brown *et al.*, 1996, utilized *Chironomus riparius* to study the biological effects of phthalate ester plasticizers in *Chironomus*, while Ali *et al.*, 1998, employed Chironomid larvae to study the toxicity of a phenyl pyrazole insecticide. This project was chosen to take a molecular biological approach and develop a gene specific probe for *Chironomus riparius* pepck mRNA with the intention of using the probe as a biomarker for exposure to environmental pollutants. This approach was similar to that of Cochrane *et al.*, 1994, who employed PCR to develop probes specific for stress protein mRNA.

Primers specific for pepck were designed for PCR using PCGene software. The primers were derived via the comparison of three invertebrate species. Pepck sequences from *Ascaris suum*, *Haemonchis contortis*, and *Drosophila melanogaster* were compared and homologous sites among the three

invertebrates were identified. It was hypothesized that *Chironomus sp.* would share the same homologous sites. Nucleotide consensus among all three organisms was not consistent throughout the span of each primer region (Figure 4). *Drosophila* nucleotides were chosen by default in instances where all three bases were not in agreement because it is the most closely related to *Chironomus*. The proposed inside and outside primer pairs were screened using the available software and found to be compatible with each other.

The initial purpose of the project focused on determining whether or not the newly designed primers would recognize and amplify a coding segment of *pepck*. The PCR primers were first run using genomic *Drosophila* DNA. The outside primer pair generated a single DNA fragment that was excised and purified. The purified fragment was used as a template for the inside primer pair and the PCR yielded a band that was within the target size range, indicating that the primers successfully amplified a coding segment of the gene.

The task was to prove that the band was, in fact, a *pepck* segment. The band was excised from the gel and purified. The purified band was then exposed to Sanger sequencing. The sequence generated was compared to the known *Drosophila pepck* sequence taken from the database. The alignment showed very good consensus between the isolated band and the known sequence (Figure 10) There were a few sites that did not match. The disagreement at these locations could be attributed to a misread of the gel, the

subtle sequence differences associated with *Drosophila* subspecies, and the fact that Taq polymerase is somewhat error prone. The results proved that the primers were specific for pepck and that an experimental control had been established.

Now that a control was established, experimentation was conducted on *Chironomus* genomic DNA using the same inside and outside primer pairs. After several PCR parameter modifications, the primers yielded a fragment that was within the target size range. The fragment was excised, purified and used as a template for the inside primer pair. The PCR of the template generated a fragment that was virtually identical to the size of the sequenced *Drosophila* fragment (Figure 9).

The *Chironomus* band was sequenced and aligned with the known *Drosophila* sequence from the database and the comparison yielded sporadic sequence homology (Figure 10). The inconsistent homology suggested that the band was not derived from pepck. This suggestion could be disputed because the size of the fragment was practically identical to that of *Drosophila*. Perhaps the fragment was actually a pepck sequence. The lack of homology with *Drosophila* could be attributed to the fact that this region of pepck does not share significant homology between different species. Inconsistencies were observed between the three species during the primer generation process (Figure 4). Frequent attempts to definitively isolate, amplify and sequence a coding segment

of pepck in *Chironomus* proved inconclusive, so a decision was made to modify the experimental objectives and proceed with developing an mRNA probe for *Drosophila* in an attempt to identify an alternative approach for pepck isolation in *Chironomus*.

The minus inside primer, KM 37, was augmented by adding additional bases and used as a probe for *Drosophila* pepck mRNA. Dexamethasone was chosen as the treatment to induce pepck, however, dexamethasone exposure concentrations needed to be determined. Watford and Mapes, 1990, used dexamethasone to induce pepck mRNA in rat hepatocytes. The exposure concentrations used to induce pepck mRNA in *Drosophila* larvae were extrapolated from the values cited in Watford's experiment. The same experimental design used by Hickey and Benkel, 1982, was implemented for the dexamethasone exposure. Each exposure was conducted in triplicate.

The goal of the dexamethasone exposure experiment was to induce transcription of pepck. Increasing levels of pepck mRNA were expected over time. The lowest levels of pepck mRNA were expected in the one hour exposure group and the highest levels of pepck mRNA were anticipated in the six-hour exposure group. The data showed that the three and six hour control groups were statistically different from the one-hour control group (Figure 12). The mRNA levels for the three control groups were not sustained throughout the

entire exposure period. The declining mRNA levels for the control group over time indicated that there was a treatment effect occurring that was not expected.

The data for the three and six hour exposure groups were statistically different from the one hour exposure group for both treatments (Figure 12). Increasing exposure time resulted in decreasing mRNA levels for both the 0.1 mM and 1.0 mM treatment groups. There was a sharper decline in mRNA levels for the 1.0 mM treatment versus the 0.1 mM treatment at the six hour exposure time. The disparity is attributed to the higher dose having a greater adverse impact on the larvae. Interestingly, there was a slight increase in mRNA levels for the 0.1 mM treatment group at three hours. This spike was statistically different in a positive manner. The increase in mRNA levels was most likely the result of experimental error. The data obtained from both treatment groups indicated that the concentrations of dexamethasone used for the exposures had an acutely toxic effect on the *Drosophila* larvae.

Further research regarding this subject needs to be conducted. Exposure studies using less concentrated dexamethasone should be investigated. A less concentrated dose of dexamethasone would most likely produce gene induction. In addition, a different exposure regimen needs to be explored. Spiking *Drosophila* growth medium with dexamethasone may prove to be a viable alternative to the approach used in this work. Finally, gene induction using differential display may provide an alternate means of identifying pepck in

Chironomus riparius. Differential display is a method that enables the researcher to clone and separate mRNA via PCR (Liang and Pardee, 1992).

Conclusion

An inside and outside primer pair specific for pepck were designed using the programs available in PCGene. Both primer pairs successfully amplified a coding segment of pepck in a PCR using *Drosophila sp.* genomic DNA. Amplification of a pepck coding segment in *Drosophila sp.* enabled the establishment of a control band.

Initial PCR experiments using genomic DNA of *Chironomus riparius* with these primers produced a significant amount of non-specific bands. A modified PCR protocol, developed to minimize non-specific amplification, produced two bands of the desired size, one virtually identical in size to the *Drosophila* pepck coding segment. Although the size of the band suggested that it was a potential coding segment of pepck, the sequence homology was less than fifty percent.

An alternative approach was developed to isolate the gene using reverse PCR. *Drosophila sp.* was chosen to explore this alternative because a coding segment of pepck had already been generated using *Drosophila* DNA as a template. An experimental protocol was designed to accomplish the goal and dexamethasone was chosen to induce the gene in *Drosophila sp.* The concentrations of dexamethasone used for the treatment proved to be acutely toxic as indicated by the decreasing mRNA levels over time.

Alternative experimental approaches need to be conducted to isolate pepck in *Chironomus sp.*. Once the gene is finally identified progress can be made investigating the gene for use as a potential biomarker.

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Appendix

Exposure Group Results

1.0 mM Dexamethasone Treatment		0.1 mM Dexamethasone Treatment	
Exposure Time (hrs)	Results (cpm)	Exposure Time (hrs)	Results (cpm)
1	2406	1	1518
	2296		2089
1	2347	1	957
	2750		1151
1	2032	1	825
	2161		2796
3	1822	3	712
	1465		2665
3	1919	3	2606
	1647		3653
3	1780	3	840
	599		579
6	899	6	666
	643		1416
6	1417	6	607
	1221		1945
6	3295	6	2761
	897		806

Treatment Control Group Results

Exposure Time (hours)	Results (cpm)
1	3873
	6007
3	2841
	2571
6	2023
	2310

Solution Hybridization Control Results

Sample	Result (cpm)
Probe TPC	3812 4170
Mouse TPC	95,632 141,753
Probe S ₁	1710 1510
Mouse S ₁	3156 5175
Mouse RNA	6375 29283

Data Analysis

Treatment (mM)	Time (hrs)	Average Result (cpm)	Average minus S1 (cpm)	Triplicate Average minus S1 (cpm)
1.0	1	2351	741	
1.0	1	2548	938	737*
1.0	1	2096	486	
1.0	3	1644	34	
1.0	3	1783	173	126*
1.0	3	178	170	
1.0	6	771	-839	
1.0	6	1319	-291	-614*
1.0	6	897	-713	
0.1	1	1804	194	
0.1	1	1054	-556	-54*
0.1	1	1811	201	
0.1	3	1689	79	
0.1	3	3130	1519	233*
0.1	3	710	-900	
0.1	6	1041	-569	
0.1	6	1276	-334	-486*
0.1	6	1784	174	
Control	1	4940	3330*	
Control	3	2706	1096*	
Control	6	2167	557*	

Oligos Etc.

Oligo Code: VH-1

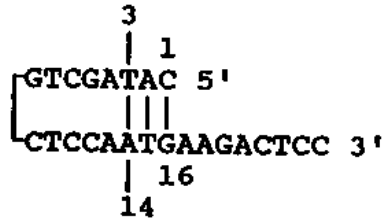
Synthesis Code: VH-1-H.DNA

SEQUENCE:

1 4 7 10 13 16 19 22
5'- CAT AGC TGC TCC AAT GAA GAC TCC -3'

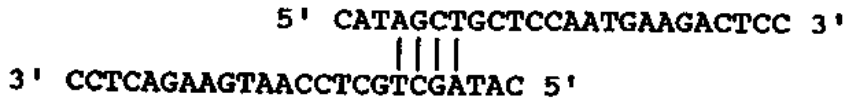
4.42 nmol/OD
32.50 µg/OD
Mw = 7.4 k (one strand)
Primer to target Tm(by %GC) = 73.9°

STEM LOOP STRUCTURE:



G = 0.9 kcal/mol
loop Tm = 1°

HOMODIMER:



Homodimer Not Stable

Modifications: 50nm

Total A₂₆₀: 9.0

Oligo Name: KM 44

3 Oligos Etc.

Oligo Code: VH-2

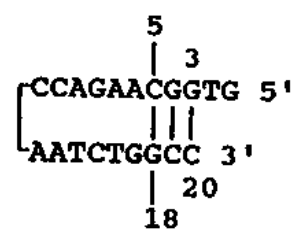
Synthesis Code: VH-2-H.DNA

SEQUENCE:

1 4 7 10 13 16 19
5'- GTG GCA AGA CCA ATC TGG CC -3'

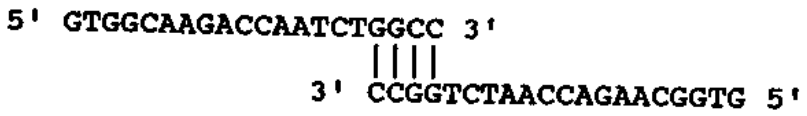
5.24 nmol/OD
32.53 µg/OD
Mw = 6.2 k (one strand)
Primer to target Tm (by %GC) = 72.3°

STEM LOOP STRUCTURE:



G = -1.3 kcal/mol
loop Tm = 50°

HOMODIMER:



Homodimer Not Stable

Modifications: 50nm
Total A₂₆₀: 6.8
Oligo Name: KP 25

Oligos Etc.

Oligo Code: VH-3

Synthesis Code: VH-3-H.DNA

SEQUENCE:

1 4 7 10 13 16 19 22
5'- AAG GTG GAA TGC GTG GGT GAC G -3'

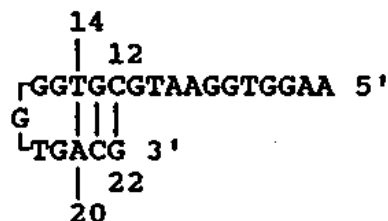
4.46 nmol/OD

31.25 µg/OD

Mw = 7.0 k (one strand)

Primer to target Tm (by %GC) = 75.0°

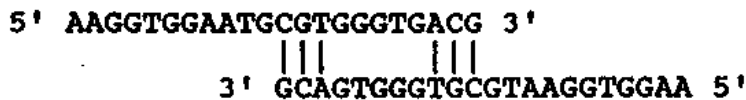
STEM LOOP STRUCTURE:



G = -0.5 kcal/mol

loop Tm = 37°

HOMODIMER:



Homodimer Not Stable

Modifications: 50nm

Total A₂₆₀: 11.0

Oligo Name: KP 9

5. Oligos Etc.

Oligo Code: VH-4

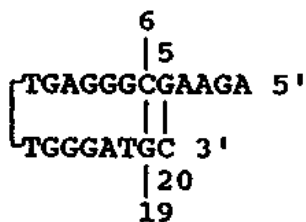
Synthesis Code: VH-4-H.DNA

SEQUENCE:

1 4 7 10 13 16 19
5'- AGA AGC GGG AGT TGG GAT GC -3'

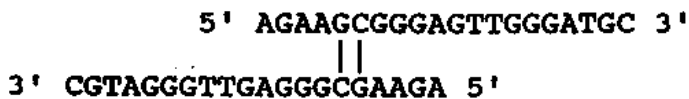
4.88 nmol/OD
31.04 µg/OD
Mw = 6.4 k (one strand)
Primer to target Tm (by %GC) = 72.3°

STEM LOOP STRUCTURE:



G = 1.8 kcal/mol
No Stable Secondary Structure

HOMODIMER:



Homodimer Not Stable

Modifications: 50nm

Total A₂₆₀: 9.4

Oligo Name: KM 37

; Oligos Etc.

Oligo Code: VH-5

Synthesis Code: VH-5-H.DNA

SEQUENCE:

1 4 7 10 13 16 19
5'- TTC TGG GCA TCA CCG ATC CC -3'

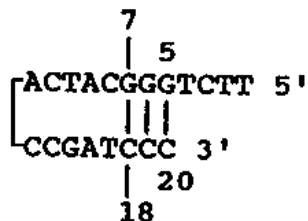
5.62 nmol/OD

34.36 μ g/OD

Mw = 6.1 k (one strand)

Primer to target Tm (by %GC) = 72.3°

STEM LOOP STRUCTURE:



G = -1.9 kcal/mol

loop Tm = 60°

HOMODIMER:

5' TTCTGGGCATCACCGATCCC 3'

```
      |||
3' CCCTAGCCACTACGGGTCTT 5'
```

Homodimer Not Stable

Modifications: 50nM

Total A₂₆₀: 7.5

Oligo Name: KP4

Oligos Etc.

Oligo Code: TS

Synthesis Code: TS-L.DNA

SEQUENCE:

1 4 7 10 13 16 19 22 25
5'- GGT GCA GAA GCG GGA GTT GGG ATG C -3'

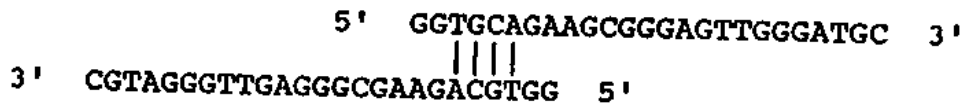
4.00 nmol/OD
31.79 µg/OD
MW = 7.9 k (one strand)
Primer to target Tm (by %GC) = 80.7°C

STEM LOOP STRUCTURE:



G = 1.1 kcal/mol
loop Tm = 0°C

HOMODIMER:



Homodimer Not Stable

Modifications: 50nM

Total A₂₆₀: 11.6

Oligo Name: DPK.37