INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

**DIFFERENTIAL GENE EXPRESSION IN TWO COLD HARDY
INSECTS IN RESPONSE TO LOW TEMPERATURES**

by

TOLGA BILGEN

B.Sc Concordia University, 1990
M.Sc University of British Columbia, 1994

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

**Carleton University
Ottawa, Ontario, Canada**

August 1998

© copyright
1998, Tolga Bilgen

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file *Votre référence*

Our file *Notre référence*

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-37042-9

Canada

The undersigned hereby recommend to the Faculty of Graduate Studies and
Research acceptance of the thesis

**DIFFERENTIAL GENE EXPRESSION IN TWO COLD HARDY
INSECTS IN RESPONSE TO LOW TEMPERATURES**

submitted by

TOLGA BILGEN

B.Sc Concordia University, 1990
M.Sc University of British Columbia, 1994

in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisor

External Supervisor

Carleton University
August, 1998

ABSTRACT

The two cold hardy gall insects, *Epiblema scudderiana* and *Eurosta solidaginis*, employ freeze avoidance and freeze tolerance, respectively, in coping with low seasonal temperatures. Biochemical and metabolic changes in these animals in response to low temperatures have been well documented, but the possibility that cold and subzero temperatures could upregulate genes in these animals has not been investigated. In this study, cDNA library screening and differential display PCR were used to address this question. Animals were sampled over a successive cold (2 weeks at 4°C) and subzero temperature (one week at -20°C) time course. Differential screening/PCR against RNA from control (15°C) animals and Northern analyses revealed a number of upregulated transcripts. All clones were sequenced, and all but two were unidentifiable through GenBank database comparisons, due to the novel and incomplete nature of their sequences. The two identifiable sequences had strong homologies to *Drosophila* genes *mlp60A* and *rpA1*, encoding respectively, a factor crucial for myogenesis and an acidic ribosomal protein. Implicated in embryonic and adult development, the upregulation of these genes suggests a unique developmental regime in *E. scudderiana* and *E. solidaginis*, both of which overwinter as final instar larvae. These animals may be preparing in advance for impending morphogenic changes, allowing for a head-start with the onset of spring.

ACKNOWLEDGEMENTS

My sincerest thanks go to Ken Storey, whose peculiar brand of encouragement and infectious *joie de science* proved indispensable both to this project and to my time here. Many thanks also to Jan Storey, for her generous advice and wry sense of editing. The Mighty QY (Quinyin Cai) was a brilliant guru of molecular biology-- I thank him and wish him very well.

Now for you, you crowds of people in my memory, who also contributed so much. To those in the lab, many of whom became dear friends, who chipped in year after year to collect galls in cold, soggy fields with nary a doughnut for reward, who were quick to share a laugh-- my thanks to you. To those further away, you were far more than mere e-mail messages and too-infrequent sightings, my friends. I appreciate the time you have given me, you are heroes all.

Speaking of heroes, the wondrous Bilgens certainly make the grade. My deepest gratitude for their endless support and their superhuman ability to lift my spirits.

Certainly not least is Rinda, she of impish charm and quick wits, who merits all the thanks I can muster.

TABLE OF CONTENTS

	Page
Acceptance Sheet.....	ii
Abstract.....	iii
Acknowledgements.....	iv
Table of Contents.....	v
List of Tables.....	vi
List of Figures.....	vii
List of Abbreviations.....	x
 CHAPTER ONE : Introduction.....	 1
Hazards of cold exposure and insect survival strategies.....	2
Mechanisms of insect coldhardiness.....	4
Proteins conferring coldhardiness.....	7
Stress-induced proteins.....	9
Other protein responses to low temperature.....	13
 CHAPTER TWO: Materials and Methods.....	 14
Animals.....	15
cDNA library construction and differential screening.....	16
Differential display PCR.....	32
 CHAPTER THREE: Results.....	 42
<i>Epiblema scudderiana</i> cDNA library.....	43
Differential display PCR.....	69
<i>Eurosta solidaginis</i>	78
<i>Epiblema scudderiana</i>	120
Cross-Northern analysis.....	154
 CHAPTER FOUR: Discussion.....	 165
 References.....	 191

LIST OF TABLES

Table		Page
1	Primers used in differential display PCR.....	36
2	Pairwise amino acid sequence similarities, <i>E. scudderiana</i> clone P3 and assorted Muscle-LIM proteins.....	59
3	Pairwise nucleotide sequence similarities, <i>E. scudderiana</i> clone P6 and assorted insect 28S rRNAs.....	65
4	Summary of total banding in all <i>E. solidaginis</i> DD-PCR reactions.....	85
5	All potential candidates from <i>E. solidaginis</i> DD-PCR.....	86-87
6	Positively confirmed <i>E. solidaginis</i> candidates.....	95
7	Pairwise amino acid sequence similarities, <i>E. solidaginis</i> clone U1 and assorted ribosomal P-proteins.....	109
8	Summary of total banding in all <i>E. scudderiana</i> DD-PCR reactions.....	129
9	All potential candidates from <i>E. scudderiana</i> DD-PCR.....	130-31
10	Positively confirmed <i>E. scudderiana</i> candidates.....	137
11	Summary of all positively confirmed clones from both <i>E. scudderiana</i> and <i>E. solidaginis</i>	157
12	Some genes induced at low temperatures in coldhardy animals..	167

LIST OF FIGURES

Figure		Page
1	Restriction digest and size estimation of <i>E. scudderiana</i> clones P3, P6 and P8.....	46
2	Northern hybridization of clone <i>E. scudderiana</i> clone P3 and size estimation of bands P3a and P3b	48
3	Northern hybridizations of <i>E. scudderiana</i> clones P3, P6 and P8..	50
4	Results of densitometric analysis of Northern autoradiographs, <i>E. scudderiana</i> clones P3, P6 and P8	52
5	Nucleotide and deduced amino acid sequence of <i>E. scudderiana</i> clone P3	55
6	Amino acid sequence alignment of <i>E. scudderiana</i> clone P3 and various Muscle-LIM protein homologues.....	58
7	Nucleotide and deduced amino acid sequence of <i>E. scudderiana</i> clone P6	61
8	Nucleotide sequence alignment of <i>E. scudderiana</i> clone P6 and insect 28S rRNA sequences	64
9	Nucleotide sequence of <i>E. scudderiana</i> clone P8	67
10	Northern hybridizations of <i>E. scudderiana</i> clones P3, P6 and P8 versus five-week, 15°C time course	71
11	DD-PCR test: individual and pooled insect RNA.....	74
12	DD-PCR test: DNase and RNase treatments.....	76
13	<i>E. solidaginis</i> DD-PCR reaction, primer combination HT ₁₁ -A, AP4.....	80
14	<i>E. solidaginis</i> DD-PCR reaction, primer combination HT ₁₁ -C, AP4.....	82
15	<i>E. solidaginis</i> DD-PCR reaction, primer combination HT ₁₁ -G, AP4.....	84

16	<i>E. solidaginis</i> DD-PCR candidate U1.....	89
17	<i>E. solidaginis</i> reamplifications, candidates U51-U55.....	92
18	Northern hybridization of <i>E. solidaginis</i> candidates.....	94
19	Partial sequencing (C-tracking) of <i>E. solidaginis</i> clone U1.....	98
20	Restriction digest of <i>E. solidaginis</i> U1 clones.....	100
21	Northern hybridization of <i>E. solidaginis</i> clone U1-2.....	102
22	Nucleotide and deduced amino acid sequence of <i>E. solidaginis</i> clone U1.....	104
23	Amino acid sequence alignment of <i>E. solidaginis</i> clone U1 and assorted ribosomal P-proteins homologues.....	107
24	Nucleotide and deduced amino acid sequence of <i>E. solidaginis</i> clones U19,U20.....	111
25	Nucleotide and deduced amino acid sequence of <i>E. solidaginis</i> clones U44, U47.....	113
26	Nucleotide and deduced amino acid sequence of <i>E. solidaginis</i> clones U51, U52.....	115
27	Nucleotide and deduced amino acid sequence of <i>E. solidaginis</i> clone U53.....	117
28	Northern hybridization and of <i>E. solidaginis</i> clone U1 versus five-week, 15°C time course.....	122
29	<i>E. scudderiana</i> DD-PCR reaction, primer combination HT ₁₁ -A, AP4.....	124
30	<i>E. scudderiana</i> DD-PCR reaction, primer combination HT ₁₁ -C, AP4.....	126
31	<i>E. scudderiana</i> DD-PCR reaction, primer combination HT ₁₁ -G, AP4.....	128
32	<i>E. scudderiana</i> DD-PCR candidates P11, P12, P43.....	133
33	<i>E. scudderiana</i> reamplifications, candidates P10, P11.....	136
34	Results of Northern hybridizations of Northern autoradiographs, <i>E. scudderiana</i> candidates P11, P12, P43.....	139
35	Partial sequencing (C-tracking) of <i>E. scudderiana</i> clone P11.....	142

36	Restriction digestion of <i>E. scudderiana</i> clone P11.....	144
37	Northern hybridization and of <i>E. scudderiana</i> clone P11-3.....	146
38	Nucleotide and deduced amino acid sequence of <i>E. scudderiana</i> clone P11.....	148
39	Nucleotide and deduced amino acid sequence of <i>E. scudderiana</i> clone P12.....	151
40	Nucleotide and deduced amino acid sequence of <i>E. scudderiana</i> clone P43.....	153
41	Northern hybridization of <i>E. scudderiana</i> clones P11, P43 versus five-week, 15°C time course	156
42	Cross-species Northern hybridizations, <i>E. scudderiana</i> clones versus <i>E. solidaginis</i> blots.....	160
43	Cross-species Northern hybridizations of <i>E. solidaginis</i> clone U1 versus <i>E. scudderiana</i> blot.....	163

LIST OF ABBREVIATIONS

cpm-	counts per minute
CRIP-	cysteine-rich intestinal protein
CRP-	cysteine-rich protein
CSP-	cold-shock protein
DAP-	diapause-associated protein
DD-PCR-	differential display polymerase chain reaction
DEPC-	diethylpyrocarbonate
DMSO-	dimethylsulfoxide
DNA-	deoxyribonucleic acid
EDTA-	ethylenediamine tetraacetate
EF-	elongation factor
EtBr-	ethidium bromide
FP-	freezing point
HD-	homeodomain
HLH-	helix-loop-helix
HSE-	heat-shock element
HSF-	heat-shock factor
HSP-	heat-shock protein
INP-	ice-nucleating protein
Kb-	kilobase
kDa-	kiloDalton

MLP- muscle LIM protein

MOPS- 3-[N-morpholine] propanesulfonic acid

mRNA- messenger RNA

nt- nucleotide

OD- optical density

ORF- open reading frame

pfu- plaque-forming units

RACE- rapid amplification of cDNA ends

RNA- ribonucleic acid

rRNA- ribosomal RNA

RT-PCR- reverse-transcription polymerase chain reaction

SCP- supercooling point

THP- thermal hysteresis protein

UTR- untranslated region

UV- ultraviolet

CHAPTER ONE:

INTRODUCTION

Overwintering insects have well-characterized biochemical responses to low temperature exposure, but similar information at the molecular level is scant. This study aims to determine whether low temperature acts to upregulate any genes in cold hardy insects, and to gain insight into their adaptations to cold. Molecular biology offers a number of avenues for this kind of investigation, including the construction and screening of cDNA libraries and the differential display polymerase chain reaction (DD-PCR) method. Coupled with Northern hybridization analysis, potentially cold-upregulated genes can be assessed via differential transcript accumulation, and subsequent sequencing may offer clues as to their identity.

Hazards of cold exposure and insect survival strategies

The extreme cold of winter in temperate and northern climes poses a significant challenge to insects and other arthropods. Subzero temperatures can inflict a variety of cellular injuries that are lethal to nonhardy species. The cold-induced perturbation of lipid and protein conformation can lead to membrane and metabolic failure (Bale, 1987; Lee et al., 1987). The growth of extracellular ice crystals can disrupt membranes through physical contact and by the dehydration and shrinkage of cells (Storey and Storey, 1988).

To successfully endure low temperature exposure, overwintering insects have adopted one of two general strategies-- freeze avoidance or freeze tolerance. The two insects that serve as subjects for this study, the gall moth *Epiblema scudderiana* and the gall fly *Eurosta solidaginis*, represent each of these strategies, respectively. Both species overwinter as last instar larvae within galls

formed on the stems of goldenrod plants. The galls are often found above the snowline, and as such, the insects may be exposed to temperatures as low as -30°C (Rickards et al., 1987).

The formation of internal ice is lethal for freeze-avoiding insects. These animals have evolved mechanisms that allow them to maintain a liquid state throughout the winter by depressing both the freezing and supercooling points of body fluids. The supercooling point (SCP) of *E. scudderiana* falls from -15°C to -38°C as the insect cold-hardens over the autumn months (Rickards et al., 1987). By contrast, freeze tolerant species permit ice to form in extracellular spaces, while taking steps to prevent the freezing of intracellular fluids. Larvae of *E. solidaginis* supercool only to about -9°C, can tolerate the freezing of up to 64% of total body water, and can endure weeks in the frozen state (Baust and Nishino, 1991; Storey and Storey, 1985; Storey and Storey, 1988).

The contributing elements that compose these opposing strategies are several and, perhaps surprisingly, some factors are common to both. The mechanisms include elimination of endogenous ice nucleators, reduction of freezable body water, production of low molecular weight cryoprotectants, and a shift to a hypometabolic state (Hoffman, 1984; Storey and Storey, 1988).

Unique types of proteins are also involved in cold hardiness in some insect species. The best characterized of these are two types of haemolymph proteins, the thermal hysteresis proteins that act as antifreezes for freeze avoiding insects and the ice-nucleating proteins found in freeze tolerant species (Duman et al., 1991b). These proteins are synthesized in response to seasonal cues, including cold exposure (Duman, 1982), suggesting that some genes may be activated by low temperatures. Further evidence of cold-inducible genes in insects is provided by the synthesis of heat shock proteins in response to cold shock (Denlinger et al., 1991). Other, as yet unknown, cold-inducible gene products may also occur in

cold hardy insects, and the detection and identification of these are the focus of this project.

Mechanisms of insect cold hardiness

Elimination of endogenous ice-nucleators

Minute volumes of pure water can supercool to -40°C before spontaneous ice formation occurs through homogeneous nucleation. Solutes and impurities in solutions or body fluids, however, act as heterogeneous ice nucleators so that most solutions show little or no supercooling below the equilibrium freezing point (FP; which is determined by the concentration of dissolved solutes). In insect body fluids, this heterogeneous nucleation can be caused by various nonspecific molecules and particles which provide sites that order water at high subzero temperatures, promoting the formation of embryonic ice crystals (Duman et al., 1991b). In order to achieve the deep supercooling necessary for overwintering, freeze avoiding insects take steps to specifically eliminate endogenous ice nucleators. In different species, endogenous ice nucleators have been traced to gut contents (bacteria, food particles) and haemolymph proteins. A common part of winter cold-hardening, therefore, is a seasonal clearing of gut contents and a change in haemolymph protein composition, by mechanisms that could include evolutionary changes in the structure of individual proteins or the seasonal elimination of some haemolymph protein types (Storey and Storey, 1988; Duman et al., 1991b). Supercooled insects are also susceptible to seeding by contact with ice in their external environment and, thus, many waterproof themselves with a waxy cuticle or silk cocoon (Bale, 1987).

Reduction of bulk water

A reduction in the amount of freezable water in organisms is a necessary component of cold-hardening in both freeze avoiding and freeze tolerant insects (Rickards et al., 1987; Storey and Storey, 1988). Freeze avoiding insects reduce bulk water content directly through dehydration during autumn cold hardening; for example, *E. scudderiana* larvae lose up to 25% of their fresh weight due to water loss (Rickards et al., 1987). This reduction in bulk water increases solute concentrations (including low molecular weight cryoprotectant polyols, see below) in body fluids and, hence, depresses both freezing point and SCP. Freeze tolerant species show no significant change in total body water content over the winter, but an increase in the amount of unfreezable or bound water occurs during cold acclimation that is closely associated with changes in the composition of macromolecules or other subcellular components (Storey and Storey, 1988; Lee, 1991). This osmotically inactive, ordered water forms 'shells' around proteins and membranes, and in doing so, is rendered unfreezable itself and also offers protection for macromolecules against denaturation (Storey, 1983). In the case of *E. solidaginis*, a three-fold increase in the amount of bound water was estimated during cold-hardening (Storey et al., 1981). Part of the increase was attributed to the accumulation low molecular weight cryoprotectants, but a significant portion was associated with the macromolecular subcellular fraction suggesting that structural changes to one or more components during cold hardening increased their capacity to order water (Storey, 1983).

Cryoprotectants

Low molecular weight cryoprotectants, most often polyhydric alcohols, are accumulated by most insects during cold-hardening (Zachariassen, 1991). The advantages of polyol accumulation are many. They can be produced from stored glycogen through existing metabolic pathways, they diffuse readily across

membranes, and their high solubility and relative nonreactivity allow the accumulation of high concentrations in cells without toxic effects. Polyols thus exert a colligative effect on SCP and FP depression, and also stabilize native protein conformation at extreme temperatures (Duman et al., 1991b; Storey and Storey, 1988, 1991). This is instrumental in freeze avoidance, where glycerol can accumulate to molar concentrations; for example, 2 M and 5 M have been measured in larvae of *E. scudderiana* and the wasp, *Bracon cephi*, respectively (Lee, 1991).

In freeze tolerant insects, polyols serve to render intracellular water unfreezable and to limit the extent of cell dehydration and shrinkage that occurs as a high percentage of total body water is converted into extracellular ice. Concurrently, freeze-concentration effects can be reduced, protein structures stabilized and extracellular ice-crystal growth retarded (Lee, 1991; Storey and Storey, 1988). Glycerol is the most common polyol employed by overwintering insects, though multicomponent systems also exist. For example, *E. solidaginis* utilizes both glycerol and sorbitol, and accumulates them in a temperature-sensitive manner; glycerol is produced during autumn cooling and sorbitol production is triggered only by cold exposures in the 5 to -5°C range (Duman et al., 1991b; Storey and Storey, 1991).

Hypometabolism

Insects typically respond to the unfavorable growth conditions of winter (cold temperature, lack of food) with metabolic rate depression. Many species enter a programmed diapause that may last several months, others experience quiescence. Given limited endogenous fuel reserves (particularly for species that will not eat again in their life cycle, such as the two studied here), insects must rebalance ATP-utilizing and ATP-producing reactions to ensure energy

conservation for long-term survival. Over long term exposure to subzero temperatures, *E. scudderiana* exhibits a reduced but stable energy state, characterized by the use of lipid reserves for basal metabolism and a reduction in TCA cycle enzyme activities (Churchill and Storey, 1989; Joannis, 1995). Larvae of *E. solidaginis* also show suppression of TCA cycle enzyme activities that indicates reduced metabolic rate (Joannis, 1995), but in the frozen state the larvae must depend on anaerobic glycolysis (with the accompanying accumulation of lactate and alanine) to support ATP needs (Storey and Storey, 1986). Mechanisms of metabolic arrest include changes in enzyme activities and in numbers of mitochondria, reversible control over the activities of various regulatory enzymes via allosteric modifiers, reversible protein phosphorylation and differential effects of temperature (high Q_{10} values) on selected loci of metabolism (Kukal, 1991; Storey and Storey, 1988).

Proteins conferring cold-hardiness

Thermal hysteresis proteins

Thermal hysteresis proteins (THPs), also known as antifreeze proteins, depress the freezing point of a solution below its colligative melting point. The proteins are thought to adsorb to embryonic ice crystals by hydrogen-bond formation between hydrophilic residues and the O and H in the crystal lattice, thereby inhibiting crystal growth until the temperature is lowered further (DeVries, 1986 ; Duman et al., 1991a). These proteins can stabilize a supercooled state for extended periods of time, and are used by freeze avoiding insects, often in conjunction with cryoprotectants, to stabilize deep supercooling (for example, in midwinter *E. scudderiana* supercool to -38°C) (Storey and Storey, 1992). THPs have also been detected in freeze tolerant organisms, including the beetle, *Dendroides canadensis* and the centipede *Lithobius forficatus* (Knight and

Duman, 1986; Tursman and Duman, 1995). It has been suggested that, in freeze tolerant species, THPs act to inhibit the recrystallization of ice, a process by which small crystals redistribute and merge to form larger ones as subzero temperatures fluctuate (Duman et al., 1991a; Knight et al., 1995).

Ice-nucleating proteins

In order to initiate the formation of extracellular ice, freeze tolerant insects often employ haemolymph ice-nucleating proteins (INPs). The many hydrophilic residues of these proteins may serve as a template to order water into an embryonic ice crystal at high subzero temperatures, with more ice developing with subsequent cooling (Duman et al., 1991a). In concert with polyols and THPs, the action of INPs ensures that extracellular ice forms in a controlled and noninjurious manner. Haemolymph INPs have been identified in a large number of freeze tolerant insects, however, they have not been detected in *E. solidaginis*. Although the larvae freeze at -9°C, their haemolymph supercools to -18°C. These observations have led to suggestions that either INPs in *E. solidaginis* are placed differently (such as embedded in cell membranes), or that the insect is nucleated by contact with external ice (Bale et al., 1989).

Diapause associated proteins

Diapause is a state of arrested development common in cold hardy insects, and diapause associated proteins (DAPs) have been detected in many overwintering larvae (De Kort and Koopmanschap, 1994; Salama and Miller, 1993; Sula et al., 1995). These proteins typically accumulate in the haemolymph and in fat body cells in anticipation of diapause, and are quickly eliminated upon resumption of development after diapause is broken. This, along with their hexameric nature and amino-acid content, suggests that these proteins are

arylphorins. Arylphorins are employed by final instar larvae as a reservoir of amino acids and used for adult tissue formation during metamorphosis (Telfer and Kunkel, 1991). Some species may also employ these storage proteins as INPs (Duman et al., 1991a).

Stress-induced proteins

The aim of this study is to investigate cold-inducible genes in coldhardy insects. This warrants a review of the best characterized of the thermally-induced genes, those encoding the heat shock proteins (HSPs).

Heat shock proteins

The HSP family is encoded by a highly conserved group of genes present in organisms ranging from bacteria to vertebrates. Although constitutively expressed at low levels, HSP synthesis is drastically accelerated in response to stress, and is often accompanied by a shut down of normal protein synthesis. The production of constitutive HSPs is also inhibited during stress, to be replaced by the inducible members of the family. This stress response is rapid and appears to be universal, having been demonstrated in every organism thus far investigated. The inductive stresses include temperature shift (both heat and cold shock), exposure to anoxia, to heavy metals and to toxins that uncouple electron transport and oxidative phosphorylation, the accumulation of denatured proteins and viral infection (Becker and Craig, 1994; Denlinger et al., 1991; Morimoto et al., 1990).

The HSPs can be grouped into four major classes based on molecular weight, hsp70, hsp60, hsp90, and the small hsps (22-27 kDa). Their functions vary, not only between groups, but also depending on the state of the cell. In unstressed cells, some HSPs act as chaperones to nascent proteins, ensuring their proper folding, assembly and translocation (hsp70, hsp60). They may also keep

various transcription factors inactive in the absence of hormonal signals (hsp90). Stress may compromise the native conformation of extant proteins in the cell, exposing otherwise hidden regions which may cause the formation of unwanted protein aggregates. HSPs serve to bind to these exposed regions, preventing aggregate formation and keeping the denatured protein inert until the recovery period, when it will be degraded. The massive amounts of HSPs produced in response to a primary stress confers thermotolerance, increasing survival of cells and organisms to subsequent and often more severe thermal stresses (Becker and Craig, 1994; Denlinger et al., 1991; Lindquist, 1987; Yost et al., 1990).

Regulatory responses during heat shock

HSPs take their name from the conditions under which they were first detected, as a cellular response by *Drosophila* to a sudden increase in temperature. Several aspects of HSP regulation during heat shock are known including both transcriptional and translational controls. Heat shock genes have conserved regulatory regions called heat shock elements (HSEs). It is generally agreed that, with a sharp rise in temperature, a transcriptional protein factor called the heat shock factor (HSF) binds to the HSE, allowing transcription of the gene by the RNA polymerase II enzyme (Wu et al., 1990).

HSF is present in cells at all times, albeit in a transcriptionally inactive state. In yeast, the HSF-HSE complex is also constitutive, though transcription still remains blocked. Upon heat shock, the HSF undergoes multiple phosphorylations, after which it initiates transcription of the HSP gene (Sorger and Pelham, 1988; Wu et al., 1990). In higher eukaryotes, HSF does not complex with HSE until heat shock occurs. The eukaryotic HSF activation mechanism is not well characterized, but since HeLa HSF shows no response to *in vitro* treatment with protein phosphatases, it is unlikely to be modified by protein

kinases (Sorger et al., 1987). It has been suggested that constitutive HSP may bind HSF, rendering it inactive until the cell experiences heat stress (Somero, 1995).

In *Drosophila*, RNA polymerase II is not only bound to heat shock genes in unstressed cells, but it is also engaged in transcription. The progress of the enzyme is halted, however, and requires activation by the formation of the HSF-HSE complex for the completion of the transcript (Gilmour et al., 1988; Rougvie and Lis, 1988; Lis et al., 1990). This is consistent with the appearance, in *Drosophila*, of HSP messenger RNA (mRNA) within 4 minutes of experiencing heat stress (Lindquist, 1986). It has been shown that the kinetics of transcriptional activation of HSP genes differ depending on the stress imposed on the cell. This may suggest the involvement of either different HSFs or multiple HSE-binding proteins, conferring various rates of transcriptional activation (Morimoto et al., 1990).

HSP mRNA is selectively translated upon heat stress. Upon transcription, most genes produce mRNA precursors which require subsequent splicing before translation can begin. During heat stress, this post-transcriptional splicing mechanism is inhibited, preventing the production of new mRNA. HSP transcripts, however, require no such processing, hence they can undergo translation as soon as they are produced (Yost et al., 1990). Also, any extant non-HSP mRNA in the cell appears to complex with intermediate filaments upon heat shock, and are thus blocked from translation (Yost et al., 1990). All of this ensures the high, and almost exclusive, production of HSP in heat stressed cells, and indeed, HSP remains the main product of protein synthesis in *Drosophila* so long as high temperatures are maintained (Lindquist, 1986).

HSP expression and cold shock

Low temperatures can induce HSP synthesis, though the response to cold appears to differ from that described above for heat shock. In *Drosophila*, for example, expression was delayed and relatively low compared with the response to heat shock; hsp70 was only produced after an 11 hour exposure to 0°C, and the expression was only five times that of constitutive levels (Burton et al., 1988). Otherwise, HSPs and their encoding mRNAs do not appear during cold shock at 0°C, but are synthesized during recovery at 25°C (Burton et al., 1988 ; Peterson et al., 1990). Similar studies report the same response in the fleshfly, *Sarcophaga crassipalpis* and the gypsy moth, *Lymantra dispar* (Joplin et al., 1990; Yocum et al., 1991). In all cases, the intensity of HSP expression is directly proportional to the severity of the cold shock and HSP accumulation offers increased tolerance to subsequent low temperature exposures (Denlinger et al., 1991).

These observations raise questions as to what the low temperature cue is for HSP synthesis. As HSPs were not synthesized during the cold shock itself, it is thought that proteins were not being denatured (Burton et al., 1988; Peterson et al., 1990). It has also been suggested that the shift from the cold to the recovery temperature constitutes a relative heat shock and, thus, stimulates the expression of HSPs (Peterson et al., 1990; Denlinger et al., 1991). In either case, it appears that the role of HSPs during cold exposures may differ from their function during heat stress.

Current information does not suggest that HSP synthesis is a part of the response to low temperature stress in cold hardy insects. Initial studies in our lab assessed HSP expression in *E. scudderiana* and *E. solidaginis*, not during cold shock, but rather in animals subjected to laboratory acclimation to different temperatures as well as to outdoor conditions. The latter group was sampled at different intervals from fall through to spring. Although constitutive HSPs were detected at all times, no induced forms were detected, except for hsp70, which

was found in the March-April outdoor acclimated *E. scudderiana*. This observation suggested that with the coming of spring, *E. scudderiana* may have required greater amounts of HSP to cope with the major protein synthesis typical of metamorphosis (Storey et al., unpublished). In a separate study, overwintering larvae of *E. solidaginis* were found to produce HSPs in response to heat stress, but not during low temperature exposure (Lee et al., 1995).

These observations imply that other adaptations, such as polyol accumulation, may play more significant roles in insect overwintering strategies than do HSPs. Indeed, rapid glycerol synthesis in response to cold shock has been observed in *S. crassipalis* (Chen et al., 1987; Lee et al., 1987). An alternative explanation may be that the temperature induction of HSPs requires the rapid fluctuation of thermal conditions rather than the more gradual temperature changes typically encountered in nature.

Other protein responses to low temperature

A cold-inducible gene encoding sorbitol dehydrogenase has been found in eggs of the silkworm, *Bombyx mori* (Niimi et al., 1993). Diapause is maintained at 25°C in these eggs, and is broken by acclimation to 5°C. The sorbitol dehydrogenase gene is activated at this temperature, and the resulting enzyme is instrumental in the conversion of accumulated sorbitol back into glycogen for the continuation of embryonic development. It is suggested that the cold-induction is via some unknown protein factor, or alternatively, that the gene may be inhibited at 25°C by a DNA-binding protein which is itself rendered inactive at 5°C (Niimi et al., 1993).

CHAPTER TWO: MATERIALS AND METHODS

Animals

Cold acclimation in insects is induced by the chilling temperatures of late autumn, and is anticipatory of the lower temperatures which accompany winter's onset. Polyol production is a convenient indicator of the time-course of acclimation. Larvae of *E. solidaginis* employ glycerol and sorbitol in a dual cryoprotectant system, and the synthesis of the two polyols is triggered differentially by low temperatures. Glycerol is produced at relatively high chilling temperatures of 23 to 10°C, whereas sorbitol synthesis only begins below 5°C (Storey and Storey, 1983). Under laboratory conditions, the two polyols accumulate to maximal concentrations after 10-14 days of sustained cold exposure. Glycerol is the major polyol cryoprotectant in *E. scudderiana*, and is accumulated to 2M levels by midwinter. Optimal synthesis occurs between -10 and 0°C, with half-maximal concentrations accumulated within 2-4 days. Insects chilled at 3°C achieve the same production in 6 days (Churchill and Storey, 1989).

These observations prompted the design of a three-step temperature regime which allowed for optimal cryoprotectant accumulation and, presumably, the development of other aspects of cold acclimation, prior to subzero temperature exposure.

Galls containing *E. solidaginis* and *E. scudderiana* larvae were collected from goldenrod fields in the Ottawa region in the early autumn of 1994 and 1995. Once in the lab, the galls were placed for two weeks in an incubator set at a constant temperature of 15°C. Animals sampled after this exposure were considered controls. The remaining 15°C galls were then moved to another incubator set at 4°C, where they remained for a further two weeks. Samples were

taken at the end of this period, and the remaining galls were transferred to -20°C. Animals were sampled after 8, 24 and 48 hours of exposure, and one final sample was taken after 1 week of exposure to -20°C. Sampling was carried out by removing insects from their galls, immediately freezing them in liquid nitrogen, then storing them at -70°C until further use.

Chemicals

All chemicals used were purchased from Sigma Chemical Co. (St.Louis, MO), and were of molecular biology grade or their equivalent and of the highest purity.

PART I : cDNA LIBRARY CONSTRUCTION AND DIFFERENTIAL SCREENING

Overview

Two composite cDNA libraries were constructed, one for each species, using a pool of mRNA from all cold-exposed samples excluding the 15°C control group. Differential screening of the *E. scudderiana* cDNA library was carried out to reveal the presence of any transcripts whose abundances increase with low temperature exposure. These differentially expressed clones were used as templates in the synthesis of radiolabeled probes, which were used to probe Northern blots of total RNA from all time. Through Northern blot analysis against total RNA from the individual time-points, the clones were confirmed as representing differentially expressed transcripts, and the sizes of these could be determined. Further details of the above outlined plan follows.

General precautions

All plastic and glassware, including bottles and pipette tips, were autoclaved and gloves were worn at all times during operations involving nucleic acid manipulation. For RNA preparation and manipulation, DEPC (diethyl pyrocarbonate) was added to double-distilled water at a ratio of 0.1% v/v, to destroy any RNases that may have been present. Water so treated was left overnight, then autoclaved to eliminate any lingering DEPC. DEPC-treated water was used to make up all stock solutions and to dissolve RNA samples.

Total RNA isolation

Samples of frozen larvae were quickly weighed out and placed in a 50ml oak ridge tube. Trizol™ reagent (Gibco-BRL, Bethesda, MD) was added to 1ml per 50-100mg tissue. The samples were then homogenized and incubated for 5 minutes at room temperature. Chloroform was added (0.2ml/ml of reagent), and after 15 seconds of shaking, samples were incubated at room temperature for 2-3 minutes. The samples were then centrifuged at $12\,000 \times g$ at 4°C for 15 minutes. Centrifugation separates the samples into three layers: a lower organic layer containing proteins, an interphase layer which contains DNA, and an upper aqueous layer containing RNA. The upper aqueous layer was removed and transferred to autoclaved 1.5ml Eppendorf tubes, and isoamyl alcohol was added to approximately 1:1 (v/v). After gentle shaking, the tube was left to incubate at room temperature for 10 minutes to allow RNA precipitation. RNA was pelleted by centrifuging the samples at $12\,000 \times g$ for 10 minutes at 4°C . The supernatant was discarded and 70% ethanol was added to the pellets to remove any lingering chemicals. After gentle shaking and then centrifugation at $7500 \times g$ for 5 minutes, the pelleted RNA was either a) dissolved in DEPC-treated water and used

immediately in quantitative/qualitative analyses or b) stored at 4°C in the 70% ethanol for up to 1 week. Subsequent storage of water-dissolved RNA was at -20°C in 50µl aliquots..

RNA quality control

The concentration and purity of RNA samples was determined spectrophotometrically using absorbances at 260 and 280nm. At 260nm, an absorbance of 1.0 is equivalent to 40µg of nucleic acid per ml. An A_{260}/A_{280} ratio of approximately 1.6 indicated an RNA sample relatively free of impurities, such as proteins or phenols.

Total RNA sample quality was also assessed through denaturing agarose gels. Stock running buffer, 10x MOPS-- 0.2M MOPS (3-[N-Morpholino] propanesulfonic acid), 1 mM EDTA (ethylenediamine tetraacetate), and 50 mM sodium acetate-- was diluted for use to 1x . A 1.5% agarose denaturing gel was prepared by dissolving solid agarose in a boiling solution of 1x MOPS and 2.2M formaldehyde. Once cooled to 55°C, the agarose solution was poured as a horizontal slab (10 x 6 x 0.5 cm) and allowed to solidify. The gel was covered with 1x MOPS running buffer. RNA samples (2-4µg, as determined by spectrophotometry) was added to loading buffer (1x MOPS, 1.8M formaldehyde, 3.8% deionized formamide and 200 ng ethidium bromide), heated to 65°C to eliminate any secondary structures and subsequently cooled on ice. Samples were added to RNA loading buffer stock (prepared as a 6x stock containing 50% deionized formamide, 1x MOPS, 40% glycerol, 0.5% w/v bromophenol blue), diluting it to 1x, and were then loaded onto the gel and run at 50 volts until the bromophenol blue dye migrated two-thirds the length of the gel (approximately 60 minutes). The quality of obtained RNA can be assessed by viewing the gel with a

UV transilluminator (310 nm). Photography under UV light provided a permanent record.

Isolation of mRNA

Poly (A)+ mRNA was purified from total RNA samples using oligo(dT) cellulose chromatography. Dry oligo(dT) cellulose (New England Biolabs, Beverly, MA) was combined with 0.1M NaOH. This slurry was poured into a sterile column (previously washed with NaOH and DEPC-dH₂O) and equilibrated with loading buffer, composed of 1M NaCl and 2 mM NaPO₄ (pH 7.2). Total RNA was heated to 65°C and mixed with loading buffer (1:1 v/v) before loading onto the column. Elution with loading buffer and re-loading of the eluate was repeated twice. The column was then washed successively with loading buffer, middle wash buffer (loading buffer + 0.3M NaCl) and finally elution buffer (10 mM Tris-HCl and 1 mM EDTA). This last step eluted the poly (A+) mRNA, to which 3M Na-acetate (pH 5.2) and 100% EtOH was added. This was then left to precipitate overnight at -20°C, after which RNA was recovered by centrifugation at 13,000 × g for 45 min at 4°C. The supernatant was discarded and the pellet washed with 75% EtOH and re-centrifuged. The resulting pellet of mRNA was air-dried and resuspended in DEPC-ddH₂O. After spectrophotometric determination of its concentration and quality (A_{260}/A_{280}), the purified mRNA was stored at -70°C until further use.

cDNA library synthesis

cDNA library construction was carried out using the Uni-ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) according to instructions. In the construction of a composite cDNA library, an equal amount (1µg) of mRNA from

each cold-exposed group was combined before heating and subsequent cold-snapping to eliminate secondary structure. An oligonucleotide linker-primer was then introduced which, via a poly (dT) region, annealed to the pooled mRNA. This linker-primer also contained an *Xho* I restriction site and protective GAGA sequence. With the subsequent addition of nucleotides (dATP, dGTP, dTTP and a 5-methyl analog dCTP) and the enzyme reverse transcriptase, a complimentary first strand of cDNA was synthesized using the pooled mRNA as template. The methylated cytosines of the new strand offer protection from restriction enzymes. Addition of the RNase H enzyme fragmented or nicked the mRNA hybridized to the first strand cDNA. The leftover fragments of mRNA then served as primers for the enzyme DNA polymerase I which, when added along with supplemental nucleotides, synthesized the second strand of cDNA. The uneven termini of the double-stranded cDNA were then filled in by the *Pfu* DNA polymerase enzyme, afterwhich *Eco*R I adapters were ligated to the blunt termini via T₄ DNA ligase. Phosphorylation of these adapters allowed the ligation of the cDNA to the vector arms, which was accomplished via T₄ polynucleotide kinase. Digestion of the linker primer's *Xho* I site followed, yielding complete cDNA which allowed for the directional insertion into the vector, Uni-ZAP XR. This was carried out with the introduction of vector arms, and T₄ DNA ligase. After insertion, the ligated cDNA/vector arms was packaged into phage with packaging extract, then the phage was used to infect host *E. coli* (XL1-Blue).

The insertion of the cDNA into the vector disrupts the β -galactosidase gene, causing the formation of clear plaques on a plate of host cells growing on media containing X-gal (5-Bromo-4-chloro-3-indoyl- β -D-galactopyranoside). Vector which has not incorporated a cDNA insert synthesises active β -galactosidase, which makes for blue plaques.

The titer of the library, the approximate number of plaque forming units/ml (pfu/ml), was performed. Serial dilutions of the library were prepared in SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄·7H₂O, 0.01% w/v gelatin). XL1-Blue host cells were cultured at 37°C in LB broth (10g tryptone, 5g yeast extract, 5g NaCl/L, pH 7.5). A 100µl aliquot of each library dilution was mixed with an equal volume of cell culture in separate tubes and incubated at 37°C for 20 minutes. The cells of each dilution were added to 5ml of molten NZY top agar (5g NaCl, 2g MgSO₄·7H₂O, 5g yeast extract, 1g peptone/L, 0.7 % w/v agarose, pH 7.5) containing IPTG (140µg/ml) and X-gal (350µg/ml), were thoroughly mixed and then poured over an NZY (5g NaCl, 2g MgSO₄·7H₂O, 5g yeast extract, 10g peptone/L, 15g agar, pH 7.5) plate pre-warmed to 37°C . After incubating these plates overnight at 37°C and counting the numbers of plaques on each, titer was determined using the following formula:

$$\text{pfu/ml} = (\text{number of plaques} \times \text{dilution factor}) \div \text{volume of extract plated.}$$

Amplification of a cDNA library results in aliquoted samples of phage, ready for long-term storage. The following was carried out for both libraries. A series of 10 NZY plates (24 × 24 cm) was prepared, as well as an overnight LB broth culture of XL1-Blue cells-- this latter was aliquoted into 10 sterile 1.5ml tubes. An aliquot of packaged phage mixture (containing approximately 50 000 phage particles) was added to each tube, followed by a 15 minute incubation at 37 °C. Each aliquot of incubated bacteria was then mixed with 7ml of molten NZY top agar, then spread evenly onto one of the NZY plates. The plates were incubated at 37°C overnight to allow plaque formation on the bacterial lawn, then 10ml of SM buffer was overlayed onto each. The plates were then incubated at 4° C overnight with gentle shaking-- this brought phage off the solid media and into solution. The bacteriophage solution was then pooled and chloroform was added to 5% v/v. This mixture was incubated at room temperature for 15 minutes,

centrifuged at $500 \times g$ for 10 minutes, then the supernatant was transferred to a new sterile container. After adding DMSO (dimethylsulfoxide) to 7% v/v, the amplified library was aliquoted into sterile 1.5ml tubes and stored at -80°C . The titre of each amplified library was determined as described above.

Differential screening of the cDNA library

This process allows for the detection of genes differentially induced by exposure to cold. Primary screening of the cDNA library revealed a number of potential positive clones. These candidates were subjected to a second round of screening.

After determining the titer of the amplified cDNA library, a sufficient number of NZY plates (24×24 cm) were prepared so that each plate contained approximately 20 000 pfu and each clone was represented at least once. Plaques were lifted onto Hybond-N $0.45 \mu\text{m}$ nylon membranes (Amersham, Cleveland Ohio) after two minutes adsorption time. Plaque lift membranes were then soaked in denaturation (1.5M NaCl, 0.5M NaOH), neutralization (1.5M NaCl, 0.5M Tris-HCl, pH 8.0) and rinse (0.2M Tris-HCl, pH 8.0, $2 \times \text{SSC}$) buffers for 2, 5 and 0.5 minutes, respectively; SSC solution was prepared as a $20\times$ stock containing 3.0M NaCl, 0.3M sodium citrate, pH 7.0. Membranes were then placed on Whatman 3M filter paper, after which UV cross-linking using a UVP Ultraviolet Crosslinker Model CL-1000 (VWRCanlab, London, Ont.), set for $120\,000 \mu\text{joules}/\text{cm}^2$ (254nm) fixed the phage DNA to the membrane. Further fixing was accomplished by drying the plaque lifts in a Biorad Model 583 gel dryer for 2 hours at 80°C between two fresh pieces of Whatman paper.

Primary screening involved the synthesis of two sets of probe-- one from mRNA of control (15°C -acclimated) and another from the pooled mRNA of cold-exposed (4°C , -20°C --8h, 24h, 48h, 1 week) animals. Essentially similar to first

strand cDNA synthesis, outlined above for cDNA library construction, this process yielded, via the use of oligo(dT) primers, reverse transcriptase and the incorporation of radiolabeled nucleotide (α - ^{32}P -dCTP), labeled probe for hybridization against the DNA bound to the plaque-lift membranes.

The plaque-lifts were prehybridized in a solution containing 50% v/v formamide, 0.25M Na_2PO_4 , pH 7.2, 0.25M NaCl, 1mM EDTA, 7% w/v SDS, 10% w/v PEG for one hour in a Labline Model 308 hybridization oven (VWRCanlab, London, Ont.) at 42°C. After this period, the radiolabeled probe was added directly to the solution bathing the plaque-lifts to a concentration of 1×10^6 cpm/ml. Prior to its addition to solution, the radiolabeled probe was boiled for 5 minutes with sheared salmon sperm DNA (Gibco-BRL, 0.2mg/ml), then cold-snapped on ice for 2 minutes, to eliminate secondary structures. The first hybridization was carried out with primer synthesized from control (15°C) mRNA. Hybridization occurred overnight (approximately 16 hours) at 42°C.

After hybridization, the plaque-lift membranes were washed in $6 \times \text{SSC}$, 0.2% w/v SDS, shaken slowly for 30 minutes at room temperature. Repeated washes were carried out as necessary until the radioactivity in the membrane was reduced to a localized region of approximately 1000 cpm (as determined with a Geiger counter). Washed membranes were sandwiched between aluminum foil and plastic wrap, then exposed to Kodak XAR5 x-ray film at -70°C for one or two days, depending on the cpm of the membranes (>1000 cpm, 1 day; 500-1000 cpm, 2 days). The x-ray film was then developed manually using Kodak chemicals (Rochester, NY). The resulting pattern of spots on the autoradiograms, representing plaques where probe had hybridized to complementary DNA, revealed clones common to both stressed (cold exposed) and control (15°C) insects.

The second hybridization utilized the same plaque lift membranes, only this time they were exposed to labeled probe synthesized from pooled mRNA from the stressed (cold exposed) groups. First however, probe from the previous hybridization (control group probe) had to be removed or stripped from the plaque-lifts. The membranes were washed in $0.2 \times \text{SSC}$ at $45\text{-}50^{\circ}\text{C}$ for 15 minutes, with as many additional washes as was necessary to reduce the radioactivity on the membranes to background levels (<100 cpm). The membranes were then pre-hybridized and hybridized as before, only the labeled probe in the hybridization solution was from the cold exposed groups. Autoradiograms were made as before, by developing x-ray film that was exposed to the plaque-lifts. Autoradiograms from each hybridization were compared in order to reveal the presence of spots unique to each. Any spots unique to the cold exposed autoradiogram indicated putatively positive cDNA clones, representing genes that may be differentially expressed under stress.

Secondary screening began with the tracing of cold-induced candidate clones back to their corresponding phage plaques on the NZY plates. Each candidate plaque was excised using sterile Pasteur pipettes and placed in a 1.5ml Eppendorf tube with 500 μl SM buffer. After the addition of chloroform (to 5% w/v), the tubes were vortexed to mix their contents and then left to sit at room temperature for 30 minutes. Tubes were then centrifuged for 15 minutes at $5\,000 \times g$ at 4°C , and the supernatants containing isolated phage were transferred to new Eppendorf tubes and stored at 4°C . Spot titers were carried out for each candidate clone, to determine the approximate pfu/ml. For each clone, 10 μl of fresh XL1-Blue cell culture was added to 5ml of molten NZY top agar. This was poured onto fresh 100×15 mm NZY plates and allowed to solidify. The plates were then incubated for 1 hour at 37°C . Serial dilutions were made of each candidate clone, and 10 μl of each dilution were spotted onto an NZY plate, then replaced at 37°C

for an overnight incubation. Plaques were then counted and the titer of each phage preparation was determined using the same formula given above for the cDNA library titer procedure.

Each potential cold-induced clone was then re-plated on separate small (10 cm diameter) NZY plates to 50-100 pfu/plate. Plaque lifts were made of these, and screening against labeled control and cold exposed probes were carried out as described above for primary screening. Membranes hybridized with radiolabeled probe were exposed to x-ray film for a minimum of two days at -70°C , and examination of the resulting autoradiograms strongly implicated some of the candidate clones as representing genes induced by cold exposure.

Northern blotting

Once identified through differential screening, confirmation of the cold-induced clones was carried out by probing Northern blots. These blots contained total RNA from all time points, including the 15°C control. This procedure also served to localize the clones to specific points in the time course of cold exposure.

Total RNA from control (15°C), cold exposed (4°C) and subzero exposed (-20°C : 8h, 24h, 48h, 1 week) larvae was separated on a 1.5% agarose formaldehyde denaturing gel (20 μg per lane). After electrophoresis, the gel was rinsed in ddH₂O, then stained with ethidium bromide (0.3mg/400ml). The stained gel was exposed to UV light and photographed using Polaroid 667 film. The ribosomal RNA (rRNA) band was used to ensure equal loading of the wells, and band intensity was quantified using Scan Jet3C scanner with Deskscan II software (Hewlett Packard).

The RNA on the gel was then blotted onto a 0.2 μm nylon membrane by the neutral transfer method. The gel and a piece of NYTRAN 0.2 μm nylon membrane (Schleicher & Schuell, Keene, NH) were washed in $10 \times \text{SSC}$, then

stacked in a transfer sandwich as follows (bottom to top): a Whatman filter paper wick, soaked in $20 \times \text{SSC}$, the gel, the nylon membrane, 2 pieces of Whatman filter paper (soaked in $10 \times \text{SSC}$), a 15 cm stack of dry paper towels, a flat piece of plexiglass and a 500g weight. Care was taken to prevent air-bubble formation between the nylon membrane and the gel. The ends of the paper wick, at the bottom of the stack, was soaking in a tray filled with $20 \times \text{SSC}$. The transfer was allowed to take place for at least 14 hours, after which the nylon membrane was washed in $2 \times \text{SSC}$. The RNA was then UV crosslinked to the membrane following the same protocol described above for differential screening.

In vivo excision of clones

Before radiolabeled probe could be made from the potential positive clones, phage had to be converted to a circular double-stranded plasmid (pBLUESCRIPT) which can replicate independently in host cells. This was accomplished using a different strain of *E.coli*, SOLR. This amplification of plasmids containing inserts of interest was also useful in subsequent sequencing operations.

From a fresh culture of XL1-Blue cells in LB broth (incubated at 37°C), mid-log phase cells ($\text{OD}_{600} = 0.2-0.5$) were centrifuged at $3000 \times g$ for 5 minutes. Resuspension of the cell pellet in 10 mM MgSO_4 (to an $\text{OD}_{600} = 1.0$) was followed by the introduction of Uni-ZAP XR phage stock (representing a clone of interest) and of ExAssist helper phage. After a 15 minute incubation at 37°C , LB broth was added to the mixture and incubation continued for approximately 3 hours with gentle shaking. Cells were pelleted by a 15 minute centrifugation at $400 \times g$, and the supernatant containing the intermediate filamentous phage was transferred to a new container and incubated at 70°C for 15 minutes. After pelleting any leftover host cells by another 15 minute centrifugation at $3000 \times g$

the supernatant containing the filamentous phage stock (the clone of interest) was transferred to 1.5ml Eppendorf tubes and stored at 4°C. A separate 200µl of fresh SOLR cells ($OD_{600} = 1.0$) was then infected with 100µl of each filamentous phage stock in 1.5ml Eppendorf tubes and incubated at 37°C for 15 minutes. A 100µl aliquot of this mixture was used to create a bacterial lawn on an LB-ampicillin (25µg/ml) plate, which was incubated at 37°C overnight. By streaking a single colony from this plate onto a fresh LB-ampicillin plate, a master plate of SOLR cells containing the pBLUESCRIPT plasmid of interest was produced. These were incubated overnight at 37°C.

Miniprep plasmid isolation

Probe synthesis and sequencing required that pBLUESCRIPT plasmids be purified from their SOLR cell hosts and the latter's genomic DNA. A single colony from a master plate was used to inoculate 3ml of LB-ampicillin (25µg/ml) broth and incubated overnight at 37°C with shaking. A 1ml aliquot of this culture was then used to inoculate 9ml of fresh LB-ampicillin in a 15ml plastic screwcap tube-- this was incubated for 3 hours under the same conditions as the first culture. The 10ml culture was then centrifuged at $3000 \times g$ at 4°C for 6 minutes, afterwhich the supernatant was discarded and the cell pellet was resuspended in 1ml of GTE (50mM glucose, 25mM Tris-HCl, pH 8, 10mM EDTA). Two ml of lysis buffer (0.2M NaOH, 1% SDS) was then added, and after gentle shaking and a 10 minute incubation on ice, the tube was centrifuged for 10 minutes at $10\,000 \times g$ at 4°C.

The supernatant from this last centrifugation was transferred to a new tube and 4.5ml isopropanol was added. A thorough mixing and 15 minute room temperature incubation was followed by another centrifugation at $10\,000 \times g$ for 10 minutes at 4°C. The supernatant was discarded and the pellet air-dried for a

few minutes before resuspension in 1ml T₅₀E₁₀ (50mM Tris-HCl, pH 8, 10mM EDTA). A 15µl aliquot of RNase A (SIGMA, 10mg/ml) was added and the mixture was incubated at 37°C for 30 minutes. This was followed by a phenol:chloroform extraction, entailing successive extractions with one volume of phenol, 1:1 phenol:chloroform and chloroform, always carrying on with the upper, aqueous phase. To the final aqueous extract, 0.1 volumes of 3M Na-acetate (pH 5.2) and 2 volumes of EtOH was added— this was left on ice for a one hour incubation, followed by a 30 minute centrifugation at $10\,000 \times g$ at 4°C. The supernatant was discarded, the plasmid pellet was washed with 70% EtOH, then left to air dry. Plasmid DNA was resuspended in 30µl of sterile ddH₂O, quantified using spectrophotometry (1 OD= 50µg/ml of double stranded DNA), and stored at -20°C until further use.

Insert isolation

The inserts carried in the isolated plasmids represent genes that are potentially induced by cold as determined by differential screening of the cDNA library. These inserts must be separated from the plasmid DNA before further manipulations can be carried out.

The first step was digestion with *EcoR* I/*Xho* I (New England Biolabs, Beverly, MA). A 1-2µg aliquot of plasmid DNA was incubated with up to 5 units of enzyme in 50µl 2× One-Phor-All Buffer PLUS (Pharmacia) at 37°C for 2 hours. The reaction was deactivated by the addition of 0.2 volumes of DNA loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 15% w/v Ficoll Type 400) and heating the solution at 65°C for 20 minutes. After cooling to room temperature, sample was loaded onto a 1.5% TAE agarose gel (0.5 × TAE; 20 mM Tris-acetate, 0.5 mM EDTA, pH 8.3) and the restriction

fragments separated— gels were run at 80V until the first tracking dye front has traversed 2/3 of the gel. A 1Kb DNA ladder (premixed to 1 μ g/ μ l) was used as a molecular weight standard. After staining in ethidium bromide solution as described above for formaldehyde gels, the gel was viewed with a UV transilluminator.

The insert band was cut out of the gel and was purified using GENECLAN III (BIO101, Vista, CA) according to the kit instructions. The gel slice was weighed and then soaked in 3 volumes of 6M NaI in a sterile 2ml Eppendorf tube. This was heated to 55°C for 5 minutes to dissolve the agarose, afterwhich EZ-GLASSMILK (silica beads) was added to at least 2 μ g/ μ g DNA. DNA binding was allowed to occur over a 10 minute incubation on ice, afterwhich the silica was pelleted by centrifugation at 10 000 $\times$ g for 30 seconds. The supernatant was discarded and the pellet washed twice with NEW Wash (NaCl, Tris and EDTA, pH 7.2). Elution of the DNA from the silica was carried out by resuspending the pellet in sterile ddH₂O, another centrifugation as before, discarding of the supernatant and resuspension of the DNA pellet.

Synthesis of labeled probe

The DNA insert isolated in the above step represents possible cold-induced genes. These inserts will serve as templates for the synthesis of radiolabeled probes.

Ten μ l of the above gene-cleaned DNA was transferred to a sterile, 500 μ l Eppendorf tube, boiled for 2 minutes, then centrifuged at 14 000 $\times$ g for 30 seconds. The following was added; 1 μ l dNTPs without dCTP (5 mM each), 1 μ l random primer (100 mM, d(N)₆, New England Biolabs, Beverly, MA), 2 μ l 10 $\times$ random primer buffer (0.5M Tris-HCl, pH 7.6, 20mM DTT, 50mM MgCl₂, 0.4M KCl), 5 units Klenow fragment of DNA polymerase I (New England Biolabs,

Beverly, MA), 5 μ l α -³²P dCTP (3000 ci/mol, Amersham). After mixing, the reaction proceeded at 37°C for 30 minutes, then 1 μ l 0.5M EDTA was added. The labeled mixture was loaded onto a small column of Sephadex G-50 (approximately 1ml, equilibrated in T₁₀E₁ buffer; 10mM Tris-Cl, 1mM EDTA, pH 8.0) and centrifuged at 400 $\times$ g for 3 minutes. T₁₀E₁ buffer was added to the eluant to a volume of 500 μ l, and 1 μ l of this was used for scintillation counting.

Probing Northern blots

Pre-hybridization of Northern blots was carried out at 42°C with pre-warmed solution (50% v/v formamide, 0.25M Na₂PO₄, pH 7.2, 0.25M NaCl, 1mM EDTA, 7% w/v SDS, 10% w/v PEG) in a hybridization oven for a minimum of 30 minutes. Radiolabeled DNA probe was boiled with salmon sperm DNA (10 mg/ml) for 5 minutes, chilled on ice, then added to the pre-hybridization solution. Hybridization was conducted at 42°C overnight (at least 14 hours), afterwhich blots were washed in successive dilutions of SSC: 2 $\times$ SSC, then 0.5 $\times$ SSC, both with 0.2% SDS and at room temperature for 15 minutes. Further washes were required for blots which had background radiation greater than 250 cpm as determined by Geiger counter— a localized area (band) of high cpm was optimal. Autoradiography was carried out as previously described, by exposing blots to x-ray film at -70°C. Development of the x-ray film was also carried out as previously described. Northern blots were used multiple times (maximum 5), and for each re-use, probe from the previous hybridization had to be stripped off the membrane. This was done in the same manner as with plaque lift membranes, described above.

Densitometric analysis of the developed autoradiograms entailed the use of computer imaging software. A Scan Jet3C scanner (Hewlett-Packard, Palo Alto, CA) was used in conjunction with Deskscan II v2.2 software (Hewlett-

Packard, Palo Alto, CA) to render an autoradiograph into a form recognizable by another software package, Imagequant v3.22 (Innovative Optical Systems Research). This latter was used to measure relative band intensity, reflecting transcript levels, across the various lanes of the autoradiograph image. This analysis not only gave final confirmation of the potential candidate clones from cDNA screening, but it also gave a profile of relative transcript levels throughout the cold/subzero temperature exposure as compared to controls.

Sequencing reactions

Clones positively identified by Northern analysis as being cold-upregulated were sequenced in both directions using the Sequenase 2.0 kit (USB, Arlington Heights, IL) according to manufacturer's instructions. A 5 μ l aliquot of plasmid DNA (or 3-5 μ g) was mixed with 1 μ l of 1.27M NaOH, 1 μ l sequencing primer (T₃ or T₇, 5 pmol/ μ l) then incubated at 37°C over 10 minutes. After cooling on ice, 1 μ l of 1.33M HCl and 2 μ l of sequenase reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl) was added and the mixture was incubated again at 37°C for ten minutes. This allowed the primer to anneal to the plasmid DNA.

A 2.5 μ l aliquot of each termination mixture (G, A, T and C, each containing 80 μ l of dNTPs, 50 mM NaCl, and 80 μ l of ddGTP, ddATP, ddTTP and ddCTP, respectively) was placed into separate tubes and warmed to 37°C. Labeling mixture stock (5 $\times$ stock contains 7.5 μ l dGTP, 7.5 μ l dCTP, 7.5 μ l dTTP) was diluted 5-fold with sterile ddH₂O. Sequenase polymerase was diluted 8-fold with sequenase dilution buffer (10mM Tris-HCl, pH 7.5, 5mM DTT, 0.5 mg/ml BSA). To the ice-cold annealed DNA mixture, the following was added; 1 μ l 1M DTT, 2 μ l dilute labeling mixture, 0.5 μ l ³⁵S-dATP and 2 μ l dilute sequenase. This was mixed and allowed to sit at room temperature for 5 minutes. A 3.5 μ l aliquot

of the cooled labeling reaction was then transferred to each of the termination mixtures, mixed and incubated at 37°C for 5 minutes. The addition of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) deactivated the reaction.

These samples were heated to 75°C immediately before loading onto a 35 × 43 cm, 0.4mm thick sequencing gel (6% acrylamide, 7M urea, 0.5 × TBE). Gels were electrophoresed at a constant power of 60 watts for up to 6 hours. After fixing in a solution of 10% v/v methanol, 5% v/v glacial acetic acid, the gels were dried onto Whatman 3M filter paper at 80°C for one hour in a Bio-Rad 583 gel dryer. The dried gel was exposed to x-ray film overnight at room temperature, then the film was manually developed and the sequence read. The initial sequence of approximately 400 bp was used to design custom oligomers (synthesized at Queen's University's DNA synthesis facility), with which further sequence was obtained.

Sequences of potential clones were entered into the GenBank BLAST server (available via the World Wide Web) for comparison and analysis. Blastn was used for the comparison of nucleotide data to known nucleotide sequences. Blastx, which translates obtained nucleotide data into amino acid sequences, was used to compare them to known amino acid sequences.

Sequence alignments for figures were carried out using DNASTAR MegAlign software (DNASTAR Inc., Madison, Wisconsin). Sequence similarities between pairs of homologues were calculated using this software, according to the formula: (number of matching residues)/ (length of compared sequence)-(total number of gaps introduced into both compared sequences).

PART II : DIFFERENTIAL DISPLAY PCR

Overview

Differential display PCR (DD-PCR) was carried out for both *E. scudderiana* and *E. solidaginis*, in order to detect any genes upregulated in response to cold and subzero temperature exposure. DD-PCR is an emerging technique that offers a powerful alternative to cDNA library screening. Apart from its relative simplicity, DD-PCR allows for the simultaneous examination of numerous samples for differential gene expression. Briefly, the process involves the reverse transcription of poly (A)+ mRNA from control and stressed tissues, followed by PCR reactions to amplify and radiolabel the products (amplicons). These are then separated on a sequencing gel and the resulting autoradiogram is compared. Bands unique to or prevalent within the stress lane potentially represent genes transcribed in response to stress (while this study was concerned with stress-induction of genes, any genes potentially downregulated in response to stress can also be detected by focusing on those bands unique to or prevalent in the control lane). The DNA in these differentially displayed bands is then recovered, reamplified, screened via Northern blot analysis, cloned and sequenced. A detailed description of the above outline follows.

Precautions and total RNA isolation

All precautions taken during DD-PCR experiments were as described above for cDNA library work. Also, total RNA was isolated using the same technique described above.

DNase treatment of total RNA

Total RNA samples were treated with DNase I to eliminate any genomic DNA contaminants which could be amplified during PCR and would produce

false banding on the sequencing gel. Reaction mixtures contained the following: (up to) 20µg total RNA, DNase I reaction buffer (1 ×, diluted from stock 200mM Tris-HCl, pH 8.4, 20mM MgCl₂, 500mM KCl), DNase I (0.3 units/µg RNA), RNAsin (2.5 units/µg RNA). Reactions were carried out at 37°C for 30 minutes, then stopped by the addition of EDTA to a final concentration of ≥ 2mM. A further incubation at 75°C was carried out to inactivate the DNase, but this was not always found to work-- lingering DNase activity digested subsequent PCR products. This necessitated a phenol:chloroform extraction, following the same procedure outlined in total RNA isolation, above.

Reverse transcription

After DNase treatment, poly (A)+ mRNA within the total RNA sample was selectively reverse-transcribed by way of primers complementary to the characteristic polyadenosine tail of the former-- these are referred to as anchored primers (see Table 1). Reactions contained the following in a final volume of 20µl: total RNA (0.25µg/µl), DTT (10mM), first strand buffer (1 ×, diluted from 10 × stock: 250mM Tris-HCl, pH 8.3, 375mM KCl, 15mM MgCl₂), dNTP (50µM), anchored primer (1.25µM), RNAsin (0.25U/µl), reverse transcriptase (10U/µl). Reactions were incubated in a thermocycler (PTC-100, MJ Research Inc.) as follows: 65°C, 5 mins → 37°C, 60 mins → 75°C, 5 mins → 4°C. The RNAsin and reverse transcriptase were not added to the reactions until after 10 minutes at 37°C. At the end of the thermocycle, each reaction was briefly centrifuged at 14 000 × g and either placed on ice for immediate PCR, or stored at -20°C for later use. This was repeated for each of the three anchored primers, such that transcripts beginning with an adenosine, guanine or cytosine residue after the poly (A)+ tail were each selectively reverse-transcribed.

Table 1: Primers used for DD-PCR. The HIND III sequence ('AAGCTT'), is common to all. Each anchored primer was used in combination with each random primer (total of 24 PCR reactions/sample).

Primer	Sequence
Anchored :	
H-T ₁₁ A	5' AAGCTTTTTTTTTTTTA 3'
H-T ₁₁ C	5' AAGCTTTTTTTTTTTTC 3'
H-T ₁₁ G	5' AAGCTTTTTTTTTTTTG 3'
Random:	
AP1	5' AAGCTTGATTGCC 3'
AP2	5' AAGCTTCGACTGC 3'
AP3	5' AAGCTTTGGTCAG 3'
AP4	5' AAGCTTCTCAACG 3'
AP5	5' AAGCTTAGTAGGC 3'
AP6	5' AAGCTTGCACCAT 3'
AP7	5' AAGCTTAACGAGG 3'
AP8	5' AAGCTTTTACCGC 3'

PCR, electrophoresis and autoradiography

A 1 μ l aliquot of the reverse-transcription reaction was used in a 10 μ l PCR which also included: PCR buffer (1 $\times$, diluted from 10 $\times$ stock: 150mM Tris-HCl, pH 8.3, 500mM KCl, 15mM MgCl₂), dNTP-C (20 μ M), dCTP (2 μ M), anchored primer (the same used for the reverse transcription, 1.25 μ M), random primer (1.25 μ M), AmpliTaq DNA polymerase (Perkin-Elmer: 0.5 U/ μ l), and 0.2 μ l of ³²P-dCTP (Amersham: 3000 Ci/mol). A drop (18-20 μ l) of mineral oil was added to the surface of the reaction. Each individual reaction was run in duplicate. Eight random primers were used (see Table 1), one at a time in combination with each of the three anchored primers. Hence, each RNA sample from control and cold-exposed insects was amplified 24 times.

The PCR reaction was carried out in the thermocycler as follows : 94°C, 1 min $\rightarrow$ 94°C, 30 seconds $\rightarrow$ 40°C, 2 mins $\rightarrow$ 72°C, 30 seconds $\rightarrow$ repeat steps 2 to 4, 30 times $\rightarrow$ 72°C, 5 mins $\rightarrow$ 4°C.

After thermocycling, a 4 μ l aliquot of each PCR reaction was mixed with 4 μ l of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). These were heated to 75°C for 5 minutes, cooled on ice, then loaded onto a denaturing polyacrylamide sequencing gel. Gels were electrophoresed at 60 watts for approximately 4 hours, then transferred directly onto an appropriately sized piece of Whatman blotting paper directly from the glass plate. Gels were then dried in a gel dryer for 40-60 minutes at 80°C.

Gels generally had high counts ($\geq 10\,000$ cpm) as measured by a hand-held Geiger counter, allowing short exposures to x-ray film in an autoradiography cassette-- 2-5 hours at -70°C (or room temperature) was often quite sufficient. Gels were oriented with x-ray film by poking needle holes through both.

Autoradiograms were developed manually and the resulting banding patterns were examined for two features: 1) reproducibility between duplicate reactions and 2) bands unique to or enhanced in the cold/subzero exposure lanes as compared to control. Reproducibility was very high, with $\geq 95\%$ of bands appearing in both duplicate lanes. Bands appearing exclusively in the stress lanes were rare, so bands in which signal intensity was far greater in the stress lanes were chosen as candidates potentially representing upregulated genes.

Band isolation and reamplification

The developed autoradiogram was oriented with the dried gel and bands of interest were located. Once located, bands were cut out of the gel with a clean razor blade. Each gel slice was soaked along with its Whatman paper backing in 50 μ l sterile ddH₂O in a sterile 1.5ml Eppendorf tube for 30 minutes, followed by 15 minutes of boiling. After centrifugation at 14 000 $\times$ g for 5 minutes, the supernatant was transferred to a fresh tube. The gel and paper pellet was not discarded, in case another extraction proved necessary.

Reamplification was carried out using this eluted DNA and the same combination of primers (anchored and random) used in the initial PCR. Five μ l of eluted DNA was included in a 40 μ l reaction containing: PCR buffer (1 $\times$), dNTP (100 μ M), anchored primer (0.2 μ M), random primer (0.2 μ M), AmpliTaq polymerase (0.04 U/ μ l), sterile ddH₂O to volume. A drop of mineral oil was applied to the top of the reaction and reamplification was carried out using the same conditions as in the initial PCR, only for 40 cycles instead of 30. After reamplification, products were aliquoted and either used immediately or stored at -80°C until further use.

To determine the results of the reamplification, the PCR products were resolved on a 1.5 % agarose gel. A 10 μ l aliquot of PCR product was mixed with 2

μl DNA loading buffer (same as stop buffer, above), heated to 75°C for 5 minutes. Running conditions and ethidium bromide staining were carried out as described above in Part I. Reamplification products were visualized under UV light and photographed.

Evaluation of candidates by Northern analysis

Northern blot probing was used to eliminate false positive DD-PCR candidates-- blotting, radiolabeled probe synthesis (using reamplification products as template), hybridization and autoradiography were all carried out as described above in Part I.

Cloning and sequencing

Candidates confirmed positive via Northern analysis were inserted into a plasmid vector to allow for sequencing. This was facilitated by the fact that Taq polymerase adds a single deoxyadenosine to the 3' end of the DNA it synthesizes. This A-overhang can be exploited with a T-vector, a plasmid with a 3'-T overhang at its insertion site. Cloning was carried out using the Original TA Cloning Kit (Stratagene, San Diego) following the manufacture's instructions.

As the A-overhangs are unstable and may not be present in amplicons older than 24 hours, reamplification of the confirmed candidates was required. This was carried out using the procedure described above. Ligation was carried out in a reaction volume of 10μl containing: 3μl fresh amplification product, 1μl ligation buffer (10 × stock: 60mM Tris-HCl, pH 7.5, 50mM NaCl, 60mM MgCl₂, 1mg/ml BSA, 70mM β-mercaptoethanol, 1mM ATP, 20mM DTT, 10mM spermidine), 2μl pCR 2.1 plasmid vector (25ng/μl), 1μl T4 DNA ligase (4U/μl), sterile ddH₂O to volume. This reaction mixture was incubated at 14°C overnight.

Transformation was carried out using supplied INV α F' cells in 50 μ l aliquots in 1.5ml tubes. β -Mercaptoethanol was added to the cells to approximately 20mM, followed by 2 μ l of the ligation reaction. This mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds, then replaced on ice for 2 minutes. A 250 μ l aliquot of SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) was then added, afterwhich the tubes were incubated at 37°C for 1 hour with vigorous shaking. Master plates for each clone were made by plating this mix onto LB agar plates containing 50 μ g/ml ampicillin and X-Gal, then the plates were left to incubate overnight at 37°C. Colonies were selected by color development-- vectors that had not incorporated an insert gave rise to blue colonies, while successful ligations resulted in white colonies.

One aspect of DD-PCR is that each band, as shown on the original sequencing gel, is likely to contain a number of different cDNA species-- generally 3-5. This is due to the limited power of the gel to resolve DNA fragments larger than 300bp, as well as the fact that some amplicons are going to be similar in size to the one that truly represents an upregulated gene. Prior to sequencing then, it becomes necessary to: 1) determine how many different sequences are present within one positive clone and 2) determine which among these represents the upregulated gene.

The first objective was met by a process called 'C-tracking', which involved the same culturing, miniprep, and sequencing procedures outlined above in Part I. Up to 10 separate bacterial colonies were selected from a clone's master plate, and each was grown overnight in an LB broth + ampicillin culture. Plasmid preparations were made and verified by restriction digest analysis. The separated inserts were extracted from the agarose gels via the GENECLEAN kit and stored at -20°C. Some of the remaining plasmid preparation was used in a partial

sequencing reaction that followed the same procedure described in Part I, only using one termination mixture-- for example, ddCTP. When resolved on a sequencing gel, each plasmid carrying a different insert displayed a unique banding pattern, or C-track. In this way, the number of different sequences present within one positive clone was determined.

The second objective was met by using the same radiolabeled probe synthesis and Northern hybridization protocol as described in Part I. Insert DNA, extracted from the restriction digest agarose gel, was used to make separate probes representing each individual sequence as determined by C-tracking. Each sequence was then used to probe separate Northern blots, and analysis of the subsequent autoradiograms revealed which among them represented an upregulated gene. The plasmid preparation carrying this particular insert was then either used in an in-lab manual sequencing reaction, or was sent away to be sequenced by an automated procedure (either the Core Facility for Protein/DNA Chemistry, Queen's University, Kingston, or Bio S&T, Lachine, Que.). All clones were sequenced completely in both directions (from the plasmid's T7 and M13 primer sites), and sequence analysis was carried out via the BLAST website, as described in Part I.

5'RACE

The amplification of cDNA ends (RACE) technique allows the isolation of sequences at the 5' termini of mRNA given partial sequence data from the 3' terminus. In this way, primers designed from known sequence can be used in the reverse transcription and subsequent PCR of a specific transcript. 5'RACE was applied to gain greater sequence data from one of the DD-PCR clones, which

represented an incomplete transcript sequence. This was carried out by Canadian Molecular Research Services (Orleans, Ontario) in the following manner.

Reverse transcription was carried out to obtain a cDNA of the specific sequence to be extended. reaction conditions were : Tris-HCl (pH 8.4) 20mM, KCl 50mM , $MgCl_2$ 2.5mM , DTT 10mM, Gene Specific Primer 1 (GSP1) 10-25ng, each dNTP 400 μ M, total insect RNA 1 μ g, reverse transcriptase 200 U. This reaction was incubated for 50 minutes at 42°C, then underwent RNase digestion (10 minutes at 37°C). The reaction was purified of proteins, unincorporated nucleotides and primers through a silica spin column elution, then the cDNA, representing the specific transcript of interest, was tailed at the 3' terminus with poly C under the following conditions: Tris-HCl (pH 8.4) 10mM, KCl 25mM, $MgCl_2$ 1.5mM, dCTP 200 μ M, cDNA of specific sequence, terminal deoxynucleotide transferase (TdT). This reaction was incubated for 10 minutes at 37°C. The dC-tailed cDNA, now containing known regions of sequence at either end, was then subjected to PCR: Tris-HCl (pH 8.4) 20mM, KCl 50mM, $MgCl_2$ 1.5mM, GSP₂ 400nM, each dNTP 200 μ M, tailed cDNA, TAQ 2.5 U. the PCR conditions were 94°C, 1 min $\rightarrow$ 94°C, 30 seconds $\rightarrow$ 55°C, 1 min $\rightarrow$ 72°C, 2 mins for 30-35 cycles $\rightarrow$ 72°C, 7 mins $\rightarrow$ 4°C. The PCR products were evaluated on 1.5% agarose gels with EtBr staining, then candidate amplicons were cloned in a TA vector and sequenced as described.

CHAPTER THREE:

RESULTS

PART I : *EPIBLEMA SCUDDERIANA* cDNA LIBRARY

Differential screening

Using a set of 7 NZY plates (24 × 24 cm) and the plaque lifts made from them, approximately 140 000 plaques from the *E. scudderiana* cDNA library were differentially screened. In an initial attempt at primary screening, stress probe was synthesized from a pool containing equal amounts of transcript from each cold (4°C) and subzero (-20°C) time-point. This was used to probe plaque lifts and was compared with hybridizations using probe synthesized from mRNA of control (15°C) insects. This round of primary screening yielded no discernable cold-upregulated plaques, and may have been due to the dilution of rare messages--mRNA unique to a single time-point, or transcripts present at very low copy number, or a combined effect. It is possible that such messages could have been diluted to undetectable levels, given the five-fold increase in relative sample volume.

A second attempt at primary screening compared plaque lift hybridizations between control (15°C) and a single stress time-point, cold (4°C) exposure. Labeled probe was synthesized from mRNA from each of these samples, and hybridization comparison yielded 11 candidate plaques, named P1-P11. Each of these plaques was isolated and grown on bacterial lawns on individual plates (10cm diameter, approximately 200 plaques). A plaque lift was made for each of these plates (i.e. for each candidate plaque) and subjected to a secondary round of differential screening; mRNA from the same two time points as the primary screen (15°C and 4°C) was used to synthesize labeled probe. In this round of

screening, three of the 11 candidates-- P3, P6 and P8-- displayed more intense signal when hybridized with 4°C probe as compared to control. The other candidates did not show any differences between hybridizations.

Northern analysis

The three positive candidates then underwent *in vivo* excision and plasmid isolations were carried out for each. Restriction digests showed single inserts for each candidate and allowed for an estimation of their sizes-- 596, 818 and 613 bp for candidates P3, P6 and P8, respectively (see Fig. 1). Using the digested inserts as templates for radiolabeled probe synthesis, northern blots were probed with each candidate. Blots contained equal amounts of total RNA from each of the cold/subzero time-points as well as control. Analysis of the subsequent autoradiograms revealed banding in all time-points, with band intensities varying degrees in the cold/subzero treatments. Transcript sizes were estimated through the use of RNA size standards (see Fig. 2 for an example).

Candidate P3 hybridized to a pair of transcripts of approximately 1400 and 700 nt, respectively (see Figs. 2 and 3). Figure 4 shows the results of densitometric analysis of these bands. Though present in the control lane, the bands were upregulated by cold exposure. Both transcripts, P3a and P3b, appear to be predominantly upregulated during the -20°C exposure, reaching a approximate 2-fold accumulation over control values. For P3a, the lower intensity at the 24 hour at -20°C time-point might not reflect downregulation, but rather the partial degradation of larger transcripts in the total RNA sample. The smaller transcript, P3b, displayed a steady upregulation over the time-course, appearing two-fold over control at 4°C, then peaking at 24 hours at -20°C by 3-fold. Figures 3 and 4 show the results of the P6 northern probe. The revealed transcript was

Figure 1. Restriction digest and size estimation of *E. scudderiana* clones P3, P6 and P8. a) shows restriction digest products resolved on a 1.5% agarose gel stained with ethidium bromide. Lane S denotes DNA size standards. Each clone is run in five lanes: 1) is the intact plasmid, no digestion. 2) and 3) are digests run with only one of two restriction enzymes, EcoR I and Xho I, respectively, and lanes 4) and 5) are digests using both enzymes. b) shows the calibration curve of the size standards and the positions P3, P6 and P9 inserts occupy, resolving as fragments of 596, 818 and 613 bp.

Figure 2. Northern hybridization of *E. scudderiana* clone P3 versus the cold/subzero time course. a) shows the autoradiograph and the resulting bands, P3a and P3b. Positions of RNA size markers are indicated. Blot was exposed to X-ray film for approximately 72 hours. Figure represents 1 of 3 repeats. b) shows EtBr-stained rRNA band from corresponding formaldehyde gel. c) shows the calibration curve for the size markers, and the positions of bands P3a and P3b. Size estimations for the two bands are approximately 1400 and 700 bp, respectively.

Figure 3. Representative Northern hybridizations of *E. scudderiana* cDNA library clones P6 and P8 versus the cold/subzero time course (a), with representative EtBr-stained rRNA band from a formaldehyde gel (b). Blots were exposed to X-ray film for 14 hours (P6) and 7 days (P8). Autoradiographs (P6 given as example) were quantified using Imagequant v.3.22 software and graphed as histograms or relative transcript abundance.

Figure 4. Results of densitometric analyses of Northern hybridizations of *E. scudderiana* clones P3 (n=3), P6 (n=4) and P8 (n=4). Each time point represents a number of individually probed blots analysed separately, and band intensities were normalized relative to the 15°C lane band. Equal loading of the blots was determined by densitometric analysis of their corresponding EtBr-stained formaldehyde gels (n=7), also normalized against the 15°C lane. All graphs show means $\pm$ SEM.

approximately 1.6 Kb in size, and displayed a maximum accumulation during the -20°C exposure before decreasing again to approximate control levels.

The results for the P8 probe are shown in Figures 3 and 4, and display an approximately 1.6 Kb mRNA differentially accumulating during all cold/subzero exposures over control. This transcript appeared upregulated almost evenly over control levels.

cDNA sequence analysis

All three clones were sequenced in-lab using the dideoxy method and by automated sequencing at the Core facility at Queen's University or at Bio S&T (Lachine, Que.). Clones were sequenced completely in both directions (sequencing primer sites T3 and T7).

Figure 5 shows the sequence data for clone P3. The 612 bp insert contained a polyadenylation signal (AATAAA) starting at position 551 followed by a poly (A)+ tail starting at position 570. A GenBank nucleotide sequence comparison indicated that P3 belongs to a family of zinc-finger domain proteins known as LIM proteins, in particular those LIM proteins associated with muscle development. P3 encoded one long open reading frame (ORF) of 94 amino acids, framed by start (ATG) and stop codons (TGA). A number of distinguishing features in the deduced amino acid sequence of P3 are indicated in Figure 5, which demonstrate that P3 encodes one copy of the LIM motif: the conserved cysteine and histidine residues (at positions 11, 14, 32, 38, 41 and 62) that characterize a LIM consensus sequence, a glycine-rich region (residues 64, 68, 71, 73, 75, 76, 77 and 79) which is also typical of the LIM proteins, and a putative nuclear targeting signal coded by residues 62 to 67 (Stronach et al., 1996).

Though homology was found to muscle-LIM proteins from a number of vertebrates, P3 appeared most similar to two *Drosophila* genes, Mlp60A and

Figure 5. Nucleotide and deduced amino acid sequences of *E. scudderiana* clone P3, muscle-LIM protein homologue. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position. Putative start codon, stop codon and polyadenylation signal are underlined. Cysteine and histidine residues common to LIM domain are double underlined. Glycine residues common to LIM proteins are in bold. Dashed underline indicates the putative nuclear targeting signal.

65	CGAGTCTTGGCAGCACCGCCCTGCACTGTATACACGCGTTTCGGAGCTAGACTCTCGTAGCGAATCG	
125	ACGAA C A G G A T A A G T T A A G C G A G C A C A	ATG CCT TTC AAA OCT GCA GAC AAC CCG AAA TGC
1		M P F K K P A D N P K C
185	CCG AAG TGC GGC AAG TCC GTC TAC GCG GAA GAG CGC GTC GCC GGC GGA TTA AAA TGG	
12	P K C G K K S V Y A A E E R V A G G L K W	
245	CAC AAG ACC TTT TTC AAA TGC AAA TGC ATG GGT GGC AAT AAG AGT TTG GAC TCC ACC AAC TGC ACG	
32	H H F F K C G M C N C AAT AAG AGT TTG GAC TCC ACC AAC TGC ACG	
305	GAA CAC GAC GGT GAA ATT TAC TGC AAA AAT TGC CAC GGT CGC AAA TTC GGA CCC AAA GGC	
52	E H D G E I Y C K N C H C R K F G P K G	
365	TAC GGC TTC GGC GGT GGC GCT GGT GGC CTA TCG ATG GAC GCC GGC GCT CAG TTC CAG GAA	
72	Y G F G G A G C L S M D A G A Q F Q E	
442	AAC GCG AAT TGA GCGCAGCCGCCACCTGATGCAGCGCTAGCgaGTGCGCAGCACATCCCGGCCACCTGCGGACTC	
92	N A N STOP	
525	CGCCCCGACAAACAATCTGTTCTTAAAAATTACTATAGTTAGGGTTATTTATCGCTATCGCTTAATTTATTTTGTTCGGTTC	
603	TATTTATTTATAAAAAATAACAAGAAATAAAAGATCGACCATCAAAAAAATAAAAAAATAAAAAAATAAAAAA	
612	AAACTCGAG	

Mlp84B, in which amino acid alignments show a respective 76.1% and 71.6% similarity. Figure 6 shows the alignment of the deduced P3 amino acid sequence with those of Mlp60A and Mlp84B, as well as rat, mouse and human sequences. Except for Mlp60A, all homologue sequences are partial, showing only the N-terminal portion which contains a single LIM motif. Table 2 shows the pairwise sequence comparisons between the first LIM domain of all 6 muscle-LIM proteins.

Given the estimated transcript size of 700 nt and its homology to Mlp60A (which encodes a 92 amino acid protein), the P3 clone likely represents the complete sequence of an *E. scudderiana* homologue to the *Drosophila* muscle-LIM protein, and would correspond to band P3b found in Northern analyses (Fig. 2). Band P3a, approximated at 1400 nt, does not correspond to the estimated size of Mlp84B which, containing 5 repeats of the LIM motif, would suggest a transcript size of at least 3.5 Kb. Band P3a, then, might represent an as-yet uncharacterized LIM-protein, perhaps one carrying two LIM-motifs. Nevertheless, it would appear that both LIM proteins are upregulated by cold and subzero exposure in *E. scudderiana*.

The nucleotide sequence for clone P6 is shown in Figure 7. The 805 bp sequence contained a polyadenylation signal (AATAAA) starting at position 714, with the poly (A)+ tail following at position 748. P6 contained one long ORF of 110 amino acids, coded in the first reading frame. Database comparison of the deduced amino acid sequence gave no reliable matches, but nucleic acid sequence homology search indicated a similarity between P6 and the 28S ribosomal RNA (rRNA) gene of numerous insects. This similarity also extended to numerous plants (including *Arabidopsis thaliana*, citrus lemon, tomato, white mustard and rice), fungi, the yeast *Saccharomyces cerevisiae*, mouse and human 28S rRNA

Figure 6. Amino acid sequence alignment of *E. scudderiana* clone P3 and muscle LIM protein sequences from *Drosophila* (Mlp60A and Mlp84B, Stronach et al., 1996), mouse (*Mus musculus*, Hashimoto et al., 1996), human (*Homo sapiens*, Arber et al., 1994) and rat (*Rattus norvegicus*, Arber et al., 1994). Residues that do not match the P3 sequence are shaded. All sequences other than P3 and Mlp60A are partial, depicting only the N-terminal and the first of their multiple LIM domains.

P3	M P	-	F K P A D N P K C P K C G K S V Y A A E E R V A G G L	29
Mlp60A	M P	-	F V P V E T P K C P A C G K S V Y A A E E R V A G G Y	29
Mlp84B	M P	S F Q P J E A P K C P R C C G K S V Y A A E E R L A G G Y	30	
Mouse	M P	N W - - C G G A K C C G A C C E K T V Y H A E E E I Q C N G R	28	
Human	M P	N W - - C G G A K C C G A C C E K T V Y H A E E E I Q C N G R	28	
Rat	M P	N W - - C G G A K C C G A C C E K T V Y H A E E E I Q C N G R	28	
P3	K W H K T F F K C C G M C N K S L D S T N C T E H D G E I Y C	59		
Mlp60A	K F H K T C F K C S M C N K A L D S T N C T E H E K E L F C	59		
Mlp84B	V F H K N C F K C G M C N K S L D S T N C T E H E R E L Y C	60		
Mouse	S F H K T C F H C M A C R K A L D S T T V A A H E S E I Y C	58		
Human	S F H K T C F H C M A C R K A L D S T T V A A H E S E I Y C	58		
Rat	S F H K T C F H C M A C R K A L D S T T V A A H E S E I Y C	58		
P3	K N C H G R K F G P K G Y G F G G G A G C L S M D A G A Q F	89		
Mlp60A	K N C H G R K Y G P K G Y G F G G A G C L S T D T G A H L	89		
Mlp84B	K T C H G R K F G P K G Y G F G G T G A G T L S M D N G S Q F	90		
Mouse	K V C Y G R R Y G P K G I G F G G Q G A G C L S T D T G E H L	88		
Human	K V C Y G R R Y G P K G I G F G G Q G A G C L S T D T G E H L	88		
Rat	K V C Y G R K Y G P K G I G F G G Q G A G C L S T D T G E H L	88		
P3	- - Q E N A N .	95		
Mlp60A	- - N - - R E	92		
Mlp84B	- L R E N G D V	97		
Mouse	G L Q F Q Q S P	96		
Human	G L Q F Q Q S P	96		
Rat	G L Q F Q Q S P	96		

Table 2. Pairwise amino acid sequence similarities, *E. scudderiana* clone P3 and assorted muscle-LIM proteins. Except for P3 and Mlp60A, only the first (N-terminal) LIM domain of the other sequences were considered in this comparison. For sequence references, see legend for Figure E

	1	2	3	4	5	6
1. P3	-	76.1	71.6	48.4	47.4	46.3
2. Mlp60A	-	-	75	58.7	57.6	56.5
3. Mlp84B	-	-	-	47.9	46.9	45.8
4. Rat	-	-	-	-	97.9	96.9
5. Mouse	-	-	-	-	-	99
6. Human	-	-	-	-	-	-

Figure 7. Nucleotide and deduced amino acid sequence of *E. scudderiana* clone P6. Putative stop codon and polyadenylation signal are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

genes. Figure 8 shows an alignment of P6 and a number of insect 28S rRNA nucleic acid sequences. The figure shows only that portion of P6 homologous to 28S, the first 320 nucleotides of the sequence which, interestingly, encodes the potential ORF. The rest of the insect sequences are similarly truncated, showing only that portion with similarity to P6, approximately 300 bases. Table 3 shows the pairwise sequence comparisons between these regions of homology, with a range of similarities from 93.5% (P6 vs. *Manduca sexta*) to 45.1% (P6 vs. *Anopheles albimanus*).

How a ribosomal RNA sequence, theoretically never intended for translation, came to be represented in a cDNA library-- which should select only for those RNAs that have poly (A)+ tails-- may be accounted for by the finding that hundreds of eukaryotic mRNAs contain regions of homology to the 28S and 18S rRNA genes (Mauro and Edelman, 1997). These rRNA-like sequences, often hundreds of base-pairs long, can be located within the 5' untranslated region (UTR), the 3'UTR and within protein coding regions. The nature of Clone P6, in the context of the insect, cannot be known prior to obtaining more or complete transcript sequence data.

The *Drosophila* 28S rRNA, though 4.1 Kb, runs with the 18S band at approximately 1.6 Kb. (Darnell et al., 1990). This corresponds to the determined transcript size of P6. This then raises the possibility that P6 could be an artefact of cDNA library synthesis, involving the splicing of AT-rich regions of mitochondrial DNA with other transcripts.

The sequence of Clone P8 is shown in Figure 9. The 581 bp sequence did not show any identity to sequences within the GenBank database, nor did it encode any substantial ORFs-- indeed, termination codons (TAA, TAG, TGA) are found in all three reading frames within the first 25 bases of sequence. There are also numerous stop codons interrupting all other reading frames. The resulting

Figure 8. Nucleotide alignment of *E. scudderiana* clone P6 and insect 28S rRNA sequences. Only bases 1-320 are shown of the P6 sequence, and the homologues are similarly truncated to show only that portion similar to P6. Bases which do not match the P6 sequence are shaded. The sequences are, in order, *Manduca sexta*, *Archaeopsylla erinacei*, *Apis mellifera*, *Panorpa communis*, and *Tenebrio molitor*. All sequences are from Friedrich and Tautz (1997), except for *T. molitor* which was a direct submission to GenBank by Friedrich (1995).

P6
Manduca
Flea
Apis
Stonefl.
Tenebri.

P6
Manduca
Flea
Apis
Stonefl.
Tenebri.

P6
Manduca
Flea
Apis
Stonefl.
Tenebri.

-----AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAACGGGAGTGTGACGGGGTAAACCCAAAGTGGCTG 103
GAGCGA/AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAATAGGAGTGTGACGGGGTAAACCCAAAGTGGCTG 109
-----A/AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAAGAGTGTGACGGGGTAAACCCAAAGTGGCTG 97
GGCGA/AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAAGAGTGTGACGGGGTAAACCCAAAGTGGCTG 102
GCGCGA/AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAAGAGTGTGACGGGGTAAACCCAAAGTGGCTG 102
GCGCGA/AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAAGAGTGTGACGGGGTAAACCCAAAGTGGCTG 102
GCGCGA/AGGGAATCGGTTCTATTTCCGGACCGGTTCAATATATGTTCCCTCGTTTAAAGAGTGTGACGGGGTAAACCCAAAGTGGCTG 102

P6
Manduca
Flea
Apis
Stonefl.
Tenebri.

-----AAGAGCGCGCGGAGGTTCCGGGAGAGTTTCTTTTCTGCTGAGGTTCCGAGTTCCATGGAATCTATATAGAGGAGATATGTTCCGAPACGGAGAGCAACCCGCTT 213
AAGAGCGCGCGGAGGTTCCGGGAGAGTTTCTTTTCTGCTGAGGTTCCGAGTTCCATGGAATCTATATAGAGGAGATATGTTCCGAGAGAGCAACCCGCTT 218
GAGAGCGCGGAGGTTCCGGGAGAGTTTCTTTTCTGCTGAGGTTCCGAGTTCCATGGAATCTATATAGAGGAGATATGTTCCGAGAGAGCAACCCGCTT 206
GAGAGCGCGGAGGTTCCGGGAGAGTTTCTTTTCTGCTGAGGTTCCGAGTTCCATGGAATCTATATAGAGGAGATATGTTCCGAGAGAGCAACCCGCTT 211
GAGAGCGCGGAGGTTCCGGGAGAGTTTCTTTTCTGCTGAGGTTCCGAGTTCCATGGAATCTATATAGAGGAGATATGTTCCGAGAGAGCAACCCGCTT 211
GAGAGCGCGGAGGTTCCGGGAGAGTTTCTTTTCTGCTGAGGTTCCGAGTTCCATGGAATCTATATAGAGGAGATATGTTCCGAGAGAGCAACCCGCTT 211

P6
Manduca
Flea
Apis
Stonefl.
Tenebri.

-----TGGCGGCGGTGTCGCGGTAACCTTGAATTCAGGTGAGGCGTGTACGTGGAGTGTGCGCGCGGTGCTGTAACCATATATGCGACAGGTCTCTCCMAGG 319
TGGCGGCGGTGTCGCGGTAACCTTGAATTCAGGTGAGGCGTGTACGTGGAGTGTGCGCGCGGTGCTGTAACCATATATGCGACAGGTCTCTCCMAGG 321
TGGCGGCGGTGTCGCGGTAACCTTGAATTCAGGTGAGGCGTGTACGTGGAGTGTGCGCGCGGTGCTGTAACCATATATGCGACAGGTCTCTCCMAGG 306
TGGCGGCGGTGTCGCGGTAACCTTGAATTCAGGTGAGGCGTGTACGTGGAGTGTGCGCGCGGTGCTGTAACCATATATGCGACAGGTCTCTCCMAGG 270
TGGCGGCGGTGTCGCGGTAACCTTGAATTCAGGTGAGGCGTGTACGTGGAGTGTGCGCGCGGTGCTGTAACCATATATGCGACAGGTCTCTCCMAGG 319
TGGCGGCGGTGTCGCGGTAACCTTGAATTCAGGTGAGGCGTGTACGTGGAGTGTGCGCGCGGTGCTGTAACCATATATGCGACAGGTCTCTCCMAGG 296

Figure 9. Nucleotide sequence of *E. scudderiana* clone P8. Numbers indicate nucleotide position.

ATTGAATAATATTTTGTAGGCAJTTTTTGGAGGAAGGTTGGGAGATGCTAAATAATTGAACAACCAACTTGGCAGGCTCTGGTAAATATTCACTTAC 98
 ACTCTATTATGAAATGICITTTATTAJTCITTTTAAACACAGGAACCAAGITCAAGACAAATTATAAGCACTGCTTGTACAGTCAGCATCATGAACATAT 196
 TTGGACCTTATTTCTAAOCCAAATTAAGTGGGATACCTCTTAATGAACTGTAAOCCGTAACCTGAACGGAAATGTAAACGAATTTAAGTGTATAT 294
 CACTGAGAAAGTGCCCAATCATTTGGTTCGTTGGGAOCCGTAGGCCAACCGGTTGCGGATGTACTATACGGGTACTGTAACTGTCTGTACGGTTACA 392
 GCTCGCTAGTGAGTACCCACCTTAAAGTTAATGATGTGAACTATTATAAAATCGTTACACCAATAGATTTAAGGTTAAACGGTTGATTATATAAT 490
 AATGTTAGITTTAAGTAGAATAAAGATTTTTTGTACTGAAAAAATAAAAAATAAAAAATAAAAAATAAAAAATAAAAAATAAAAAATAAAAAAT 581

deduced amino acid sequences, such as they were, did not show any similarity to any known protein sequence. Clone P8 nevertheless did include a polyadenylation signal (AATAAA) starting at position 512, followed by a poly (A)+ tail starting at position 531. As it is a fragment of a larger sequence (1.6 Kb), it is possible that clone P8 represents a 3' untranslated region (UTR) of a cold-upregulated transcript.

Further Northern analysis

As clone P3 represents a muscle-LIM protein that is crucial during myogenesis, it raised the possibility that *E. scudderiana* larvae might be expressing this and other genes according to a developmental time-table independently of cold exposure. The insect is, after all, in its last larval instar, and the cold/subzero time course allowed 3 extra weeks of potential developmental time which the 15°C control group does not experience.

In light of this, a second 5 week time course was constructed using *E. scudderiana* larvae held at a constant 15°C. Larvae were collected within the same seasonal period (late September) as animals taken for the cold/subzero time course. Samples were taken as previously described at time-points corresponding to those of the cold/subzero time course. From the time of collection, then, in-gall larvae were kept at 15°C and sampled at 2 weeks, 4 weeks, 4 weeks and 8 hours, 4 weeks plus a day, 4 weeks plus 2 days, and 5 weeks. Each of these sampling times corresponded to, respectively, the Control (2 weeks at 15°C), 4°C (two weeks), and the four -20°C time-points (8 hrs, 24 hrs, 48 hrs, 1 week) of the cold/subzero temperature time course.

Total RNA was isolated from insects from each time-point using the Trizol protocol described in the Materials and Methods. Equal amounts of RNA were loaded onto formaldehyde gels and Northern blots were made. Radiolabeled

probe from each of the *E. scudderiana* clones was synthesized and hybridized. The results of these hybridizations are shown in Figure 10. Both P3 transcripts appeared during the 15°C time course, though their band intensities did not change as time progresses. P6 also appears evenly at each time-point. That P8 even appeared during the 15°C time course is debatable, though within the size range expected for its transcript (approximately 1.6 Kb), a band may be present, though it is most obscure. These results support the initial finding that these transcripts are upregulated as part of *E. scudderiana*'s response to cold temperature exposure.

PART II : DIFFERENTIAL DISPLAY

Preliminary testing of the DD-PCR technique was required in order to address a number of questions, including : 1) are the differences between individual animals within a control or treatment group enough to cause differential banding, 2) can any such differences be reduced or checked by using total RNA pooled from a number of individuals from the same group and 3) how crucial is DNase treatment of total RNA samples prior to reverse transcription?

Using the Trizol protocol described in the Materials and Methods, total RNA was isolated from 3 individual control (15°C) larvae of *E. solidaginis* and *E. scudderiana*. The concentration of each was determined spectrophotometrically, and then pooled mixes, one for each species, were made using equal amounts of total RNA from the 3 individual samples. Aliquots of each (individual and

Figure 10. Northern hybridizations of all *E. scudderiana* cDNA library clones versus a five-week 15°C time course. Each hybridization was carried out once, and blots were exposed to X-ray film for approximately 14 hours. Representative EtBr-stained rRNA band from one of the corresponding formaldehyde gels is shown.

pooled) were DNase treated and their concentrations determined again. Each individual and pooled sample was then used in reverse transcription and subsequent PCR reactions. The resulting banding pattern is shown in Figure 11, where few differences between individual insects could be seen (lanes 1-3). Pooling these samples (lanes P) did not eliminate the possibility of false positives arising from differences between individuals, but whatever differences there are appear to be largely evened out by the pooling.

The question of genomic DNA contamination of total RNA samples was addressed using total RNA isolated from bulk *E. solidaginis* larvae. Aliquots of this pooled RNA were used in reactions that included and excluded DNase treatment, in combination with/without reverse transcription, prior to PCR amplification. Figure 12 shows the results from these four reactions: the banding pattern in lane 1 (-DNase/-RT) demonstrates that the RNA sample is contaminated with enough DNA to be amplified by PCR. The absence of bands in lane 2 (+DNase /-RT) demonstrates that treatment with DNase effectively eliminates DNA contamination. Without subsequent reverse transcription, no amplification takes place and no bands appear. Lanes 3 and 4 compare the results of reverse transcription with and without prior DNase treatment. Although no major differences can be seen, DNase treatment prior to reverse transcription should reduce the frequency of false positives in subsequent DD-PCR experiments. In all following experiments, each control and treatment group was represented by pools of ten individual insects, and RNA samples from these were

DNase treated prior to PCR

Figure 11. Differential display PCR test. Total RNA from both individual (numbered lanes) and pooled (lanes marked P) insects (of each species, *E. scudderiana* and *E. solidaginis*) were reverse transcribed and amplified using primers HT₁₁-G and AP8.

E. scudderiana *E. solidaginis*

Figure 12. Differential display PCR test. Total RNA from *E. scudderiana* was reverse transcribed and amplified with and without (+ or -) prior DNase (pre-reverse transcription) and RT (reverse transcription) treatments.

	1	2	3	4
DNAse	-	+	+	-
RT	-	-	+	+

NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

UMI

Eurosta solidaginis

Pooled total RNA samples from control (15°C) and each of the cold treatment time points (4°C 2 weeks, -20°C 8 hrs, 24 hrs, 48 hrs 1 week) were selectively amplified using the 24 combinations possible between the three oligo (dT) anchored primers and the 8 random primers (Table 1). Each reaction utilized the same amount of total RNA and was run in duplicate. The amplification products were then separated on polyacrylamide gels and displayed as autoradiograms. Figures 13, 14 and 15 show the results of three such gels, using anchored primers HT₁₁-A, HT₁₁-C and HT₁₁-G, respectively, each in combination with random primer AP4. Table 4 shows a summary of the results of all primer combinations. Overall, each PCR reaction revealed between 50 and 90 bands, ranging in size from 100 to 600 bp. Overall, approximately 1500 displayed bands were assessed, among which 62 were chosen as candidates upregulated in response to cold exposure. These are listed in Table 5. Sixty-one bands appeared to be downregulated in response to low temperatures, these being exclusive or most prominently displayed in the control lane. Only the former group of candidates were considered in this project.

All potentially upregulated candidates were traced back to their positions on their respective gels, using the autoradiographs as described in the Materials and Methods. Examples of candidate bands can be seen in Figures 14-16. Each band was then cut out of the gel and the cDNA eluted as described.

Figure 13. *E. solidaginis* DD-PCR reactions, covering all time-points of the cold/subzero temperature time course. Primer combination HT₁₁-A and AP4 and amplification products were resolved on a polyacrylamide gel. U-marked bands indicate upregulated candidates, while D indicates potentially downregulated bands.

Figure 14. *E. solidaginis* DD-PCR reactions, covering all time-points of the cold/subzero temperature time course. Primer combination was HT₁₁-C and AP4 and amplification products were resolved on a polyacrylamide gel. U-marked bands indicate upregulated candidates, while D indicates potentially downregulated bands.

Figure 15. *E. solidaginis* DD-PCR reactions, covering all time-points of the cold/subzero temperature time course. Primer combination was HT₁₁-G and AP4 and amplification products were resolved on a polyacrylamide gel. U-marked bands indicate upregulated candidates, while D indicates potentially downregulated bands.

Table 4. Summary of total banding in all *E. solidaginis* DD-PCR reactions. Averages presented as mean $\pm$ SE. Potentially upregulated and downregulated bands are indicated by upward and downward arrows, respectively.

	<u>Anchored Primers</u>			<u>Total Bands</u>
	HT ₁₁ -A	HT ₁₁ -C	HT ₁₁ -G	
Bands, all APs	331	592	577	1500
Bands/AP	41.4 $\pm$ 14	74 $\pm$ 26	72.1 $\pm$ 25	-
Total $\uparrow$	27	17	18	62
Total $\downarrow$	26	19	16	61

Table 5. Summary of all *E solidaginis* potentially upregulated candidates. Anchored and arbitrary primers are referred to by their letters (A,C,G) and numbers (1-8) respectively. A dash (-) indicates failure to reamplify candidate in question. Table continues on next page.

Band	Primer Combination	Estimated Band Size(bp)
U1	A-1	371
U2	A-1	222
U3	A-2	318
U4	A-2	-
U5	A-2	-
U6	A-2	189
U7	A-2	156
U8	A-3	355
U9	A-3	-
U10	A-4	850
U11	A-4	484
U12	A-4	341
U13	A-5	807
U14	A-5	461
U15	A-5	-
U16	A-5	327
U17	A-6	628
U18	A-6	509
U19	A-6	325
U20	A-6	312
U21	A-6	160
U22	A-7	324
U23	A-7	222
U24	A-7	222
U25	A-8	358
U26	A-8	348
U27	A-8	252
U28	C-1	350
U29	C-2	324
U30	C-2	127
U31	C-3	288
U32	C-4	231
U33	C-4	-
U34	C-4	-
U35	C-5	912

Table 5, continued. Summary of all *E solidaginis* potentially upregulated candidates. Anchored and arbitrary primers are referred to by their letters (A,C,G) and numbers (1-8) respectively. A dash (-) indicates failure to reamplify candidate in question.

<u>Band</u>	<u>Primer Combination</u>	<u>Estimated Band Size(bp)</u>
U36	C-5	-
U37	C-5	-
U38	C-6	230
U39	C-6	212
U40	C-6	207
U41	C-7	880
U42	C-8	567
U43	C-8	480
U44	C-8	260
U45	G-1	312
U46	G-1	178
U47	G-2	262
U48	G-3	257
U49	G-3	238
U50	G-3	229
U51	G-4	479
U52	G-4	351
U53	G-4	312
U54	G-4	278
U55	G-4	224
U56	G-5	388
U57	G-5	367
U58	G-6	351
U59	G-6	-
U60	G-7	486
U61	G-7	213
U62	G-8	328

Figure 16. *E. solidaginis* candidate U1, from DD-PCR polyacrylamide gel.

Reamplification of each candidate followed, using the same combination of anchored and random primers as in the original PCR reaction. Not all reamplification reactions were successful. Ten candidates (see Table 5) failed to reamplify despite numerous PCR reactions as well as further attempts at eluting cDNA from the original DD-PCR band. Where reamplifications were successful the amplicons were resolved on 1.5% agarose gels using ethidium bromide staining. Size estimations of the amplicons were also carried out at this time. Figure 17 shows the results of a few such reamplifications, those for candidates U51-55.

Each reamplified candidate was cut out of their agarose gels and the band DNA isolated using the Geneclean kit as described in the Materials and Methods. The DNA was then used to synthesize ^{32}P -labeled probe for Northern hybridizations. The results of these hybridizations are presented in Figure 18--only 8 of the candidates appeared to be upregulated upon exposure to low temperatures. The rest either did not show prominent banding at all on the Northern autoradiographs, or they showed no increase in band intensity during cold exposure as compared to the control time-point. Table 6 summarizes the results of size estimations of both the PCR products and their respective transcripts.

Each positive candidate was cloned as described in the Materials and Methods, including ligation into a T-vector plasmid and transformation of host cells with these. Each clone was plated, and color-selection of the resulting

Figure 17. Reamplification and size estimation of *E. solidaginis* candidates U51-U55. a) shows the amplicons resolved on a 1.5% agarose gel stained with ethidium bromide. Lane S denotes DNA size standards. b) shows the calibration curve of the size standards and the positions the amplicons occupy, resolving as fragments of 479, 351, 312, 310 and 224 bp.

Figure 18. Northern hybridization of *E. solidaginis* DD-PCR candidates. Autoradiographs represent single hybridizations. Northern blots were exposed to X-ray film for 14 hours (U19, U47), 48 hours (U1, U44, U52, U53) and 72 hours (U20). EtBr staining of rRNA bands from corresponding formaldehyde gels is presented as Mean $\pm$ SEM (n=7).

Table 6. *E. solidaginis* DD-PCR candidates positively confirmed by Northern analysis. Anchored and arbitrary primers are referred to by their letters (A,C,G) and numbers (1-8) respectively.

Candidate	Primer Combination	Estimated size (bp)	Estimated Transcript size (bp)
U1	A-1	371	650
U19	A-6	325	650
U20	A-6	312	600
U44	C-8	189	1050
U47	G-2	262	850
U51	G-4	479	600
U52	G-4	351	130
U53	G-4	312	300

colonies showed ligations were successful in 85-98% of the reactions. Each clone was then C-tracked, or partially sequenced, in order to determine how many different sequences the original DD-PCR band contained. Figure 19 shows the results of one such C-track, in which 4 separate colonies of clone U1 revealed 3 different sequences. Three sequences was the average number among the 8 candidate clones, with a range from 2 to 6. Each sequence contained within each of the 8 clones was isolated by restriction digest and radiolabeled for Northern probing (see Figure 20). Radiolabeled probe was synthesized from each of these inserts and Northern hybridizations were carried out against total RNA from all time-points in the cold/subzero time course, including controls. Autoradiography was employed to determine which of these represented the original upregulated DD-PCR band. In the case of U1, two sequences showed little or no banding, while U1-2 showed increased transcript accumulation over the cold and subzero temperature time-points (Fig. 21). Any differences in banding intensity may be accounted for by the slight banding by the contaminating sequences in the original band.

After identifying the inserts that represented the actual upregulated clone, each underwent automated sequencing. The nucleotide and deduced amino acid sequence of clone U1 is shown in Figure 22-- the 578 bp sequence encodes one long ORF of 113 amino acids. This ORF is bound by a start codon (ATG) and stop codon (TAA) at nucleotide positions 75 and 411, respectively. One ambiguous nucleotide (position 324) made for an untranslatable codon (amino acid position 84). A polyadenylation signal (AATAAA) is present starting at position 546, and

Figure 19. Partial sequencing ('C-tracking') of sequences from four separate bacterial colonies transformed with clone U1. Plasmid preparations from each colony were partially sequenced, revealing that two of them contained the same sequence (lanes 1, representing clone U1-1). The two other reactions (lanes 2 and 3) show unique sequences, U1-2 and U1-3.

1 2 1 3

Figure 20. Restriction digests of the three representative *E. solidaginis* U1 clones, U1-1, U1-2 and U1-3. Plasmids were digested with EcoR I, resolved on a 1.5% agarose gel and stained with ethidium bromide. Lane S denotes DNA size standards.

Figure 21. Northern hybridization of *E. solidaginis* clone U1-2 versus cold/subzero time course, representing one hybridization. Blot was exposed to X-ray film for 5 days. EtBr-stained rRNA band from the corresponding formaldehyde gel is shown.

Figure 22. Nucleotide and deduced amino acid sequence of *E. solidaginis* clone U1-2. Putative start codon, stop codon and polyadenylation signal are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

1	ATG	CGT	TAC	GTG	GCT	GCA	TAC	CTG	TTG	GCT	GCT	CTT	GGC	GGT	AAT	GAG	AAC	CCG	AAA	AAT	74
	<u>M</u>	R	Y	V	A	A	Y	L	L	A	A	L	G	G	N	E	N	P	K	N	134
21	GCC	GAT	ATC	GAG	AAG	ATT	CTC	AGC	TCA	GTG	GGT	ATT	GAG	GCT	GAA	GCC	GAA	CGC	CTA	AGC	194
	A	D	I	E	K	I	L	S	S	V	G	I	E	A	E	A	E	R	L	S	
41	AAA	GTT	ATC	AAG	GAA	TTA	AAT	GGC	AAG	TCT	ATT	GAA	GAA	TTG	ATT	GCC	CAG	GGT	OGC	GAG	254
	K	V	I	K	E	L	N	G	K	S	I	E	E	L	I	A	Q	G	R	E	
61	AAA	CTC	TCG	TCC	ATG	CCT	GTT	GGT	GGT	GGT	GCT	GCT	GCT	GCC	GCC	GCG	CCC	GCA	GCT	GCT	314
	K	L	S	S	M	P	V	G	G	G	V	A	A	A	A	A	P	A	A	A	
81	GCT	CCG	GCC	TAG	GGT	GAC	AAA	AAA	GAA	GCT	AAG	AAA	GAA	GAG	AAG	AAG	GAA	GAA	TCT	GAG	374
	A	A	A	X	G	D	K	K	E	A	K	K	E	E	K	K	E	E	S	E	
101	TCT	GAA	GAC	GAT	GAT	ATG	GGC	TTC	GGT	CTC	TTC	GAA	TAG	GAT	GCT	GGC	AGT	GGT	AGT	AGT	445
	S	E	D	D	D	M	G	F	G	L	F	E	STOP								
	CCTTCCGTTACCGTGAAGCTCGAGTCAACAGTGTGTCAGCATGGGGTGGATTGTTGTAACCGCATATATCTTACATACACAAATGGATTCTTTTATTCTAT																				543
	GAAATAAAGTATATGTTTACACCGTTTAAAAAAA																				578

the sequence terminates with a string of 7 adenosines, representing a complementary portion of the anchored primer used in this clone's amplification, HT₁₁-A. This last feature is also likely to represent the start of a poly (A)+ tail.

The original length of the U1 sequence, reflected by the insert size (Fig. 20), was 334 nucleotides, and encoded an ORF of 62 amino acids. A GenBank comparison of this sequence yielded a match to the *Drosophila* ribosomal protein (r-protein) known as rpA1. An alignment between the deduced amino acid sequences of clone U1 and rpA1 revealed a number of similarities, but most prominently it suggested that the U1 sequence was incomplete. Specifically, about half of the amino acid sequence comprising the N-terminal region appeared to be lacking compared to rpA1 and other homologues. The incomplete nature of the U1 sequence was further evidenced by the lack of a start codon and the discrepancy of its corresponding transcript's size, determined by Northern analysis as approximately 650 nt.

Complete sequence data, as shown in Figure 22, was obtained by Canadian Molecular Research Services (Orleans, Ontario) using the 5' RACE (rapid amplification of cDNA ends) technique.

The alignment of the U1 deduced amino acid sequence with those of other r-protein homologues is shown in Figure 23. The *Drosophila* rpA1 encodes a 113 residue ORF, which includes two distinctive domains-- one hydrophobic (residues 58-86) and the other hydrophilic (residues 87-107) (Quian et al., 1987). Both bear strong similarities to corresponding regions in the U1 sequence-- U1's hydrophobic region lies between residues 58-85 and in it shows the characteristic

Figure 23. Amino acid sequence alignment of *E. solidaginis* clone U1 and assorted ribosomal P-proteins. All sequences are complete. The homologues are from *Drosophila* (rpA1, Qian et al., 1987), *Branchiostoma floridae* (Tweedie et al., 1996), *Bos taurus* (Tan, 1994), *Rattus norvegicus* (Wool et al., 1991) and *Cryptochiton stellari* (Snyder, 1998). Residues that do not match the U1 sequence are shaded.

U1 M R Y V A A Y L L A A L G G N E N P K N A D I E K I L S S V 30
 rpA1 M R Y V A A Y L L A V L G G K D S P A N S D L E K I L S S V 30
 Branchiostome M R Y V A A Y L L A V L G G N A N P S A G D I K K I L G S V 30
 Rat M R Y V A S Y L L A A L G G N S N P S A K D I K K I L D S V 30
 Human M R Y V A S Y L L A A L G G N S S P S A K D I K K I L D S V 30
 Bovine M R Y V A S Y L L A A L G G N S S P S A K D I K K I L D S V 30

U1 G I E A E A E R L S K V I K E L N G K S I E E L I A Q G R E 60
 rpA1 G V E V D A E R L T K V I K E L A G K S I D D L I K E G R E 60
 Branchiostome G I D A E R L N K V I G E L K G K D I E E V M A A G R 60
 Rat G I E A D D E R L N K V I S E L N G K N I E E D V I A Q G V G 60
 Human G I E A D D D R L N K V I S E L N G K N I E E D V I A Q G I G 60
 Bovine G I E A D D D R L N K V I S E L N G K N I E E D V I A Q G I G 60

U1 K L S S M P V G G G V A A A A A A - P A A A A A A X G D K K - 89
 rpA1 K L S S M P V G G G G A V A A A A D A A P A A A A G G D K K - 90
 Branchiostome K L S S M P S G G G V A A A A G G G A A A G A P A 90
 Rat K L A S V P A G G A V A V S A A P C S A A P A A P A A 90
 Human K L A S V P A G G A V A V S A A P C S A A P A A P A A 90
 Bovine K L A S V P A G G A V A V S A A P C S A A P A A P A A 90

U1 - E A K K E E K K E E K K E E S E S E - D D D M G F G L F E . 113
 rpA1 - E A K K E E K K E E S E S E - D D D M G F A L F - E 113
 Branchiostome A E K K E E K K E E S E S E E E S - D D D M G F G L F D D 117
 Rat A E K K E E K K E E S E S E E E - S D D D M G F G L F D D 115
 Human A E K K E E K K E E S E S E E E - S D D D M G F G L F D D 115
 Bovine A E K K E E K K E E S E S E E E - S D D D M G F G L F D D 115

alanine-rich zone. U1's hydrophilic region is contained within residues 86-106, and displays lysines and glutamates corresponding exactly to the rpA1 sequence. The highly conserved N-terminal region contains a putative GTP binding site and has been demonstrated to be crucial for the association of rpA1 to the ribosome (Olson et al., 1993, Remacha et al., 1995b). Also like the rpA1, the U1 sequence does not contain any histidine, cysteine or tryptophan (Quian et al., 1987). Pairwise comparisons of the amino acid sequences of r-protein homologues from other species are shown in Table 7. Similarities ranged from 77.9% (U1 vs. *Drosophila* rpA1) to 54% (U1 vs. yeast).

The sequence data for the rest of the positive clones are shown in Figures 24-27. None of these corresponded to any known sequences, according to a GenBank comparison. Most of these clones contained the sequence of at least one of the primers used in the original reverse transcription and amplification reactions. Incomplete primer sequences, or the lack of them, may be the result of degradation of the amplicon prior to ligation into the vector.

In comparing cDNA size with corresponding transcript sizes, most of these seven clones are likely to represent partial sequences of the original mRNA (see Table 11). Clone U52 however, presents an inconsistency in this respect, in that it exceeds its corresponding transcript in length by approximately 3.5 times. In light of this, U52 might represent a portion of immature, unspliced transcript, introduced into the total RNA pool via nuclear disruption (Luce and Burrows, 1998). For the rest of the U-series clones, complete sequence data may be obtained by the 5'RACE method, as was done in the case of U1.

Figure 24. Nucleotide and deduced amino acid sequence of *E. solidaginis* clones U19 and U20. Putative stop codons are underlined, as are DD-PCR primer sequences. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466
---	---	---	---	---	---	---	---	---	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

	1	21	41	61	122	182	198
AAT	AAA	AGC	CAT	AAA	ANK	ATC	AAA
N	K	S	H	K	?	I	K
	E						K
ACT	GAA	KTA	CIT	AAA	CTC	TAC	AAA
TTT	F	L	Y	CTT	CTC	CTC	AAA
ATC	I	A	F	TTC	GCT	TTC	CTT
ATT	I	R	F	TTT	CGA	F	A
CTG	L	C	F	TTC	TGC	F	A
AAA	K	K	Q	CAG	AAG	CAG	AAA
TAT	Y	Q	F	TAT	CAG	TTT	Y
TGG	W	V	G	GTA	GTA	GGA	W
CGC	R	L	Y	CTT	CTT	TAT	R
CAA	Q	R	R	CGT	TTT	AGA	Q
CAG	Q	F	P	CAG	TTT	CCC	Q
NGC	?	V	R	GTC	GTC	AGA	?
GTG	V	F	F	TTC	TTC	TTC	V
GTT	V	T	T	ACT	ACT	ACA	V
GCC	A	H	I	CAC	CAC	ATA	A
AAG	K	H	V	CAT	CAT	GTT	K
GTG	V	Q	N	CAA	CAA	AAT	V
GA							

Figure 25. Nucleotide and deduced amino acid sequence of *E. solidaginis* clones U44 and U47. Putative stop codons and DD-PCR primer sequence complements are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

U44

1	TTA	CCC	GCA	TTA	GGG	ATG	ATG	AGT	AGG	AGT	ATG	CGT	TAT	CIT	TTT	AGG	AAA	ATA	TGG	TCG	TT	2
	L	P	A	L	G	M	M	S	R	S	M	R	Y	L	F	R	K	I	W	S	GAT	62
21	GAG	CGC	ATC	CCG	TCG	ATT	ACG	AAT	GCA	AAT	TAT	GTC	CAA	GAA	CTA	TTT	TCC	TAA	CTGGTCTACAAGG			126
	E	R	I	P	S	I	T	N	A	N	Y	V	Q	E	L	F	S	STOP				
	TCGTTTTCGAAAAA																				145	

U47

AAGCTTIOGACITGIGGCAATTTCATGTAACTAGATTGGAATGATGTTTIOGTCATCCCTTGTCAAAATTTGGTTTGGAGACAACAGCITTCGGCTTTTGT 98

GCCATCTTCAAGGTAAATTCATACITCTTAOCGAAAAATCTTAATTTGTAAOGTAAACATAGTTTATATAGAACAGTCACACTATATCCATCTACACAATC 197

CTGTGATCTGTATATAATGOCAAAAA 231

Figure 26. Nucleotide and deduced amino acid sequence of *E. solidaginis* clones U51 and U52. Stop codons and DD-PCR primer sequence complements are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

U51

1 TTTTAACAGACTCTAATTATTCACAATGAACGNTATAAGGTGGTTCCGACAAAGTGACGCGATATTCGCAAAATAAGACTTATTAGCGAAGTAAAAAG 31
AAGCTTCTCAACGCAAAAGAACAGGAATTGTT
AIG TTT NCA GAA AAT AGT ATA GAA CTA TTT CAG CAC CAT ATA TAC TTT TAT AAT TTC AAT 128
M F ? E N S I E L F Q H H I Y F Y N F N 188
21 GTT TTT TAT AAT TAT TCG AAG ATG GCG TTC GGT TTC ATT TCA AGA ATA TTT GCA AAA CTT 248
V F Y N Y S K M A F G F I S R I F A K L
41 ATT GCT GCT GTA ATC ATA CAT CAA CAC TCA ACT TTT TTG TGA GTTTCTTACTTTTCTAATCTTTTGTGCT 319
I A A V I I H Q H S T F L STOP
TATGTGTGTTATTGACCATAATTTTAAATGGCGGACGTTTATTTTATTAAATTATTGGATTTCATTAAATGAATAAGACCAATATGGATGTCAAAAGAAA 417
GGTATTATTTCGTAGTTTTCAAAAAAGCTTAA 455

U52

CTGGAATTCGGCTTANNCTTCTACCGTTACGGATGATCAGCCATGACTGAAGATAGACTACAGCCTGTGTCTGTGTTTCGJCAATTAATTCACCCCTAG 99
AACAAAGTTTCCANAATGTTTATTTCAAATCAATATATGTATANNNAATAGTTTCATTAACTTGATAATCAAAATTTCTAATAATTGGA 197
ATTATTGAATTATTGTGTGCTTATAITGTGATAATAACCCGATGAARGATCGGAAGTGGATTTGGCTGGATACAGCTGGATTTTCCATTTCCTCGCTC 296
GATTCTAGAATTGACACGGGGCCAGTCCCTAGTTCTACGATTATGCCTACTTTAGAGAGATATTGTACGGGAATTCAGGGCAGGCACTAACTATGAG 393
AACCCTATACCAAAAAAATAAACCTTAA 419

Figure 27. Nucleotide and deduced amino acid sequence of *E. solidaginis* clone U53. Putative stop codon and DD-PCR primer sequence complements are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

U53

1	TTG	ATA	TAT	GTA	GCA	CTT	GCA	TAC	TGT	TCG	AAA	TTT	CCG	CAC	TTC	AAGCTTCTCAACGTCGATATTAT				22
	L	I	Y	V	A	L	A	Y	C	S	K	F	P	H	F	ACT	TAC	ATT	CTA	82
																T	Y	I	L	
21	AAC	CCC	CGT	GAT	GCC	ATT	ATT	TCA	TCA	TCA	GCA	TCT	ACT	TTC	AAA	TTC	TTC	ATA	CTA	142
	N	P	R	D	A	I	I	S	S	S	A	S	T	F	K	F	F	I	L	V
41	AAA	GTA	CAA	TTA	TTG	CAC	ATG	TCA	TTA	TGT	GGA	GTT	CAC	CTT	CAG	TAC	TGA	ATGGCTTTTAAAT	206	
	K	V	Q	L	L	H	M	S	L	C	G	V	H	L	Q	Y	STOP			
	ACTCGACGTTATGGACTAGGAACCTCCCTGGTTGGTTATTATTAAAGTGTGAAGATATCTCCCGATATTTCGAGTCTCAGTTGCTTTCGGCGCCTCACACG																			304
	TTGTAGTTGGCGGTCTTCCG																			324

The deduced amino acid sequences based on the nucleotide data revealed a number of possible ORFs, but as most of the clones are incomplete transcript sequences, those presented are speculative at best. U19 (see Fig. 24) represents approximately 1/3 of its transcript, and contains a putative 34 residue ORF in its second reading frame. This was found to contain an N-myristoylation site at residues 7-12. Clone U20 (Fig. 24), also representing an approximate third of its transcript, encoded a 65 amino acid ORF over its entire length, suggesting that perhaps it is the result of anchored primer HT₁₁-A's annealing to a short poly A stretch in mid-transcript sequence, as opposed to the poly (A)+ tail. No motifs were found in this ORF.

Figure 25 shows sequence and deduced ORF data for clones U44 and U47. Clone U44 contains a 145 bp sequence, and its corresponding transcript has been estimated at 1050 nt. U44 has a possible ORF of 36 amino acids. U47, however, contained stop codons in all reading frames within the first 20 bases. It represents 231 bp of an estimated 850, and might represent a 3'UTR of this transcript.

Sequences and possible ORFs for clones U51 and U52 are shown in Figure 26. U51 contains a 455 bp sequence, and as its transcript is an estimated 600 nt, it may possibly represent a complete sequence. A 53 amino acid ORF is encoded by U51 in its third reading frame-- this putative peptide is approximately 6.4 KDa, and may be particularly non-polar as its hydrophobic residue content is high (25 A, I, F, L, W and V residues), though these are not concentrated in any regions. A nucleotide ambiguity makes for an unreadable codon at position 3.

This clone may represent a small, novel protein, upregulated in *E. solidaginis* in response to cold. Clone U52 contains a 419 bp sequence which does not appear to have any long ORFs, owing to a number of termination codons throughout all reading frames. This, coupled with the size of its corresponding transcript (200 nt) suggests that U52 may contain portions of true cold-induced transcript, but they remain indistinguishable within its sequence.

Lastly, clone U53 is a 324 bp sequence which approximates the estimated transcript size of 300 nt (see Fig. 27). U53 contains a number of ORFs, but the longest, 56 residues, is shown in Figure 27. The lack of a start codon suggests that either this is not the correct ORF or that the sequence is incomplete. U53 does not, however, contain a polyadenylation signal (AATAAA), nor the sequence for anchored primer HT₁₁-G, which would likely represent the start of the poly (A)+ tail. This suggests some degree of degradation of the amplicon prior to cloning, and also highlights the speculative nature of this deduced amino acid sequence.

Further Northern analysis was undertaken along the same lines as with the *E. scudderiana* clones, in order to address the possibility that the U-series clones were upregulated by the passage of time rather than by cold exposure. A 15°C time course was contracted with *E. solidaginis* larvae as previously described for *E. scudderiana*, with time-points corresponding to those from the cold/subzero time course.

The results from probing the 15°C time course with radiolabeled probe synthesized from clone U1 are shown in Figure 28. The transcript abundance does increase over time, and this may suggest that both temporal and thermal controls may be involved in the expression of U1. Further studies on this clone might involve Western immunoblotting, in order to determine the presence of rpA1 protein during changes in transcript abundance.

Epiblema scudderiana

Differential display experiments on *E. scudderiana* were carried out using pooled samples of total RNA from each control and cold-exposed time point, in the same manner as the *E. solidaginis* experiments described above: equal amounts of the RNA samples were amplified using all 24 combinations possible between the anchored and random DD-PCR primers. Reactions were run in duplicate, and the amplicons were resolved on a polyacrylamide gel. Figures 29, 30 and 31 show the results of three such runs, where random primer AP4 was respectively paired with anchored primers HT₁₁-A, HT₁₁-C and HT₁₁-G. Table 8 shows a summary of the results of amplifications using all primer combinations. Each PCR reaction's autoradiograph revealed approximately 70 bands, with a size range of 100 to 900 bp. Approximately 1630 displayed bands were assessed-- 66 of these were either exclusively or more prominently represented in the cold/subzero lanes, and were considered as candidates (listed in Table 9). Three such differentially displayed bands, P11, P12 and P43 are shown in Figure 32.

Figure 28. Northern hybridization and densitometric analysis of *E. solidaginis* clone U1 versus a five-week 15°C time course. Autoradiograph represents one hybridization. Blot was exposed to X-ray film for 10 days. EtBr-stained rRNA band of the corresponding formaldehyde gel is shown.

Figure 29. *E. scudderiana* DD-PCR reactions, covering all time-points of the cold/subzero temperature time course. Primer combination was HT₁₁-A and AP4 and amplification products were resolved on a polyacrylamide gel. P-marked bands indicate upregulated candidates, while D indicates potentially downregulated bands.

Figure 30. *E. scudderiana* DD-PCR reactions, covering all time-points of the cold/subzero temperature time course. Primer combination was HT₁₁-C and AP4 and amplification products were resolved on a polyacrylamide gel. P-marked bands indicate upregulated candidates, while D indicates potentially downregulated bands.

Figure 31. *E. scudderiana* DD-PCR reactions, covering all time-points of the cold/subzero temperature time course. Primer combination was HT₁₁-G and AP4 and amplification products were resolved on a polyacrylamide gel. P-marked bands indicate upregulated candidates, while D indicates potentially downregulated bands.

Table 8. Summary of total banding in all *E. scudderiana* DD-PCR reactions. Averages presented as mean $\pm$ SE. Potentially upregulated and downregulated bands are indicated by upward and downward arrows, respectively.

	<u>Anchored Primers</u>			<u>Total Bands</u>
	HT ₁₁ -A	HT ₁₁ -C	HT ₁₁ -G	
Bands, all APs	557	577	496	1630
Bands/AP	69.6 $\pm$ 7.5	72.1 $\pm$ 8.6	62 $\pm$ 3	-
Total $\uparrow$	29	14	23	66
Total $\downarrow$	9	15	4	28

Table 9. Summary of all *E scudderiana* potentially upregulated candidates. Anchored and arbitrary primers are referred to by their letters (A,C,G) and numbers (1-8) respectively. A dash (-) indicates failure to reamplify candidate in question. Table continues on next page.

Band	Primer Combination	Estimated Band Size(bp)
P1	A-1	537
P2	A-1	502
P3	A-1	418
P4	A-1	231
P5	A-1	206
P6	A-2	619
P7	A-2	540
P8	A-2	461
P9	A-2	389
P10	A-2	330
P11	A-3	842
P12	A-3	691
P13	A-3	-
P14	A-3	680
P15	A-3	626
P16	A-3	616
P17	A-3	383
P18	A-4	870
P19	A-4	490
P20	A-4	179
P21	A-5	520
P22	A-5	-
P23	A-6	856
P24	A-6	701
P25	A-6	564
P26	A-6	411
P27	A-7	481
P28	A-7	437
P29	A-7	391
P30	C-2	-
P31	C-2	205
P32	C-2	196
P33	C-3	444
P34	C-4	223
P35	C-5	574
P36	C-5	158

Table 9, continued. Summary of all *E. scudderiana* potentially upregulated candidates. Anchored and arbitrary primers are referred to by their letters (A,C,G) and numbers (1-8) respectively. A dash (-) indicates failure to reamplify candidate in question.

<u>Band</u>	<u>Primer Combination</u>	<u>Estimated Band Size(bp)</u>
P38	C-6	444
P39	C-6	205
P40	C-7	425
P41	C-7	397
P42	C-7	253
P43	C-8	314
P44	G-1	735
P45	G-1	-
P46	G-1	431
P47	G-1	388
P48	G-1	367
P49	G-2	617
P50	G-2	578
P51	G-3	705
P52	G-3	-
P53	G-4	693
P54	G-4	415
P55	G-6	617
P56	G-6	555
P57	G-6	-
P58	G-7	417
P59	G-7	363
P60	G-7	263
P61	G-7	257
P62	G-7	214
P63	G-7	209
P64	G-8	245
P65	G-8	-
P66	G-8	239

Figure 32. *E. scudderiana* candidates P11, P12 and P43, from DD-PCR polyacrylamide gel.

Twenty-eight bands appeared potentially downregulated by the cold exposure, but were not considered in this project.

As in the *E. solidaginis* experiment, each candidate was traced back to its original position on the polyacrylamide gel, extracted and reamplified with the same primer combination used in the first PCR reaction. As in the *E. solidaginis* experiments, not all reamplifications were successful. Eight of the 66 candidates failed to reamplify. The successful amplicons were resolved on 1.5% agarose gels and detected with EtBr staining. Figure 33 shows the results of two reamplifications, those of candidates P10 and P11.

Only those candidates which were 300 bp or greater were carried further in the screening process. This was done in the hopes of obtaining sufficient sequence data from positively confirmed candidates for identification via database comparison. Also, multiple bands from the same primer combination could represent amplified fragments of the same transcript. Choosing the largest candidate(s) would reduce the possibility of carrying on with several fragments of different sizes, all of which code for the same message. Out of the original 66 candidates, 43 were found to be ≥ 300 bp. These size-selected candidates were used to synthesize radiolabeled probe for Northern hybridizations, as described in the Materials and Methods. Only three of these 43, candidates P11, P12 and P43, showed increased transcript accumulation under cold conditions. Transcript sizes were estimated at 1071, 1148 and 1202 nt, respectively (see Table 10). The results of these hybridizations are shown in Figure 34.

Figure 33. Reamplification and size estimation of *E. scudderiana* candidates P10 and P11. C1a shows the amplicons resolved on a 1.5% agarose gel stained with ethidium bromide. Lane S denotes DNA size standards. C1b shows the calibration curve of the size standards and the positions the amplicons occupy, resolving as fragments of 825 and 692 bp.

Table 10. Positively confirmed *E. scudderiana* candidates. Anchored and arbitrary primers are referred to by their letters (A,C,G) and numbers (1-8) respectively.

Candidate	Primer Combination	Estimated size (bp)	Estimated Transcript size (bp)
P11	A-3	842	1071
P12	A-3	691	1148
P43	C-8	314	1202

Figure 34. Results of Northern hybridizations of *E. scudderiana* candidates P11, P12 and P43. Each autoradiograph represents one hybridization. Blots were exposed to X-ray film 72 hours (P11, P12) and 10 days (P43). Relative EtBr staining of rRNA band in corresponding formaldehyde gels is shown as Mean $\pm$ SEM (n=3).

The three positively confirmed candidates were cloned into a T-vector as described in the Materials and Methods, then C-tracking was conducted. Figure 35 shows the results of one such C-track, that of clone P11-- six colonies were assessed, revealing four different sequences for this clone, while P12 and P43 contained four and five, respectively. For P11, restriction digests were carried out for each sequence (see Figure 36), and each was subsequently used as template for radiolabeled probe synthesis and Northern hybridization. For each clone, the one sequence representing the original, upregulated sequence was determined by autoradiography (see Fig. 37 for an example). In the case of P11, sequence P11-3 was found to be the genuinely upregulated one. P12-4 and P43-2 were similarly found to be genuine.

Each verified clone was then sequenced by an automated sequencing method. The sequence for clone P11-3 is shown in Figure 38. The 654 bp cDNA was framed by the DD-PCR primers used in its initial amplification, random primer AP3 (bases 1-9) and anchored primer HT₁₁-A (bases 637-654), the latter representing the beginning of the poly (A)+ tail (position 637). Upstream of this, three possible polyadenylation signals were found starting at positions 569, 573 (AATTAA-- see Birnstiel et al., 1985) and 632 (AATAAA). P11-3 also encoded a long ORF of 174 amino acids-- although three ambiguities in the nucleotide sequence made for 2 unreadable codons at positions 113 and 142 (see Figure 38). Although there was no start codon, the deduced sequence did include a terminal stop codon (TAA). This and the corresponding transcript size, estimated at 1070

Figure 35. Partial sequencing ('C-tracking') of sequences from six separate bacterial colonies transformed with clone P11. Plasmid preparations from each colony were partially sequenced, revealing that two sequences were represented twice (P11-1 and P11-3, lanes 1 and 3, respectively), while the others (P11-2 and P11-4, lanes 2 and 4, respectively) were unique.

1	2	3	4	1	3
1	1	1	1	1	1
2	2	2	2	2	2
3	3	3	3	3	3
4	4	4	4	4	4
5	5	5	5	5	5
6	6	6	6	6	6
7	7	7	7	7	7
8	8	8	8	8	8
9	9	9	9	9	9
10	10	10	10	10	10
11	11	11	11	11	11
12	12	12	12	12	12
13	13	13	13	13	13
14	14	14	14	14	14
15	15	15	15	15	15
16	16	16	16	16	16
17	17	17	17	17	17
18	18	18	18	18	18
19	19	19	19	19	19
20	20	20	20	20	20
21	21	21	21	21	21
22	22	22	22	22	22
23	23	23	23	23	23
24	24	24	24	24	24
25	25	25	25	25	25
26	26	26	26	26	26
27	27	27	27	27	27
28	28	28	28	28	28
29	29	29	29	29	29
30	30	30	30	30	30
31	31	31	31	31	31
32	32	32	32	32	32
33	33	33	33	33	33
34	34	34	34	34	34
35	35	35	35	35	35
36	36	36	36	36	36
37	37	37	37	37	37
38	38	38	38	38	38
39	39	39	39	39	39
40	40	40	40	40	40
41	41	41	41	41	41
42	42	42	42	42	42
43	43	43	43	43	43
44	44	44	44	44	44
45	45	45	45	45	45
46	46	46	46	46	46
47	47	47	47	47	47
48	48	48	48	48	48
49	49	49	49	49	49
50	50	50	50	50	50
51	51	51	51	51	51
52	52	52	52	52	52
53	53	53	53	53	53
54	54	54	54	54	54
55	55	55	55	55	55
56	56	56	56	56	56
57	57	57	57	57	57
58	58	58	58	58	58
59	59	59	59	59	59
60	60	60	60	60	60
61	61	61	61	61	61
62	62	62	62	62	62
63	63	63	63	63	63
64	64	64	64	64	64
65	65	65	65	65	65
66	66	66	66	66	66
67	67	67	67	67	67
68	68	68	68	68	68
69	69	69	69	69	69
70	70	70	70	70	70
71	71	71	71	71	71
72	72	72	72	72	72
73	73	73	73	73	73
74	74	74	74	74	74
75	75	75	75	75	75
76	76	76	76	76	76
77	77	77	77	77	77
78	78	78	78	78	78
79	79	79	79	79	79
80	80	80	80	80	80
81	81	81	81	81	81
82	82	82	82	82	82
83	83	83	83	83	83
84	84	84	84	84	84
85	85	85	85	85	85
86	86	86	86	86	86
87	87	87	87	87	87
88	88	88	88	88	88
89	89	89	89	89	89
90	90	90	90	90	90
91	91	91	91	91	91
92	92	92	92	92	92
93	93	93	93	93	93
94	94	94	94	94	94
95	95	95	95	95	95
96	96	96	96	96	96
97	97	97	97	97	97
98	98	98	98	98	98
99	99	99	99	99	99
100	100	100	100	100	100

Figure 36. Restriction digests of the four representative *E. scudderiana* P11 clones, P11-1, P11-2, P11-3 and P11-4. Plasmids were digested with EcoR I, resolved on a 1.5% agarose gel and stained with ethidium bromide. Lane S denotes DNA size standards.

Figure 37. Northern hybridization of *E. scudderiana* clone P11-3. Autoradiograph represents one hybridization, and EtBr-stained rRNA band of corresponding formaldehyde gel is shown.

Figure 38. Nucleotide and deduced amino acid sequence of *E. scudderiana* clone P11. Putative stop codon and DD-PCR primer sequence complements are underlined. Three potential polyadenylation signals are underlined in bold. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

nt, suggested that the N-terminal region of the putative coding region was missing from the clone sequence. A GenBank homology search did not yield any reliable matches to the P11-3 nucleotide or amino acid sequence, but the glutamine-rich ORF (18.3% Q) might suggest that P11-3 is involved in protein-protein interactions (Courey and Tjian, 1988).

The nucleotide sequences for clone P12-4 is shown in Figure 39. P12-4 was a 657 bp sequence, which included the two DD-PCR primers AP3 and HT₁₁-A. The latter represented the start of the poly (A)+ region at position 642, and is preceded upstream by a polyadenylation signal (AATAAA) starting at position 623. The sequence was interrupted in all reading frames by stop codons, though the longest ORF, starting at the 5' end in the second frame, encodes a 45 amino acid sequence. Neither nucleotide nor protein database searches revealed any homologies between P12-4 and any known sequences. The transcript size for this sequence was estimated at 1150 nt, again suggesting that the N-terminal portion of any encoded ORF was missing from the clone.

The sequence for clone P43-2 (see Figure 40) also included its particular DD-PCR primers (AP8, HT₁₁-C) within its 359 bp sequence. Though the presence of the anchored primer suggested the start of the poly (A)+ region, starting at position 346, no polyadenylation signal could be found upstream to it. The longest deduced ORF from the 5' end of P43-2 encoded a 25 amino acid sequence. Neither nucleotide nor amino acid sequences yielded similarities in any database searches. Considering that the corresponding transcript size for this clone was estimated at 1200 nt, this clone represents an incomplete sequence.

Figure 39. Nucleotide and deduced amino acid sequence of *E. scudderiana* clone P12. Putative stop codon, polyadenylation signal and DD-PCR primer sequence complements are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	52
--	---	---	---	---	---	---	---	---	---	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----

Figure 40. Nucleotide and deduced amino acid sequence of *E. scudderiana* clone P43. Putative stop codon and DD-PCR primer sequence complements are underlined. Numbers on left pertain to the amino acid sequence, while numbers on right are nucleotide position.

Further Northern analyses were undertaken to address the possibility that the three *E. scudderiana* clones P11, P12 and P43 would have increased transcript abundance with time despite any changes in temperature. The same *E. scudderiana* 5 week, 15°C time course was used as in the cDNA library investigation. Inserts of the three clones were obtained through restriction digestion, and used for the synthesis of radiolabeled probe. Northern hybridizations revealed banding in the case of clones P11 and P43, but banding for clone P12 was, like for P8, questionable (see Fig. 41).

Clone P11 band intensity did show an increase compared to the 2 week time-point. These results suggest that while P11 transcript abundance will increase with time, cold exposure serves to increase the pace and degree of induction..

Clone P43 also showed an increased transcript abundance over the 15°C time course. The band intensity approximated that of the banding in the cold/subzero time. Low temperature exposure, then, appears to affect transcript levels in terms of timing and abundance, as in P11 and P43.

PART III : CROSS-NORTHERN ANALYSIS

***E. scudderiana* clones vs. *E. solidaginis* blots**

Table 11 shows all of the positively verified clones from both *E. scudderiana* and *E. solidaginis*. Two of these, P3 and U1, contained identifiable, complete sequences of known gene products, while the rest are considered novel.

Figure 41. Northern hybridization of *E. scudderiana* clone P11 and P43 versus a five-week 15°C time course. Each autoradiograph represents one hybridization. Blots were exposed to X-ray film for 7 days (P11) and 72 hours (P43). Representative EtBr staining of rRNA band in a corresponding formaldehyde gel is shown.

Table 11. Summary of all positively confirmed clones from both *E. scudderiana* and *E. solidaginis*.

Species/ Clone	Size (bp)	Estimated Transcript size(s) (b)	Comments
<i>E. scudderiana</i>			
P3	612	700, 1400	Muscle LIM protein
P6	805	1600	Novel sequence-- 28s rRNA-like
P8	608	1600	Novel sequence
P11	654	1100	Novel sequence, Q-rich ORF
P12	657	1150	Novel sequence
P43	359	1200	Novel sequence
<i>E. solidaginis</i>			
U1	578	650	rpA1 homologue
U19	263	650	Novel sequence
U20	198	600	Novel sequence
U44	145	1050	Novel sequence
U47	231	850	Novel sequence
U51	455	600	Novel sequence
U52	419	200	Novel sequence
U53	324	300	Novel sequence

The two identified clones both represent elements of developmental import-- P3 is crucial for myogenesis while U1, as a ribosomal protein, may represent cell proliferation or a requirement for the synthesis of new proteins. As both these and the other clones appear to be upregulated by the same exposure to cold in two overwintering insects, the question arose as to whether the upregulated clones from one species might be present and similarly upregulated in the other species. This prompted cross-Northern experiments, where radiolabeled probe from the *E. scudderiana* clones were hybridized onto Northern blots of *E. solidaginis*, and vice versa.

The cross-hybridization results for all *E. scudderiana* clones isolated from cDNA library and DD-PCR techniques are shown in Figure 42. Clone P3 hybridized to only one transcript in *E. solidaginis*, as opposed to the two transcripts detected in *E. scudderiana*. The *E. solidaginis* transcript approximates 1400 nt, corresponding to the size of the larger *E. scudderiana* transcript (P3a, see Fig. 2), and shows an increased band intensity under cold/subzero conditions as compared to the 15°C control lane. These results suggest the upregulation of some LIM-encoding gene with cold exposure, possibly a homologue of the upregulated gene in *E. scudderiana*.

Clone P6 hybridized to an *E. solidaginis* transcript of 2.2 Kb, which does not correspond to the size of the transcript in *E. scudderiana* (1.6 Kb). Transcript abundance appears to increase over that of control at the 4°C time-point, paralleling that of the *E. scudderiana* transcript, shown in Figure 4. This suggests

Figure 42. Cross-species Northern hybridizations, all *E. scudderiana* cDNA library and DD-PCR clones versus *E. solidaginis* blot. Each autoradiograph represents one hybridization. Blots were exposed to X-ray film for 48 hours (P6), 72 hours (P3, P8), 7 days (P11) and 10days (P43). Relative EtBr staining of rRNA band in corresponding formaldehyde gels is shown as Mean $\pm$ SEM (n=5).

that, during low temperature exposure, *E. solidaginis* upregulates a novel gene with some sequence homology to P6-- possibly one containing a region similar to the 28S rRNA gene.

Hybridization of clone P8 revealed an *E. solidaginis* transcript of approximately 1.6 Kb, which matches the results found on *E. scudderiana* blots. This transcript also displayed a similar upregulation as in *E. scudderiana*, (see Fig. 3). Like P6, it would appear *E. solidaginis* increases the transcript abundance of a P8 homologue upon encountering cold and freezing temperatures.

The cross-hybridizations of the DD-PCR clones P11 and P43 can be seen in Figure 42. Clone P12 did not show any banding on *E. solidaginis* blots, suggesting that this sequence is unique to *E. scudderiana*. Clone P11, however, hybridized to an *E. solidaginis* transcript of approximately 2.1 Kb, which does not correspond to the *E. scudderiana* transcript size. Clone P43 hybridized to an *E. solidaginis* transcript of approximately 2.3 Kb, exceeding the *E. scudderiana* transcript size by approximately 2-fold. The autoradiographs suggest that *E. solidaginis* upregulates genes with some sequence homology to the *E. scudderiana* clones, upon cold exposure.

***E. solidaginis* clones vs. *E. scudderiana* blots**

When an *E. scudderiana* blot was probed with clone U1, a transcript of approximately 700 nt was revealed (see Fig. 43)-- this corresponds to the transcript size found on *E. solidaginis* blots and suggests that *E. scudderiana* might express an rpA1 homologue as well. However, in the case of *E. scudderiana*, the transcript did not appear to change its abundance over the

Figure 43. Cross-species Northern hybridization of *E. solidaginis* clone U1 versus *E. scudderiana* blot. Autoradiograph represents one hybridization. Blot was exposed to X-ray film for 10 days. EtBr staining of rRNA band in a corresponding formaldehyde gel is shown.

cold/subzero time course. The slight decrease in band intensity at 24 hours at -20°C may be due to RNA degradation rather than actual downregulation. Overall, these results may suggest that though an rpA1 homologue is expressed in *E scudderiana*, it is not upregulated as a result of cold exposure.

CHAPTER FOUR:

DISCUSSION

Investigations into gene-induction at low temperatures in animals has tended to focus on non-cold hardy species and their reactions to thermal stress. In mice, for instance, HSPs are induced in brown adipose tissue and during whole-animal hypothermia, though after hours of low temperature exposure (also see Chapter One). In addition, induction levels are lower than during subsequent recovery periods (Matz et al., 1995, Cullen and Sarge, 1997). In rat liver, a number of hours at 4°C leads to the induction of mitochondrial mRNAs and rRNA (Martin et al., 1993). These findings have offered some insight into gene induction during cold-shock, but have ignored the survival mechanisms of animals adapted to low temperatures.

Studies on the molecular response of cold- and freezing-tolerant animals to low temperatures, to thermal conditions they normally encounter in nature, have only recently been conducted. Table 12 offers a partial list of genes induced at low temperatures in a variety of cold hardy animals. In most cases, the induced gene suggests an unexpected aspect of the organism's coldhardiness strategy--including responses to increased plasma membrane viscosity at low temperatures and to blood vessel damage by ice-crystals (Tiku et al., 1996, Cai and Storey, 1997a). These studies also highlight the differences between responses to cold and to freezing, which in turn reflect the inherent dangers unique to both conditions (see Chapter One).

In this study, upregulation of genes at low temperatures was observed for the first time in two overwintering insect species, the freeze-avoiding *E. scudderiana* and the freeze-tolerating *E. solidaginis*. A number of novel and identifiable gene transcripts were found to be upregulated by exposure to cold and

Table 12. Some genes induced at low temperature in cold- and freezing-tolerant animals.

Source	Stress	Gene	Proposed role	Reference
<i>Bombyx mori</i>	5°C, 30-40 days	Sorbitol dehydrogenase	Post-diapause polyol clearance	Niimi et al., 1993
Rainbow trout cell culture	4°C, 1 day	<i>CDC48B</i> homologue	In yeast, crucial for low temperature survival	Yamashita et al., 1996a
	4°C, 1-3 days	Ferretin H subunit	Counter cold-induced cytosolic iron toxicity	Yamashita et al., 1996b
Carp liver	10°C, 1-3 days	Δ^9 -desaturase	Maintain plasma membrane fluidity	Tiku et al., 1996
Wood frog liver	24 hrs freezing, (-2.5°C)	fibrinogen, α and λ subunits	promote clotting of ice-induced injury	Cai and Storey, 1997a
	24 hrs freezing, (-2.5°C)	<i>FR10</i> (novel gene)	?	Cai and Storey, 1997b

subzero conditions, and these results offer a number of points to consider, including overwintering and development in these insects. The sequences of two clones bore strong similarities to recognized genes, and will be discussed in detail below.

Two major differential screening techniques, involving cDNA libraries and DD-PCR, were used in the course of this study, which provided an opportunity to assess their respective merits and shortcomings. Given the proper handling of mRNA (so as to minimize shearing) during its construction, a cDNA library might be expected to provide longer clone sequences, approaching the complete length of their representative transcripts. However, the use of a composite library constructed from the message pools of various treatment groups requires numerous rescreenings.

DD-PCR offers an advantage in this respect, in that selectively amplified messages from more than one treatment group can be compared simultaneously. The two major disadvantages of this technique, however, lie in the high frequency of false positives (approaching 90% of the potential candidate bands) and in the heterogeneous makeup of the confirmed bands (each containing up to 4 distinct sequences of similar size). Northern hybridizations and partial sequencing can be used to cope with these problems, though this can prove a lengthy process. Another aspect of DD-PCR is that, more often than not, clones representing upregulated transcripts provide truncated sequence data. This stems from the use of random primers during the amplifications, and leads to sequences unidentifiable either due to their novel nature or by the fact that the clone covers only the 3' UTR of the corresponding transcript. By imposing a size limit on candidates chosen during the screening, the pursuit of false positives and very short sequences can be minimized.

The strongest indication that *E. scudderiana* clone P3 encodes a member of the LIM protein family (also known as the cysteine-rich proteins, CRPs) is the presence of the conserved LIM motif in its 94 amino acid ORF. This cysteine-rich consensus sequence, $CX_2CX_{16-23}HX_2CX_2CX_{16-21}CX_{2-3}(C/D/H)$, where X is a variable amino acid, is contained within residues 11-62. The third cysteine of the consensus is replaced in P3 by a phenylalanine at position 35. Upon inspection, this may be the result of a misreading of nucleotide 196, where a G instead of a T at that position would give the codon for cysteine, TGT. Following the LIM motif is a glycine-rich region of consensus $GPKG(Y/F)G(Y/F)G(M/Q)GAG$, found in positions 68-79. Finally, overlapping the glycine-rich region is the putative nuclear targeting signal, RKFGPK, at positions 66-71. All of these characteristics are common to the LIM domain (Stronach et al., 1996).

The LIM domain contains a pair of zinc-finger-like structures, each of which coordinates a zinc ion. The conserved cysteine residues are key to this coordination, which has been demonstrated to be specific and is thought to play an essential role in stabilizing the tertiary structure of the LIM domain (Michelson et al., 1993). The name LIM is derived from the first three genes in which it was found: *lin-11* (*Caenorhabditis elegans*), *Isl-1* (rat) and *mec-3* (*C. elegans*). These founder members of the group each encode two LIM domains as well as a homeodomain (HD), and thus belong to the LIM-HD class of LIM proteins. Another class of proteins contains both LIM and kinase domains, and a third class encodes LIM motifs only. Proteins in this latter class contain one to five copies of the LIM domain (reviewed by Sanchez-Garcia and Rabbitts, 1994, Dawid et al., 1995).

Homology searches and alignments show that clone P3 bears a remarkable similarity to a number of LIM-containing sequences (see Fig 6), but most of all to

the *Drosophila* muscle LIM protein, Mlp60A, which has been shown to be vital for myogenesis (Arber et al., 1994, Stronach et al., 1996). Only partial sequences of the other LIM proteins are shown in Figure 6-- the first of their multiple LIM motifs closest to the N-terminus. Mlp60A is a LIM-only protein which displays all three characteristic consensi described above, contains a single LIM motif and is a predicted 92 amino acids in length. The nucleotide and amino acid similarities between Mlp60A and P3 are 71.3% and 76.1%, respectively. Other LIM proteins found in *Drosophila* include apterous, a LIM-HD protein, and Mlp84B, another muscle-specific LIM-only protein which contains 5 LIM motifs. The presence in P3 of a single LIM domain, however, and its predicted length of 94 amino acids leads to the conclusion that this clone represents an *E. solidaginis* homologue to Mlp60A.

Myogenesis can be represented by three generalized steps (reviewed by Russo et al., 1992, Ludolph and Konieczny, 1995, Abmayr and Keller, 1998), the first being the determination of a subset of embryonic mesodermal cells as myoblasts. These proliferating muscle precursor cells eventually enter a phase of differentiation, marked by a withdrawal from the cell cycle and the fusion of individual cells into syncytial myotubes. These early myotubes then undergo further differentiation and migrate to their attachment sites, until they are fully mature and innervated. Each stage in this process is marked or initiated by the activation of a number of genes, both mesoderm- and muscle-specific. In insects, a subset of myoblasts are sequestered within imaginal discs, and these undergo proliferation and differentiation with the onset of metamorphosis. Metamorphic myogenesis occurs via the complete replacement of histolyzed larval muscles by new adult muscle, and the remodeling of existing larval muscle into new adult muscle (Chapman, 1982).

The muscle-LIM proteins play key roles in the terminal differentiation of striated muscle. In *Drosophila* the temporal and spatial expression patterns of muscle-LIM proteins allude to their involvement in myotube development, while more vigorous experimentation on the rat homologue MLP has offered more detailed insights into its role. In the *Drosophila* embryo, Mlp60A and Mlp84B are highly enriched in somatic, visceral and pharyngeal muscles during later stages of differentiation— neither are found in proliferating myoblasts, but they appear just prior to fusion. The expression of Mlp60A and Mlp84B is weak in nascent syncytia, but increases as myotube maturation progresses (Stronach et al., 1996). These proteins display a second expression peak during the pupal stage, when further myogenic events occur, and even persist in the adult fly (Stronach et al., 1996). The rat MLP is a LIM-only protein containing two LIM motifs, and is found exclusively in muscle tissue. MLP expression in embryonic rats is coincident with myotube differentiation. Overexpression of MLP in a myoblast cell line accelerates myogenic differentiation, while its absence severely impairs this process (Arber et al., 1994). *In vivo*, MLP-deficient mice display cardiac and skeletal muscle defects, and do not survive long after birth (Arber et al., 1997). A nonmyogenic cell line expressing MLP, however, will not produce muscle-specific proteins, suggesting that MLP does not affect cell cycle events or myogenic determination (Arber et al., 1994).

LIM proteins have been implicated in the development of a wide range of tissues (see Curtis and Heilig, 1998, for review), and this is also reflected by their expression profiles. Though detectable in rat embryos, the cysteine-rich intestinal protein (CRIP) mRNA shows a dramatic increase in the intestine of 24 day-old rats at the transition from suckling-to-weaning behaviour (Birkenmeier and Gordon, 1986). The human and mouse CRPs are undetectable during arrested

growth in cultured fibroblasts and 3T3 cells, respectively, but are upregulated significantly once serum-induced proliferation resumes (Wang et al., 1992). Hematopoietic cells fail to differentiate into erythroid precursors in mice and humans in the absence of rhombotin 2 (RBTN2), a LIM-only protein (Wadman et al., 1994). SLIM is a LIM-only protein found in human skeletal muscle *in vivo* and is upregulated in cultured myoblasts prior to cell fusion. SLIM expression continues through to early myotube differentiation (Morgan et al., 1995, Morgan and Madgwick, 1996). The expression of CRP1 and CRP2, homologues in human and mouse myoblasts, has been shown to increase dramatically when cells cease proliferation (Louis et al., 1997). Further, transcript levels of the mouse CRP2 in vascular smooth muscle cells have been found to decrease dramatically when the cells change from a differentiated to an undifferentiated state, as during injury (Yet et al., 1998). In a variety of tissues, then, key developmental events coincide with the expression of LIM proteins. In muscle, LIM proteins appear to be involved at a number of developmental stages, spanning the proliferative phase through to myotube maturation.

The conservation of the LIM domain in a variety of proteins over a wide evolutionary range suggests its functional importance. Although their function is still under investigation, the primary mode of LIM protein action appears to involve protein-protein interactions. German et al. (1992) demonstrated the importance of the LIM domain in the synergistic activation of the insulin gene. The LIM domain of the LIM-HD transcription factor *lmx-1* interacts with the bHLH domain of the *shPAN-1* factor, when both are bound to their adjacent sites on the insulin promoter. The LIM domain of the LIM-HD protein *Isl-1* has been found to inhibit DNA binding by the homeodomain (Sanchez-Garcia et al., 1993). The rhombotin LIM-only proteins, RBTN1 and RBTN2 were found to interact

specifically with the bHLH domains of a trio of transcription factors implicated in T cell leukemia, even when the latter proteins are complexed with other partners (Wadman et al., 1994). Not only then, do LIM proteins act via protein-protein interactions, but they are also implicated in the modulation of transcription factor activity.

LIM-only proteins have also been found to interact with each other-- zyxin and CRP1, which contain three and two LIM domains respectively, are found bound to each other in the cytoplasm of avian smooth muscle cells (Crawford et al., 1994). This interaction is specific, despite the overall uniformity of LIM domains, and the first of zyxin's three LIM motifs is sufficient to carry out this function (Schmeichel and Beckerle, 1994). These proteins are also found associated with cytoskeletal elements, possibly acting as scaffolds for other protein-protein interactions (see below).

The specific interactions between essentially uniform LIM domains and other proteins suggests some form of *in vivo* LIM regulation. Indeed, *in vitro* studies show that LIM domains associate indiscriminately with each other (reviewed by Arber and Caroni, 1996). Recent work, however, has found LIM-binding factors in rat and *Drosophila* named NL1 and Chip, respectively (Jurata and Gill, 1997, Dawid et al., 1998). These proteins appear to modulate LIM-mediated homeodomain transactivation, indicating that a universal regulatory mechanism for LIM function exists based on their cellular or tissue contexts.

Though the function of the muscle-LIM proteins is unclear, their distribution in developing myotubes suggests a role in cytoarchitectural formation or maintenance. In the absence of MLP expression, rat cardiomyocytes show severe cytoskeletal disruption (Arber et al., 1997). Immunocytochemistry revealed a cytoplasmic distribution of the rat MLP in mature myotubes (Arber et al., 1994).

In *Drosophila*, both Mlp60A and Mlp84B are also found in the myotube cytoplasm, with the former showing an even cytoplasmic distribution and the latter appearing concentrated at the terminal ends of muscle fibers (Stronach et al., 1996). All three muscle-LIM proteins have been shown to specifically associate with the actin cytoskeleton (Arber et al., 1997, Stronach et al., 1996). In the case of the rat MLP, only the second of its two LIM domains is required for this association, and though similar studies have not been carried out on the *Drosophila* homologues, Mlp60A only carries one LIM domain. These cytoskeletal interactions and loss-of-function studies suggest the muscle-LIM proteins may act as scaffolds for the formation of protein complexes in the cytoplasm.

LIM proteins, as discussed above, associate with and may influence the activity of transcription factors via protein-protein interactions. Might the same be true of the muscle-specific LIM-only proteins? The distribution of the rat MLP was found to change within the maturing myotube, with an initial nuclear accumulation in immature myotubes followed by an overall cytoplasmic distribution (Arber et al., 1994). Further, the first of its LIM domains was found to increase nuclear localization of MLP, and has been shown to mediate interactions with Myo-D, a bHLH transcription factor crucial to myogenesis. The heterodimerization was found to enhance the DNA-binding of Myo-D. This may involve the stabilization of a Myo-D-cofactor complex or the LIM protein might alter Myo-D's conformation to ease recognition and DNA binding (Kong et al., 1997). Both *Drosophila* muscle LIM proteins are also found in myotube nuclei, though the accumulation is not great enough to discount passive diffusional effects (Stronach et al., 1996). Mlp60A, however, does contain a putative nuclear targeting signal, and there is conflicting evidence that this protein may accumulate predominantly in the nucleus (Kong et al., 1997). Considered in light of the

existence in *Drosophila* of nau, a Myo-D homologue (reviewed in Abmayr and Keller, 1998), there is a strong possibility that the single LIM-domain Mlp60A and *E. scudderiana* P3 may be involved in the activation of muscle-specific genes not only at terminal differentiation, but perhaps during early phases of myogenesis.

The levels of P3b transcript appear to remain elevated throughout the cold/subzero time course (see Fig. 4), and this might reflect the sequestration of mRNA for later translation. Western immunoblot analysis using radiolabeled anti-Mlp60A antibodies would be required to determine the presence of the protein in overwintering insects. Similar immunoblot or Northern hybridization analyses could be carried out for a number of other proteins associated with myogenic or more global developmental events. In addition, insects kept at 15°C do not show a change in P3b transcript abundance over the course of five weeks (see Fig. 10), suggesting that its upregulation is dependant on an exposure to low temperatures.

That a gene with such developmental implications as Mlp60A is upregulated in cold-exposed *E. scudderiana* raises questions as to its role in the overwintering insect. Most investigations of Mlp60A and the rat MLP homologue strongly implicate these muscle-LIM proteins in the terminal differentiation of myotubes, yet whether *E. scudderiana* is undergoing substantial myogenesis at the outset of or during the winter is questionable. Imaginal discs do show proliferative and internalized developmental activity prior to the pupal moult (Chapman, 1982, Milner and Bleasby, 1985), but could that account for the apparently cold-induced Mlp60A expression? Myogenesis occurs predominantly during embryogenesis and again at metamorphosis, and is dependant on ecdysteroid titre at both these stages-- this is reflected by the expression peaks of Mlp60A and Mlp84B in *Drosophila* (Stronach et al., 1996). *E. scudderiana* larvae

overwinter in their final instar, and will not continue their development without an exposure to cold. They undergo pupation and metamorphosis only in spring, yet appear to upregulate an Mlp60A homologue at the onset of cold temperatures. This raises the possibility that cold exposure causes changes in ecdysteroid titre.

In insects, final-instar larva experience commitment peaks and a subsequent prepupal peak in ecdysteroid (see Chapman, 1982 and Riddiford 1993). The former act to render larval tissues competent for a pupal moult without initiating it, while the global changes of morphogenesis are triggered soon after by the latter peak (Riddiford, 1993, Riddiford and Truman, 1993).

It is possible that in *E. scudderiana* larvae, cold temperatures trigger the commitment peaks, while the increased temperatures of spring might subsequently initiate the prepupal ecdysteroid peak. This may account for the upregulation of a selection of developmentally important genes, as well as the timing of development in these animals. The upregulation in late fall/early winter of an Mlp60A homologue in *E. scudderiana* would then be in anticipation of the global changes due in spring. Such gene expression in anticipation of a future requirement may have a precedent in the freezing-induction of fibrinogen subunits in the wood frog (Cai and Storey, 1997a). These proteins are involved in the formation of blood clots, and might be upregulated early in the winter in anticipation of damage to capillary beds during spring thaw.

E. solidaginis clone U1 appears to be a homologue of one of the acidic ribosomal proteins (r-proteins), found in all eukaryotic ribosomes studied. Eukaryotic ribosomes contain a number of acidic r-proteins, but the most acidic pair within each, P1 and P2, comprise the P proteins (Chooi et al., 1980). These are considered to be counterparts to the prokaryotic L7/L12 r-proteins (Quian et al., 1987). The P proteins of *Drosophila*, named rpA1 and rpA2, were first

recognized by their pI values (below pH 4.5) and molecular weights (11.3 and 11.7 kDa respectively), and have since been cloned (Chooi et al., 1980, Vidales et al., 1981, Quian et al., 1987, Olson et al. 1993). Both rpA1 and rpA2 are coded for by single-copy genes, present on different chromosomes, and the rpA1 gene sequence was shown to be intronless. rpA1 and rpA2 differ notably in the 5'UTR of their respective mRNAs, but they share nucleotide and amino acid identities of 53% and 39%, respectively. Both show the conserved features of P proteins, including a possible N-terminal GTP-binding site, the alanine-rich hinge region and the C-terminal region (Olson et al., 1993). U1 displays the greatest similarity to the *Drosophila* rpA1, having a 77.9% similarity in amino acid sequence (see Table 7).

Clone U1 displays a number of features that suggest it represents a complete transcript sequence (see Fig. 22): the cDNA length corresponds to that of its transcript (578 and approximately 600 nt, respectively), the sequence includes a polyadenylation signal and a portion of poly (A)+ tail, the deduced amino acid sequence is bracketed by a start and stop codon as well as 3' and 5' UTRs, and lastly, the deduced ORF of 112 amino acids approximates the lengths of other known P protein homologues.

The deduced amino acid sequence of U1 bears a number of similarities with known P proteins (see Fig. 23). Overall, it possesses a high alanine content (18%, compared with 16% in the *Drosophila* rpA1) which is concentrated in a hydrophobic, acidic stretch known as the hinge region (residues 62-83 and 62-84 in U1 and rpA1, respectively)(Olson et al., 1993). This region is followed by a lysine and glutamate-rich hydrophilic region (residues 86-106 and 87-107 in U1 and rpA1, respectively) and by the 9-residue C-terminal region, which is particularly well conserved in all known eukaryotic P protein sequences-- most

likely due to its importance in associating the P proteins with the ribosome (Olson et al., 1993, Remacha et al., 1995b).

Like their prokaryotic counterparts L7 and L12, rpA1 and rpA2 (and all P proteins) are present in the large subunit of the ribosome as a pair of dimers on the stalk, one of that subunit's three protrusions. Whether the P protein dimers constitute the stalk itself or form only a portion of it, is unclear (Hill et al., 1990). Reversible phosphorylation may be key to controlling P protein affinity to the ribosome, as phosphorylation of yeast homologues was found to increase their association with the large ribosomal subunit. It was further found that cytosolic pools of yeast P proteins are not only unphosphorylated, but are also likely to be dimerized (Vidales et al., 1984). More recent evidence, however, suggests that the phosphorylation state of the P proteins may have less to do with ribosomal affinity than with their actual role in ribosome functioning (Vard et al., 1997).

The ubiquitous nature of the P proteins in ribosomes, and the degree of their sequence conservation (in eukaryotes), indicates their importance in ribosomal functioning (Vidales et al., 1984). Early work with denuded core ribosomal particles suggested that the absence of P1 and P2 does not impede the formation of the 80S yeast ribosome on mRNA in vitro, and that polypeptide synthesis could occur, though at reduced rates (Sanchez-Madrid et al., 1979). A mutant yeast strain, deficient in P1 and P2, also showed reduced polymerizing activity (30% that of wild type) and a reduced growth rate compared to wild type strains (Remacha et al., 1995a). These observations demonstrate that the P proteins play some role in the elongation phase of protein synthesis.

There is also evidence implicating P proteins in the modulation of GTP hydrolysis during elongation, and this may be affected by their phosphorylation

state. Core yeast ribosomal particles, denuded of P1 and P2, were found to have 50% of the elongation-factor (EF)-dependant GTPase activity as complete ribosomes (Sanchez-Madrid et al., 1979). This parallels findings in *E.coli*, where the L7/L12 dimer is thought to be responsible for EF-dependant GTPase activity, possibly by relieving some conformational stress or block of EF (Hill et al., 1990). Phosphorylation, in turn, may act to enhance P protein performance in this role. Rat ribosomes containing P1/P2 dimers where only the latter protein was phosphorylated retained most of their polymerizing activity, whereas they remained functionally inhibited when only the P1 was phosphorylated (Vard et al., 1997). Hence, the P proteins, though not essential for ribosomal function, do modulate their polymerizing activity and enhance cell viability.

Another aspect of P protein function may involve selective translational activity by the ribosome. Using an *in vitro* translation method, Remacha et al. (1995b) found that P proteinless yeast ribosomes transcribed a different assortment of mRNAs from a cytoplasmic pool, compared to the profile generated by complete ribosomes. The differences suggested not only that the P proteins are essential for the translation of certain mRNAs, but that they may also act to suppress the translation of others. These transcripts and their proteins have yet to be characterized, but the role of P proteins in modulating ribosomal activity is nevertheless clear.

In cold-hardened *E. solidaginis* then, an increase in an rpA1 homologue might indicate an overall increase in protein synthesis, or in the selective translation of particular transcripts. However, the role of increased rpA1 abundance cannot be considered without first addressing r-protein gene regulation. The regulation of r-protein synthesis has been investigated at the transcriptional, post-transcriptional and translational levels, and may have

implications for the individual regulation of the P protein subset, separate from other r-proteins.

The coordinated expression of r-protein genes is well known, and suggests an 'all-or-nothing' mechanism for ribosomal synthesis in both prokaryotes and eukaryotes (reviewed by Mager, 1989 and Amaldi et al., 1995). This coordinated production of equimolar amounts of eukaryotic r-proteins may be achieved at the level of transcription via commonalities in the 5' regions of the r-protein genes. Putative protein binding sites, named HOMOL 1 and RPG boxes, have been detected in a number of r-protein gene promoter regions in yeast, suggesting that they may be under the common control of a small number of specific transcription factors. Meanwhile, r-protein gene promoters from higher eukaryotes, including vertebrates, display a similarity to the constitutive promoters of housekeeping genes (reviewed by Mager, 1989, Amaldi et al., 1995). This suggests that r-protein genes may be constantly active, with transcriptional modulation being the main form of their control. Overall, the precedent of coordinate expression would suggest that in *E. solidaginis*, all r-protein genes, including an rpA1 homologue, may be upregulated upon low temperature exposure.

Might the rpA1 homologue be the only r-protein upregulated in cold-exposed *E. solidaginis*? The P proteins, as a pair of dimers, are the only r-proteins present in multiple copies in the assembled ribosome. This may indicate their separate regulation at the level of transcription. The 5' flanking region of the *Drosophila* rpA1 gene appears to have a pyrimidine-rich region similar to those found in mammalian r-protein genes (Qian et al., 1987), but this has not been substantiated as having the constitutive-expression effect of housekeeping genes. In light of this, then, there remains the possibility of rpA1's individual regulation.

Post-transcriptional mechanisms may also contribute to the control of r-protein expression. In yeast, it has been suggested that alternative splicing of an r-

protein pre-mRNA may serve to inhibit further transcription of its gene (reviewed by Mager, 1989). This kind of negative-feedback has not been observed in other eukaryotic systems. Northern hybridizations of the *E. solidaginis* rpA1 homologue indicate an increase, peak, and subsequent decrease of its transcript over the cold/freezing time-course (see Figure 21). If these changes are indeed reflections of transcriptional activity, such a negative-feedback mechanism may be a contributing factor. However, the alternative splicing of rpA1 transcript may have resulted in the detection of multiple bands in northern blot analyses-- as this was not the case, other forms of transcriptional control may also contribute to the up and subsequent down-regulation of rpA1 transcript.

Control of r-protein abundance has also been observed at the translational level. In *Drosophila*, for example, translational control has been strongly implicated in r-protein production, and specifically in the case of rpA1 (Al-Atia et al., 1985). During embryogenesis, rpA1 mRNA abundance changes little, but its association with polysomes does, according to the developmental stage of the animal (Fruscolini et al., 1983). In the case of rpA1, the 5'-UTR of the mRNA itself may contribute to its selective translation, whether by inherent secondary structures, or by the binding of specific protein factors, all of which affect its affinity for the translational machinery (Patel and Jacobs-Lorena, 1992). This has been substantiated in *Xenopus*, where a number of proteins have been detected bound to the 5'-UTR of transcriptionally repressed r-protein transcripts (reviewed by Larson et al., 1991, Amaldi et al., 1995). This translational regulation may reflect the control of P protein production apart from the other r-proteins.

Whether by specific mRNA-binding proteins or by the r-proteins themselves, the cytoplasmic sequestration of r-protein transcripts appears to be a significant control mechanism to tailor ribosome synthesis to the cell's needs. In the case of cold-exposed *E. solidaginis*, however, selective translation of an rpA1

homologue may not play as great a role. The synthesis of the P protein would require verification by Western blot analyses, using labeled, rpA1-specific antibodies. In this way the question of U1 transcript sequestration for future or prolonged use might be addressed.

Post-translational mechanisms may also contribute to the control of r-protein expression. In order to ensure the approximately equal abundance of all r-proteins, not only are the r-protein and rRNA genes under coordinate control, but any r-proteins not associated with rRNA are quickly degraded in the cytoplasm (Gorenstein and Warner, 1976, Tushinski and Warner, 1982, reviewed by Mager, 1989 and by Amaldi et al., 1995). This degradation of non-ribosomally associated r-proteins, however, appears not to apply to the P proteins; there is a constant cytoplasmic pool of these proteins, which, possibly depending on their phosphorylation state, reversibly associate with the large subunit of the ribosome (Vidales et al., 1984, Saenz-Robles et al., 1990, Vard et al., 1997). This selective regulation of P protein abundance at the post-translational level, along with the translational regulation described above, might suggest that their transcriptional control is separate from the rest of the r-protein complement. In *E. solidaginis*, then, the rpA1 homologue may be regulated at the transcriptional level separately from the other r-proteins.

Is the *E. solidaginis* rpA1 homologue the sole r-protein upregulated in response to low temperatures? The upregulation of all r-protein genes would make for more than seventy new transcripts. Only one r-protein was found and verified in this study, and it represents a P protein that would remain stable in the cytoplasm if left unassociated with a 60S ribosomal subunit. This may suggest that the rpA1 homologue was the only r-protein transcript present among the upregulated genes.

That other r-protein transcripts were not found among the differentially displayed bands, however, may not necessarily indicate the sole upregulation of the rpA1. It is possible that the other r-protein transcripts were not amplified by the eight random primers used-- the estimated number of amplified bands from all 24 DD-PCR reactions was approximately 1500. These represent 10% of the estimated 15 000 mRNAs present in the average eukaryotic cell. This estimation does not include the frequency of repeat transcripts, those amplified by more than one primer combination. Another possibility is that some of the other *E. solidaginis* r-proteins are in fact represented among the other U-series clones, however, as these have not shown any matches in a sequence comparison search, they are classified as novel sequences. This might be feasible, as the other clones are quite short relative to their transcripts and may only represent 3' UTRs.

The further investigation of the possible upregulation of the other r-proteins would require a number of different approaches. First, steps would have to be taken to obtain complete sequences of the other verified, novel clones found in this study. 5'RACE might be employed towards this, and the complete sequence data may reveal other r-protein homologues among the cold-induced clones. Hybridization of northern blots with radiolabeled probes synthesized from other (i.e. *Drosophila*) r-protein consensus sequences may also reveal the upregulation of additional r-proteins in *E. solidaginis*. Reverse-transcription PCR (RT-PCR) could also be employed, using primers specific for known r-proteins, and RNA from control (15°C) and cold/subzero *E. solidaginis*. In addition, it would be of particular interest to know whether or not the companion P protein, rpA2, is also being upregulated during low temperature exposure.

The upregulation of an rpA1 homologue, with or without the rest of the r-protein complement, invites speculation as to what is happening during the

exposure of *E. solidaginis* to cold and freezing temperatures. Is this synthesis meeting an immediate need, or is it anticipating a future requirement?

Ribosomal synthesis is generally attuned to the cell's need for protein synthesis, and this requirement is strongly affected by cell proliferation (reviewed by Mager, 1989, Larson et al., 1991). Experimental perturbation of growth rate (i.e. changes in available nutrients or endocrine factors) has been shown to affect ribosomal synthesis-- non-growing mouse cells in culture show a 50% decrease in r-protein synthesis compared to growing controls, while the synthesis of non-ribosomal proteins changes little (Tushinski and Warner, 1982). It would seem unlikely that cell proliferation is occurring to any great degree in overwintering *E. solidaginis* larvae. Growth and protein synthesis would be substantial in spring, when pupariation and subsequent developmental changes occur. This raises the possibility that if *E. solidaginis* is synthesizing new ribosomes, it may be doing so in anticipation of upcoming developmental events. Otherwise, exposure to cold acts as a cue for the immediate synthesis of new proteins and this in turn requires a greater abundance of ribosomes.

A possible immediate use for new ribosomes may be as a compensatory measure against a decreased rate of translation at low temperatures. In *E. coli*, cold shock amounts to a transient inhibition of translational activity, affecting growth rate. After acclimation, mediated by cold shock protein (CSP) expression, growth does resume, although at a slower rate than at 37°C. Among the *E. coli* CSPs, three have been identified as being associated with the ribosome and are required for efficient ribosomal function at low temperatures (reviewed by Thieringer et al, 1998). Indeed, a decrease in translational efficiency has been postulated to play a major role in triggering the cold-shock response in bacteria (Jones and Inouye, 1994). Ribosome biogenesis appears to be part of the cold shock response in yeast, where one of its cold-induced genes, NSR-1, is involved

in rRNA processing (Kondo et al., 1992, Kondo and Inouye, 1992). Cold exposure in other eukaryotes also involves changes in the translational machinery - cold adapted toad fish show an increase in elongation factor 1 (EF1) in liver tissue, as well as a general increase in protein synthesis (Nielson et al., 1977). The accumulation of EF1 is thought to help overcome an initial decrease in translational activity upon cold exposure. In plants, r-proteins and ribosome-associated proteins have been found to increase in abundance upon cold exposure, making ribosomes from cold-adapted plants more efficient at low temperature translation *in vitro* (reviewed by Thieringer et al, 1998). Whether by increasing ribosome synthesis or by expressing ribosome-associated factors, correction of a cold-induced decrease in translational activity appears to be part of a generalized reaction to low temperatures. *E. solidaginis* may be following this pattern.

If *E. solidaginis* is not synthesizing new ribosomes, the increased abundance of P proteins in the cytoplasm may reflect a slightly different reaction to cold exposure. It has been suggested that P proteins remain free in the cytoplasm prior to translational initiation (Remacha et al., 1995b). This, coupled with the possibility that the phosphorylation state of the P proteins may not govern their affinity for the large ribosomal subunit (Vard et al., 1997), suggests that some other mechanism may be in place to affect this affinity. This as yet uncharacterized mechanism may be adversely affected by a decrease in temperature, and an increase in cytoplasmic P protein may correct for this. In this way, the sole upregulation of the P proteins may serve to restore translational activity reduced by low temperatures.

The two genes discussed above are the only complete, identifiable sequences found in the course of this study. The remaining 12 are considered as representing novel genes, and are also presumed to be incomplete, making

commentary on their putative protein products and roles in insect overwintering speculative. These clones have been described individually in Chapter Three. Further work with these incomplete clones could focus on a search for complete transcript sequences, using either 5'RACE or the probing of existing cDNA libraries. If, after more sequence data are obtained, the clones remain unidentifiable via database searches, deduced amino acid sequences can be searched for recognizable motifs or proteins expressed using a baculovirus system can be studied.

A number of these P and U-series clones have also been used in Northern hybridizations against 5-week 15°C time courses, in an attempt to investigate the possibility that chronology takes precedence over temperature in transcript upregulation. P3, P6 and P8 showed no changes in transcript abundance relative to the first time-point (see Fig. 10), indicating that low temperatures are crucial for their upregulation. P11 and U1, meanwhile, both showed a steady increase in transcript abundance over the five weeks (see Figs. 41 and 28). While transcript would accumulate with time, low temperatures might affect either mRNA stability and/or translational activity, and further studies, would be required to address these questions, including possible nuclear run-on assays and/or Western blot analysis. In a few cases then, it seems that transcript upregulation is governed by both the passage of time and changes in temperature.

Whether or not they contained identifiable homologues, all clones represented transcripts that showed changes in their abundance during cold/subzero temperature exposure, relative to control. Whether these changes were due to increased transcription or increased mRNA half-life, cannot be

determined within the scope of this study. Nuclear run-on assays might be employed to address this issue in the case of cold upregulated genes.

The initiation of changes in transcript abundance could reflect a number of alternative scenarios, including the effects of cold versus freezing. Freezing comprises two stresses which are known to induce genes, anoxia and changes in cell-volume.

The anaerobic conditions imposed by internal ice formation requires freeze-tolerant animals to have a well developed tolerance for anoxia (Storey and Storey, 1988, Storey and Storey, 1985). Anoxic conditions trigger polyol synthesis in *E. solidaginis* independently of low temperatures. Anoxic larvae held at 13°C produced both glycerol and sorbitol, the latter accumulating to concentrations comparable with those in aerobic larvae held below 5°C (Storey and Storey, 1990). Anoxic *E. scudderiana* held at -4°C accumulate glycerol, but to far lower concentrations than aerobic larvae at the same temperature (Churchill and Storey, 1989b). Anoxia, then, may also act as a gene-inducing stress in both *E. solidaginis* and *E. scudderiana*.

Cell volume changes may also contribute to gene induction in overwintering animals. Cai and Storey (1997) found the novel wood frog gene *FR10* to be upregulated during freezing as well as dehydration, but not during anoxia. In *E. solidaginis*, cell volume may affect polyol accumulation (Rojas et al., 1983), suggesting that this stress may have a role in gene-induction, including the clones found in the course of this study.

In light of the above, future work could focus on gene induction as *E. solidaginis* encounters the above mentioned stresses, anoxia and cell-volume changes. Time courses and corresponding Northern blots could be made (anoxia and dehydration) and probed with the U-series clones found in this study. In addition, these anoxia and dehydration time courses could be used in further DD-

PCR experiments to investigate the possibility of genes induced during these stresses. Any new sequences found in this manner, identifiable or novel, could be used to probe Northern blots of the cold/subzero time course.

As discussed above in relation to the expression of clone P3, the cue for changes in transcript abundance in both *E. scudderiana* and *E. solidaginis* could be hormonal in nature. Chilling stress has been implicated in affecting hormonal balance in insects (Rojas et al., 1987, reviewed in Jankovic-Hladni, 1991, Ivanovic, 1991). Indeed, cryoprotectant synthesis in *E. solidaginis*, demonstrably induced by low temperatures, has also been thought to be under hormonal control (Baust and Lee, 1981, Baust and Lee, 1982, Hamilton et al., 1986). Low temperature induced changes in hormone titre would also provide a mechanism to ensure the correct timing of spring pupation these insects. This is crucial as *E. scudderiana* and *E. solidaginis* can only overwinter as last-instar larvae, and early pupation would be fatal (Baust and Nishino, 1991, Storey and Storey, 1988). As the adult forms of both these species are non-feeding, it would be to the insects' advantage to metamorphose as quickly as possible in spring, so as to economize fuel reserves.

A number of approaches could serve to investigate the possible hormonal aspect of low temperature gene regulation in *E. scudderiana* and *E. solidaginis*. The promoter region of the cold-induced *B. mori* sorbitol dehydrogenase gene contains a putative steroid-receptor binding site, suggesting temperature-induced changes in hormonal balance (Niimi et al., 1996). Similar investigations into the promoter regions of the P and U genes found to be upregulated by cold/subzero temperatures may also yield interesting insights into their regulation, either by hormone receptor or some novel means. Radioimmune assay protocols exist which can be applied to the question of ecdysteroid titres in *E. scudderiana* and *E.*

solidaginis. (Koolman and Walter, 1985, Cassier and Cyborowski, 1991). Also, the expression of a number of ecdysteroid-induced genes, known as primary response genes, can be examined in *E. scudderiana* and *E. solidaginis* as they encounter low temperatures (see Andres et al., 1993, Huet et al., 1995 for a number of possible candidates).

The results of such studies would serve to elucidate the unique developmental regime of these two insect species, and how it might affect their respective overwintering strategies. In addition, it may open new avenues of research, such that the mechanism by which low temperatures affect hormone titer might become a key focus in the investigation of insect coldhardiness.

The aim of this study was to investigate the possible low temperature upregulation of genes in two cold hardy insect species using differential screening of a cDNA library and via DD-PCR. Any genes thus found would offer insight into the insects' winter survival strategies. A number of transcripts appear to increase in abundance upon low temperature exposure in *E. scudderiana* and *E. solidaginis* (see Table 11), though only two were identifiable by sequence database comparison. In the former insect, a homologue of the muscle-LIM protein Mlp60A was found to be upregulated upon cold exposure and in the latter species, a homologue of a ribosomal protein, rpA1 was found to be cold-upregulated. Both of these genes have developmental implications, suggesting for the first time that advance preparation for impending metamorphic changes may be part of the insects' overwintering strategy.

These findings offer an unexpected complement to previous biochemical studies in these insects, which highlighted responses to immediate needs brought about by low temperatures. As hormonal titre may effect the upregulation of these developmentally crucial genes, it raises the possibility not only that low

temperatures may serve as cues for hormone synthesis and/or release, but also that this might have direct effects on previously characterized biochemical changes conferring cold survival.

REFERENCES

- Abmayr SM and Keller CA (1998). *Drosophila* myogenesis and insights into the role of *nautilus*. *Curr. Topics Dev. Biol.* 38: 35-80.
- Abmayr SM, Erickson MS and Bour BA (1995). Embryonic development of the larval body wall musculature of *Drosophila melanogaster*. *Trends Genet.* 11: 153-159.
- Al-Atia GR, Fruscoloni P and Jacobs-Lorena M (1985). Translational regulation of mRNAs for ribosomal proteins during early *Drosophila* development. *Biochemistry* 24: 5798-5803.
- Amaldi F, Camacho-Vanegas O, Cardinali B, Cecconi F, Crosio C, Loreni F, Mariottini P, Pellizzoni L and Pierandrei-Amaldi P (1995). Structural and expression of ribosomal protein genes in *Xenopus laevis*. *Biochem. Cell Biol.* 73: 969-977.
- Andres AJ, Fletcher JC, Karim FD and Thummel CS (1993). Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. *Dev. Biol.* 160: 388-404.
- Arber S and Caroni P (1996). Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ. *Genes & Dev.* 10: 289-300.
- Arber S, Halder G and Caroni P (1994). Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell* 79: 221-231.
- Arber S, Hunter JJ, Ross J, Hongo M, Sansig G, Borg J, Perriard J-C, Chien KR and Caroni P (1997). MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell*: 88: 393-403.
- Bale JS (1987) Insect cold hardiness : freezing and supercooling - an ecophysiological perspective. *J. Insect Physiol.* 33: 899-908.
- Bale JS, Hansen, T.N. and Baust, J.G. (1989) Nucleators and sites of nucleation in the freeze tolerant larvae of the gallfly *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* 35: 291-298.
- Baust JG and Lee RE (1981). Divergent mechanisms of frost-hardiness in two populations of the gall fly, *Eurosta solidaginis*. *J. Insect. Physiol.* 27: 485-490.

- Baust JG and Lee RE (1982). Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). *J. Insect. Physiol.* 28: 431-436.
- Baust, J.G. and Nishino, M. (1991) Freezing tolerance in the goldenrod gall fly (*Eurosta solidaginis*). In: Insects At Low Temperature. Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.
- Becker J and Craig EA (1994). Heat-shock proteins as molecular chaperones. *Eur. J. Biochem.* 219: 11-23.
- Beltrame M and Bianchi ME (1990). A gene family for acidic ribosomal proteins in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 10: 2341-2348.
- Birkenmeier EH and Gordon, JI (1986). Developmental regulation of a gene that encodes a cysteine-rich intestinal protein and maps near the murine immunoglobulin heavy chain locus. *Proc. Natl. Acad. Sci. USA* 83: 2516-2520.
- Birnstiel ML, Buslinger M and Strub K (1985). Transcription termination and 3' processing: the end is in site! *Cell* 41: 349-359.
- Burton, V. Mitchell, H.K., Young, P. and Peterson, N.S. (1988) Heat shock protection against cold stress of *Drosophila melanogaster*. *Mol. Cell. Biol.* 8: 3550-3552.
- Cai Q and Storey KB (1997a). Freezing-induced genes in wood frog (*Rana sylvatica*): fibrinogen upregulation by freezing and dehydration. *Am. J. Physiol.* 272: R1480-R1492.
- Cai Q and Storey KB (1997b). Upregulation of a novel gene by freezing exposure in the freeze-tolerant wood frog (*Rana sylvatica*). *Gene* 198: 305-312.
- Cassier P and Cymborowski B (1993). Physiology of larval diapause in the wax moth, *Galleria mellonella*: and ultrastructural analysis. *Comp. Biochem. Physiol.* 105A: 679-687.
- Chapman RF (1982). The insects: structure and function. 3rd Ed. Harvard University Press. Cambridge, Mass.
- Chen, C.P., Denlinger, D.L. and Lee RE (1987) Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalis*. *Physiol. Zool.* 60: 297-304.

- Chooi WY, Sabatini LM, Macklin M and Fraser D (1980). Group fractionation and determination of the number of ribosomal subunit proteins from *Drosophila melanogaster* embryos. *Biochemistry* 19: 1425-1433.
- Churchill, T.A. and Storey KB (1989a) Metabolic responses during long term exposure to subzero temperatures by a freeze avoiding insect. *Cryo-Lett.* 10 : 197-204.
- Churchill, T.A. and Storey KB (1989b) Metabolic correlates to glycerol biosynthesis in a freeze-avoiding insect, *Epiblema scudderiana*. *J. Comp. Physiol. B* 15:461-472.
- Collins, FH (1996). *Anopheles albimanus* adult DNA. Unpublished.
- Consoulas C and Levine RB (1997). Accumulation and proliferation of adult leg muscle precursors in *Manduca* are dependant on innervation. *J. Neurobiol.* 32: 531-553.
- Courey AJ and Tjian R (1988). Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55: 887-898.
- Crawford AW, Pino JD and Beckerle MC (1994). Biochemical and molecular characterization of the chicken cysteine-rich protein, a developmentally regulated LIM-domain protein that is associated with the actin cytoskeleton. *J. Cell Biol.* 124: 117-127.
- Cullin KE and Sarge KD (1997). Characterization of hypothermia-induced cellular stress response in mouse tissues. *J. Biol. Chem.* 272: 1742-1746.
- Curtiss J and Heilig JS (1998). DeLIMinting development. *Bioessays* 20: 58-69.
- Darnell J, Lodish H and Baltimore D (1990). Molecular cell biology. 2nd Ed. Scientific American Books.
- Dawid IB, Breen JJ and Toyama R (1998). LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet.* 14: 156-162.
- Dawid IB, Toyama R and Taira M (1995). LIM domain proteins. *C.R. Acad. Sci. Ser. III Sci Vie.* 318: 295-306.
- deKort, C.A.D. and Koopmanschap, A.B. (1994) Nucleotide and deduced amino acid sequence of a cDNA clone encoding diapause protein I, an aryphorin-

type storage hexamer of the Colorado potato beetle. *J. Insect Physiol.* 40: 527-535.

Denlinger, D.L., Joplin, K.H., Chen, C-P and Lee RE (1991) Cold shock and heat shock. In: Insects At Low Temperature. Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.

DeVries, A.L. (1986) Antifreeze glycopeptides and peptides : interactions with ice and water. *Methods Enzymol.* 127: 293-303

Duman JG (1982) Insect antifreezes and ice-nucleating agents. *Cryobiology* 19: 613-627.

Duman J.G., Wu, D.W., Xu, L., Tursman, D. and Olsen, T.M. (1991) Adaptation of insects to subzero temperatures. *Quart. Rev. Biol.* 66: 387-410.

Duman JG, Xu, L., Neven, L.G., Tursman, D. and Wu, D.W. (1991) Haemolymph proteins involved in insect subzero-temperature tolerance : ice nucleators and antifreeze proteins. In: Insects At Low Temperature Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.

Friedrich M and Tautz D (1997). An episodic change of rDNA nucleotide substitution rate has occurred during the emergence of insect order Diptera. *Mol. Biol. Evol.* 14: 644-653.

Fruscoloni P, Al-Atia GR and Jacobs-Lorena M. (1983). Translational regulation of a specific gene during oogenesis and embryogenesis of *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 80: 3359-3363.

German MS, Wang J, Chadwick RB and Rutter WJ (1992). Synergistic activation of the insulin gene by a LIM-homeodomain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. *Genes Dev.* 6: 2165-2176.

Gilmour, D.S., Dietz, T.J. and Elgin, S.C.R. (1988) TATA box-dependent protein-DNA interactions are detected on heat shock and histone gene promoters in nuclear extracts derived from *Drosophila melanogaster* embryos. *Mol. Cell. Biol.* 8: 3204-3214.

Gorenstein C and Warner JR (1976). Coordinate regulation of the synthesis of eukaryotic ribosomal proteins. *Proc. Natl. Acad. Sci. USA* 75: 1547-1551.

Hashimoto N and Ogashiwa M (1996). Differential expression of three double-LIM proteins during the skeletal muscle terminal differentiation. Unpublished.

- Hill WE, Dahlberg A, Garrett RA, Moore PB, Schlessinger D and Warner JR, Eds (1990). The Ribosome: structure, function and evolution. American Society for Microbiology. Washington, D.C.
- Hoffman, K.H. (1985) Metabolic and enzyme adaptation to temperature. In: Environmental Physiology and Biochemistry of Insects. Hoffman, K.H., Ed. Springer-Verlag, Berlin, New York.
- Huet F, Ruiz C and Richards G (1995). Sequential gene activation by ecdysone in *Drosophila melanogaster*: the heirarchical equivalence of early and early late genes. *Development* 121: 1195-1204.
- Jankovic-Hladni MI (1991). Hormones and metabolism in insect stress. In: Hormones and Metabolism in Insect Stress. Ivanovic JP and Jankovic-Hladni MI, Eds. CRC Press, Ann Arbor, Boston.
- Joanisse, D.R. (1995) Metabolism during overwintering in two overwintering species of cold-hardy goldenrod gall insects. PhD Thesis, Biology, Carleton University.
- Jones PG and Inouye M (1994). The cold-shock response-- a hot topic. *Mol. Microbiol.* 11: 811-818.
- Joplin, K.H., Yocum GD and Denlinger, D.L. (1990) Cold shock elicits expression of heat shock proteins in the flesh fly, *Sarcophaga crassipalpis*. *J. Insect Physiol.* 36: 825-834.
- Jurata LW and Gill GN (1997). Functional analysis of the Nuclear LIM domain interactor NLI. *Mol. Cell. Biol.* 17: 5688-5698.
- Ivanovic JP (1991). Metabolic response to stressors. In: Hormones and Metabolism in Insect Stress. Ivanovic JP and Jankovic-Hladni MI, Eds. CRC Press, Ann Arbor, Boston.
- Kjer KM, Baldrige GD and Fallon AM (1994). Mosquito large subunit ribosomal RNA: simultaneous alignment of primary and secondary structure. *Biochim. Biophys. Acta* 1217: 147-155.
- Knight, C.A. and Duman JG (1986) Inhibition of recrystallization of ice by insect thermal hysteresis proteins : a possible cryoprotective role. *Cryobiology* 23: 256-262.
- Knight, C.A., Wen, D. and Laursen, R.A. (1995) Nonequilibrium antifreeze peptides and the recrystallization of ice. *Cryobiology* 32: 23-34.

- Koolman J and Walter J (1985). Ecdysteroids in insects: how are their concentrations regulated? In: Metamorphosis. Balls M and Bownes M, Eds. Clarendon Press, Oxford.
- Kondo K and Inouye M (1992). Yeast NSR1 protein that has structural similarity to mammalian nucleolin is involved in pre-rRNA processing. *J. Biol. Chem.* 267: 16252-16258.
- Kondo K, Kowalski L and Inouye M (1992). Cold-shock induction of yeast NSR-1 protein and its role in pre-rRNA processing. *J. Biol. Chem.* 267: 16259-16265.
- Kong Y, Flick MJ, Kudla AJ and Konieczny SF (1997). Muscle LIM protein promotes myogenesis by enhancing the activity of Myo D. *Mol. Cell. Biol.* 17: 4750-4760.
- Kukal, O. (1991) Behavioural and physiological adaptations to cold in a freeze-tolerant Arctic insect. In: Insects At Low Temperature Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.
- Larsen DE, Zahradka P and Sells BH (1991). Control points in eucaryotic ribosome biogenesis. *Biochem. Cell. Biol.* 69: 5-22.
- Lee RE (1991). Principles of insect low temperature tolerance. In: Insects At Low Temperature Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.
- Lee RE, Chen C-P and Denlinger DL (1987). A rapid cold-hardening process in insects. *Science* 238: 1415-1417.
- Lee RE, Dommel RA, Joplin KH and Denlinger DL (1995). Cryobiology of the freeze-tolerant gall fly *Eurosta solidaginis*: overwintering energetics and heat shock proteins. *Climate Res.* 5: 61-67.
- Lindquist S (1987). The heat-shock response. *Ann. Rev. Biochem.* 55: 1151-1191
- Lis JT, Xiao H and Perisic, O (1990). Modular units of heat shock regulatory regions : structure and function. In: Stress Proteins in Biology and Medicine. Morimoto, R.I., Tissieres, A. and Georgopoulos, C. Eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Louis HA, Pino JD, Schmeichel KL, Pomies P and Beckerle MC (1997). Comparison of three members of the cysteine-rich protein family reveals

functional conservation and divergent patterns of gene expression. *J. Biol. Chem.* 272: 27484-27491.

Luce MJ and Burrows PD (1998). Minimizing false positives in differential display. *Biotechniques* 24: 754-759.

Ludolph DC and Konieczny SF (1995). Transcription factor families: muscling in on the myogenic program. *FASEB J.* 1595-1604.

Mager WH (1988). Control of ribosomal protein gene expression. *Biochim. Biophys. Acta.* 949: 1-15.

Maniatis T, Fritsch ER and Sambrook J (1989). Molecular Cloning: A Laboratory Handbook. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Martin I, Vinas O, Mampel T, Iglesias R and Villarroja F (1993). Effects of cold environment on mitochondrial genome expression in the rat: evidence for a tissue-specific increase in the liver, independent of changes in the mitochondrial gene abundance. *Biochem. J.* 296: 231-234.

Matz JM, Blake MJ, Tatelman HM, Lavoie KP and Holbrook NJ (1995). Characterization and regulation of cold-induced heat shock protein expression in mouse brown adipose tissue. *Am. J. Physiol.* 269: R38-R47.

Mauro VP and Edelman GM (1997). rRNA-like sequences occur in diverse primary transcripts: implications for control of gene expression. *Proc. Natl. Acad. Sci. USA* 94: 422-427.

Michelson JW, Schmeichel KL, Beckerle MC and Winge DR (1993). The LIM motif defines a specific zinc-binding protein domain. *Proc. Natl. Acad. Sci. USA* 90: 4404-4408.

Milner MJ and Bleasby AJ (1985). The alignment of imaginal anlagen during the metamorphosis of *Drosophila melanogaster*. In: Metamorphosis. Balls M and Bownes M, Eds. Clarendon Press, Oxford. 1985.

Morgan MJ and Madgwick AJA (1996). Slim defines a novel family of LIM-proteins expressed in skeletal muscle. *Biochem. Biophys. Res. Commun.* 225: 632-638.

Morgan MJ, Madgwick AJA, Charleston B, Pell JM and Loughlan PT (1995). The developmental regulation of a novel muscle LIM-protein. *Biochem. Biophys. Res. Commun.* 212: 840-846.

- Morimoto RI., Tissieres, A and Georgopoulos C. (1990). The stress response, function of the proteins, and perspectives. In: Stress Proteins in Biology and Medicine. Morimoto, R.I., Tissieres, A. and Georgopoulos, C. Eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Nielson JBK, Plant P and Haschemeyer AE (1977). Control of protein synthesis in temperature acclimation. I. Characterization of polypeptide elongation factor 1 of toadfish liver. *Physiol. Zool.* 50: 11-21.
- Niimi T, Yamashita O and Yaginuma T (1993). A cold-inducible *Bombyx* gene encoding a protein similar to mammalian sorbitol dehydrogenase. *Eur. J. Biochem.* 213: 1125-1131
- Niimi T, Yamashita O and Yaginuma T (1996). Structure of the *Bombyx* sorbitol dehydrogenase gene: a possible alternative use of the promoter. *Insect Mol. Biol.* 5: 269-280.
- Nordin JH, Cui Z and Yin C-M (1984). Cold-induced glycerol accumulation by *Ostrinia nubilalis* larvae is developmentally regulated. *J. insect Physiol.* 30: 563-566.
- Olson PF, Salo T, Garrison K and Fessler JH (1993). *Drosophila* acidic ribosomal protein rpA2: Sequence and characterization. *J Cell. Biochem.* 51: 353-359.
- Patel RC and Jacobs-Lorena M (1992). Cis-acting sequences in the 5'-Untranslated region of the ribosomal protein A1 mRNA mediate its translational regulation during early embryogenesis of *Drosophila*. *J Biol. Chem.* 267: 1159-1164.
- Peterson, NS, Young P and Burton V (1990). Heat shock mRNA accumulation during recovery from cold shock in *Drosophila melanogaster*. *Insect Biochem.* 20: 679-684.
- Quián S, Zhang JY, Kay MA and Jacobs-Lorena M (1987). Structural analysis of the *Drosophila* rpA1 gene, a member of the eucaryotic 'A' type ribosomal protein family. *Nuc. Acids. Res.* 15: 987-1003.
- Remacha M, Jimenez-Diaz A, Bermejo B, Rodriguez-Gabriel MA, Guardinos E and Ballesta JPG (1995a). Ribosomal acidic phosphoproteins P1 and P2 are not required for cell viability but regulate the pattern of protein expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 15: 4754-4762.
- Remacha M, Jimenez-Diaz A, Santos C, Briones E, Zambrano R, Rodrigues-Gabriel MA, Guarinos E and Ballesta JPG (1995b). Proteins P1, P2, and

- P0, components of the eukaryotic ribosome stalk. New structural and functional aspects. *Biochem. Cell Biol.* 73: 959-968.
- Rich BE and Steitz JA (1987). Human acidic ribosomal phosphoproteins P0, P1 and P2: analysis of cDNA clones, *in vitro* synthesis and assembly. *Mol. Cell. Biol.* 7: 4065-4074.
- Rickards J, Kelleher MJ and Storey KB (1987). Strategies of freeze avoidance in larvae of the goldenrod gall moth, *Epiblema scudderiana* : winter profiles of a natural population. *J. Insect Physiol.* 33: 443-450.
- Riddiford LM (1993). Hormones and *Drosophila* development. in: The Development of *Drosophila melanogaster*. Bate M and Martinez-Arias A, Eds. Cold Spring Laboratory Press.
- Riddiford LM and Truman JW (1993). Hormone receptors and the regulation of insect metamorphosis. *Receptor.* 3: 203-209.
- Rojas RR, Hamilton MD and Baust JG (1987). Juvenile hormone modulation of insect cold hardening: ice-nucleating activity. *Cryobiol.* 24: 465-472.
- Rojas RR, Lee RE, Luu T-A and Baust JG (1983). Temperature dependence-independence of antifreeze turnover in *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* 29: 865-869.
- Rougvie AE and Lis JT (1988). The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of *D. melanogaster* is transcriptionally engaged. *Cell* 54: 795-804.
- Russo VEA, Brody S, Cove D and Ottolenghi S (1992). Development. Springer-Verlag. Berlin, New York, London.
- Saenz-Robles MT, Remacha M, Vilella MD, Zinker S and Ballesta JPG (1990). The acidic ribosomal proteins as regulators of the eukaryotic ribosomal activity. *Biochim. Biophys. Acta.* 1050: 51-55.
- Salama MS and Miller TA (1993). In vitro translation of diapause mRNA from the fat body of active and diapause larvae of the pink bollworm, *Pectinophora gossypiella* (Saunders). *Arch. Insect Biochem. Physiol.* 23:1-11.
- Sanchez-Garcia I and Rabbitts TH (1994). The LIM domain : a new structural motif found in zinc-finger-like proteins. *Trends Genet.* 10: 315-320.

- Sanchez-Garcia I, Osada H, Forster A and Rabbitts TH (1993). The cysteine-rich LIM domains inhibit DNA binding by the associated homeodomain in Isl-1. *EMBO J* 12: 4243-4250.
- Sanchez-Madrid F, Reyes R, Conde P and Ballesta JPG (1979). Acidic ribosomal proteins from eukaryotic cells : Effect on ribosomal functions. *Eur. J. Biochem.* 98: 409-416.
- Schmeichel KL and Beckerle MC (1994). The LIM domain is a modular protein-binding surface. *Cell* 79: 211-219.
- Snyder MJ (1998). Ribosomal proteins S27E, P2 and L37A from marine invertebrates. *Mol. Marine Biol. Biotechnol.* In press.
- Somero GN (1995). Proteins and temperature. *Ann. Rev. Physiol.* 57: 43-68.
- Sorger PK and Pelham HRB (1988). Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* 54: 855-864.
- Sorger PK , Lewis MJ and Pelham HRB (1987). Heat shock factor is regulated differentially in yeast and HeLa cells. *Nature* 329: 81-84.
- Storey JM and Storey KB (1983). Regulation of cryoprotectant metabolism in the overwintering gallfly larva, *Eurosta solidaginis* : temperature control of glycerol and sorbitol levels. *J. Comp. Physiol.* 149: 495-502.
- Storey JM and Storey KB (1985). Freezing and cellular metabolism in the gallfly larva, *Eurosta solidaginis*. *J. Comp. Physiol.* 155: 333-337.
- Storey JM and Storey KB (1986). Winter survival of the gallfly larva *Eurosta solidaginis*: profiles of fuel reserves and cryoprotectants in a natural population. *J. Insect Physiol.* 32:549-556.
- Storey KB (1983). Metabolism and bound water in overwintering insects. *Cryobiology* 20: 365-379.
- Storey KB and Storey JM (1991). Biochemistry of cryoprotectants. In: Insects At Low Temperature Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.
- Storey KB and Storey JM (1988). Freeze tolerance in animals. *Physiol. Rev.* 68: 27-84.

- Storey KB and Storey JM (1992). Biochemical adaptations for winter survival in insects. In: Advances in Low-temperature Biology. Steponkus, P.L., Ed. JAI Press, London.
- Storey KB, Park, I.R.A. and Storey JM (1981). Isozyme composition and low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *Cryo-letters* 2: 279-284.
- Stronach BE, Siegrist SE and Beckerle MC (1996). Two muscle-specific LIM proteins in *Drosophila*. *J. Cell Biol.* 134: 1179-1195.
- Sula, J., Kodrik, D. and Socha R. (1995). Hexameric haemolymph protein related to adult diapause in the red firebug, *Pyrrhocoris apterus* (l.). (Heteroptera). *J. Insect Physiol.* 41: 793-800.
- Tan D (1994). *Bos taurus* ribosomal P2 protein amino acid sequence. Direct submission to GenBank.
- Tauber MJ, Tauber CA and Masaki S (1986). Seasonal Adaptations of Insects. Oxford University Press. New York.
- Tautz D, Hancock JM, Webb DA, Tautz C and Dover GA (1988). Complete sequences of the rRNA genes of *Drosophila melanogaster*. *Mol. Biol. Evol.* 5: 366-376.
- Telfer WH and Kunkel JG (1991). The function and evolution of insect storage hexamers. *Ann. Rev. Entomol.* 36: 205-228.
- Thieringer HA, Jones PG and Inouye M (1998). Cold shock and adaptation. *BioEssays* 20: 49-57.
- Tiku PE, Gracey AY, Macartney AI, Benyon RJ and Cossins AR (1996). Cold-induced expression of Δ^9 -desaturase in carp by transcriptional and posttranslational mechanisms. *Science*. 271: 815-818.
- Tursman D and Duman JG (1995). Cryoprotective effects of thermal hysteresis protein on survivorship of frozen gut cells from the freeze-tolerant centipede *Lithobius forficatus*. *J. Exp. Zool.* 272: 249-25.
- Tushinski RJ and Warner JR (1982). Ribosomal proteins are synthesized preferentially in cells commencing growth. *J. Cell. Physiol.* 112: 128-135.
- Tweedie S, Charlton J, Clark V and Bird A (1996). *Branchiostoma floridae* P1 amino acid sequence. Direct submission to GenBank.

- Vard C, Guillot D, Bargis P, Lavergne JP and Reboud JP (1997). A specific role for the phosphorylation of mammalian acidic ribosomal protein P2. *J. Biol. Chem.* 32: 20259-20262.
- Vidales FJ, Saenz Robles MT and Ballesta JPG (1984). Acidic proteins of the large ribosomal subunit in *Saccharomyces cerevisiae*. Effect of phosphorylation. *Biochemistry* 23: 390-396.
- Vidales FJ, Sanchez-Madrid F and Ballesta JPG (1981). The acidic proteins of eukaryotic ribosomes: a comparative study. *Biochim. Biophys. Acta* 656: 28-35.
- Wadman I, Li J, Bash RO, Forster A, Osada H, Rabbitts TH and Baer R (1994). Specific *in vivo* association between the bHLH and LIM proteins implicated in human T cell leukemia. *EMBO J.* 13: 4831-4839.
- Wang X, Lee G, Liebhaber SA and Cooke NE (1992). Human cysteine-rich protein. *J Biol. Chem.* 267: 9176-9184.
- Winteroe AK, Fredholm M and Davies W (1996). Evaluation and characterization of a porcine small intestine cDNA library. *Mamm. Genome* 7: 509-517.
- Wool IG, Chan YL, Gluck A and Suzuki K (1991). The primary structure of rat ribosomal proteins P0, P1 and P2 and a proposal for a uniform nomenclature for mammalian and yeast ribosomal proteins. *Biochimie* 73: 861-870.
- Wu C, Zimarino V, Tsai C, Walker B and Wilson S (1990). Transcriptional regulation of heat shock genes. In: Stress Proteins in Biology and Medicine. Morimoto, R.I., Tissieres, A. and Georgopoulos, C. Eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Yamashita M, Ojima N and Sakamoto T (1996a). Induction of proteins in response to cold acclimation of rainbow trout cells. *FEBS Lett.* 382: 261-264.
- Yamashita M, Ojima N and Sakamoto T (1996b). Molecular cloning and cold-inducible gene expression of ferritin H subunit isoforms in rainbow trout cells. *J. Biol. Chem.* 271: 26908-26913.
- Yet SF, Folta SC, Jain MK, Hsieh CM, Maemura K, Layne MD, Zhang D, Marria PB, Yoshizumi M, Chin MT, Perrella MA and Lee ME (1998). Molecular cloning, characterization, and promoter analysis of the mouse *Crp2/SmLim* Gene. *J Biol. Chem.* 273: 10530-10537.

- Yocum GD, Joplin, K.H. and Denlinger DH (1991). Expression of heat shock proteins in response to high and low temperature extremes in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 18: 239-249.
- Yost HJ, Peterson RB and Lindquist S (1990). Posttranscriptional regulation of heat shock protein synthesis in *Drosophila*. In: Stress Proteins in Biology and Medicine. Morimoto, R.I., Tissieres, A. and Georgopoulos, C. Eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Zachariassen KE (1991). The water relations of overwintering insects. In: Insects At Low Temperature Lee, R.E and Denlinger, D.L., Eds. Chapman and Hall, London and New York.
- Zelhof AC, Yao T-P, Evans RM and McKeown M (1995). Identification and characterization of a *Drosophila* nuclear receptor with the ability to inhibit the ecdysone response. *Proc. Natl. Acad. Sci. USA* 92: 10477-10481.
- Zelhof AC, Ghbeish N, Tsai C, Evans RM and McKeown M (1997). A role for Ultraspiracle, the *Drosophila* RXR, in morphogenetic furrow movement and photoreceptor cluster formation. *Development* 124: 2499-2506.

IMAGE EVALUATION TEST TARGET (QA-3)

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved