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ISOLATION OF DIAPAUSE-ASSOCIATED CLONES FROM THE FLESH FLY, 
*SARCOPHAga CRASSIPALPIS*

DISSERTATION

Presented in Partial Fulfillment of the requirements for the degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University
1998

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ABSTRACT

To further define diapause at the cellular level, an elimination hybridization protocol was used to isolate diapause-associated clones from a cDNA library constructed against diapausing pupal brains. From this primary screening 96 clones were labeled as putative diapause-specific clones, and these clones were then used as probes against diapause and nondiapause poly (A)+ RNA blots for diapause status verification. Three clones were identified as diapause-upregulated and seven clones were diapause-downregulated. Each diapause-upregulated and diapause-downregulated clone was partially sequenced to search for possible identity with other known genes, resulting in similarities to genes linked to cell cycle progression, stress response and DNA repair.

Flow cytometric analysis performed on brains from diapausing and nondiapausing flesh flies confirmed that a G0/G1 cell cycle arrest occurs throughout diapause. This arrest is broken at diapause termination when cells begin to proliferate leading to adult development.

One diapause-downregulated clone (pScD56) was found to have high identity (85%) to the *Drosophila melanogaster* cell cycle gene, Proliferating Cell Nuclear Antigen (PCNA) at the amino acid level. Northern blot and in situ hybridization using diapausing pupae and flesh flies that had broken diapause revealed that ScPCNA is expressed at very low levels during diapause, but at diapause termination increase rapidly. Northern blots were also performed using three other G1-associated cell cycle regulatory genes *cyclin E*, *p21* and *p53*. None of these showed differential expression, suggesting that PCNA alone may be regulating the proliferation arrest observed during diapause.
Heat shock and cold shock are known to inhibit cell cycle progression, so it was of interest to elucidate the possible role of *ScPCNA* following exposure to temperature extremes. Expression of *ScPCNA* decreased in response to cold shock (-10°C) but not heat shock (45°C), which is concordant with the current model proposing that the response strategy against high temperature stress is different from the strategy for cold temperature survival.

Finally, the expression of two heat shock transcripts (hsp23 and hsp 70) was tested in response to desiccation stress in the flesh fly. Although both hsps were highly upregulated following desiccation, their expression by desiccation pretreatment was found incapable of inducing cross tolerance against heat and cold stress.
To my parents, for all the encouragement and support they have given me throughout the years, and to my wonderful wife Jamie.
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CHAPTER I

INTRODUCTION

The majority of insects can utilize one of two strategies to overcome adverse environmental periods, migration and/or diapause. Migration represents the movement of insects away from adverse conditions, while insect diapause is an alternative pathway in which development is delayed in order to synchronize the active stages of the life cycle with available food sources.

Most univoltine insect species utilize a genetically predetermined diapause (obligatory) that occurs in every generation regardless of environmental conditions, while many multivoltine species undergo facultative diapause, requiring specific environmental cues to enter the diapause program. Although these two separate diapause programs exist, they share common characteristics that make diapausing individuals biochemically distinct from their nondiapausing counterparts. Characteristics of diapausing insects include, but are not limited to, decreased metabolism, developmental or reproductive arrest and increased storage of food supply prior to diapause entrance to span the inactive stage (Denlinger, 1985).

The flesh fly, Sarcophaga crassipalpis, exhibits a facultative diapause that is induced primarily by photoperiod, coupled with decreasing temperatures to enhance diapause entrance (Denlinger 1972; Denlinger, 1985). Four days of short photoperiod (less than 13.5 hours) are necessary during the late embryonic and early larval stages for the initiation of the diapause program in S. crassipalpis (Denlinger, 1971).
The delay from initiation to diapause entrance allows the developing larvae to prepare for the onset of the overwintering period.

Several experiments have linked the neuroendocrine system to a vital role in the initiation and storage of the diapause program. Exposure of the head and abdominal regions to different photoperiods results in the insect's developing to the daylength perceived by the head, suggesting that photoperiod of the head determines diapause programming in many insects (Geispitz 1957; Williams 1963). Further experimentation with crude fiber optics showed that the brain proper, not the eyes, is responsible for photoreception and therefore is the primary site used to measure daylength in insects (Lees, 1964). Transplantation of diapause-destined brain-ring gland complexes into nondiapause-programmed individuals resulted in about 30% entrance into diapause suggesting that a factor produced and stored in the brain carries the information needed for diapause initiation, and that this active factor can be transferred between insects (Giebultowicz and Denlinger, 1986).

The arrest in development during diapause may also be linked to the neuroendocrine system. Levels of 20-hydroxyecdysone (20E) appear normal in diapause-destined larvae, until diapause initiation when titers quickly drop, increasing only after diapause termination (Walker and Denlinger, 1980). Juvenile hormone (JH) titers cycle throughout diapause and appearing to drive the O\textsubscript{2} consumption and protein synthesis cycles (Denlinger et al., 1984; Slama and Denlinger, 1992; Joplin and Denlinger, 1989). Research efforts are underway in our lab to characterize the role(s) that the protein receptors of these two hormones might play in the maintenance and termination of flesh fly diapause (Flannagan et al., 1998).

Recent research has implicated diapause not only as an extended period of inactivity, but as a unique developmental stage in which a specific subset of proteins are being synthesized. A diapause specific protein has been isolated and characterized from the
hemolymph of the pink bollworm, *Pectinophora gossypiella* (Salama and Miller, 1992). This protein makes up 38.6% of the total hemolymph proteins in diapausiing bollworm larvae, and as with many other diapause-related proteins functions as a storage protein not as a regulatory protein. The cDNA corresponding to this protein has also been cloned and its expression characterized (Salama and Miller, 1993). Diapause-specific proteins have also been isolated from larvae of the southwestern comborer, *Diatraea grandiosella*, and the codling moth, *Cydia pomonella* (Brown and Chippendale, 1978; Brown, 1980).

Differential protein expression is also present between insects destined for diapause and their non diapause-destined counterparts. Dillwith et al. (1984), isolated and described a protein expressed exclusively in fat body from diapause-destined larvae of the southwestern comborer, *Diatraea grandiosella*. Pre-diapause proteins are thought to play a role in storage and transportation of lipids and carbohydrates (Miller and Silhacek, 1982).

A neuropeptide responsible for the induction of diapause has been isolated from the silkworm, *Bombyx mori* (Yamashita et al., 1985). Diapause hormone (DH) is a peptide secreted by the subesophageal ganglion of the mother, which acts on developing oocytes to initiate diapause in the embryo (Yamashita et al., 1985). This gene encodes a common polypeptide precursor from which itself, PBAN, and three other FXPRL amide peptides are released through post-transcriptional modification (Xu et al., 1995). Suwan et al. (1994), reported that DH activity is linked to two specific structural components: the presence of hydrophobic amino acids near the carboxy terminus and amidation of the C-terminus. DH represents the only known peptide that functions as a diapause-regulator, but it fails to induce diapause in any other insects species.

Using 2-Dimensional PAGE, Joplin et al. (1990) discovered a cluster of approximately 15 proteins which are specific to brains of diapausiing *S. crassipalpis*. These proteins are found throughout the diapause period and disappear only after diapause termination. The identity and function of these proteins were not tested, but the fact that
they appear to be diapause-specific is of vital importance, suggesting that diapause-specific genes must exist.

**EUKARYOTIC CELL CYCLE REGULATION**

Another extremely interesting area of diapause physiology is the molecular mechanism underlying the developmental arrest exhibited during flesh fly diapause. Is this arrest at the organismal level or are the cells themselves arrested, leading to an inhibition of proliferation?

The division of eukaryotic cells is regulated by a family of protein kinases known as cyclin-dependent kinases (cdks) (reviewed by Nasmyth, 1996). Included in this group are cdc2, cdk2, cdk4, cdk5, and cdk6. Each cyclin-dependent kinase functions at a specific cell cycle stage directing the cell to progress through the cell cycle in the correct order (Nurse, 1994).

Individual cdks must be regulated by a specific cyclin partner in order for successful kinase activity to be ensured. Complexes necessary for successful cell cycling include cdc2-cyclin B, cdk4-cyclin D, cdk2-cyclin A, and cdk2-cyclin E. Figure 1.1 demonstrates the general temporal gene expression and activity of the main cyclins and cdks throughout the cell cycle. Cyclins D and E are primarily involved in G1 to S transition, while cyclins A and B are involved in ordering the S and G2 stages, respectively.
Figure 1.1. Eukaryotic cell cycle progression
Progression from G1 to S is highly regulated by cell "checkpoints" due to the importance of fidelity preceding DNA replication. Cell cycle checkpoints were first discovered in response to DNA damage, following irradiation of human and rat cell lines. These checkpoints arrest the cell in G1, allowing the DNA to be repaired prior to replication. Induction of DNA damage by chemicals or irradiation causes inhibition of cyclin D-cdk4 and cyclin E-cdk2 kinase activities in mammalian cell lines, resulting in failure of retinoblastoma (Rb) phosphorylation by these cyclin-dependent kinases (Sherr, 1996). Hypophosphorylated Rb sequesters transcription factor E2F, which results in G1 arrest of the cell (Chellappan et al., 1991). E2F is the transcription factor that regulates expression of DNA polymerase I and other S phase regulatory enzymes. Hyperphosphorylated Rb will not bind to E2F, allowing free E2F to initiate transcription of DNA pol. I, subsequently leading to S phase progression.

Cyclins D and E have also been extensively studied at the structural and functional levels, and both are involved in the inactivation of the growth inhibitory action of Rb via phosphorylation by their cdk partners. Although both play roles in G1-S regulation, the activation of S phase during embryogenesis in Drosophila melanogaster is linked to the expression of cyclin E and not cyclin D (Knobloch et al., 1994). Ectopic expression of cyclin E during a G1 arrest following the exit of the cell cycle in D. melanogaster embryogenesis results in progression of the cells into the S phase of the cell cycle (Richardson et al., 1995).

Another important protein in the cell cycle pathway is the proliferating cell nuclear antigen (PCNA). This protein is an essential replication factor for simian virus 40 (SV40) DNA replication in vitro and is involved in the elongation stages of DNA replication (Prelich et al., 1987), as well as in cellular chromosomal DNA replication in vivo (Jaskulski et al., 1988; Waseem et al., 1992). More specifically, PCNA seems to be a processivity factor of DNA polymerase δ, an enzyme necessary for DNA replication.
Fukuda et al. (1995) proposed the model that PCNA forms a homotrimer that acts as a molecular clamp during DNA synthesis, interacting with the DNA strand and DNA pol. In addition to its essential role in replication, PCNA is required for nucleotide excision repair of DNA (Zeng et al., 1994) and also may participate in cell cycle control as demonstrated by an interaction with a cdk-cyclin complex (Xiong et al., 1993). Therefore, PCNA is multifunctional through interaction with several specific partners, and all of the functions seem crucial for cell proliferation.

Recent research efforts have been successful in isolating and characterizing many cell cycle inhibitors (Elledge, 1996). One such inhibitor is the tumor suppressor p53 that is expressed in response to DNA damage by chemical mutagens or irradiation. While the role of p53 as a direct cdk inhibitor has not yet been established, it is an upstream regulator of waf1/cip1 (p21), a gene that encodes a protein which directly inhibits cdk-cyclin kinase activity (El Diery et al., 1993; Harper et al., 1993).

In vitro studies suggest p21 is a universal cyclin/cdk complex inhibitor, but in vivo p21 seems to function primarily as a G1 complex inhibitor (Xiong et al., 1993). p21 was originally thought to be expressed only in response to DNA damage or apoptosis, under the direct control of p53. Subsequent studies have shown that p21 can be expressed independently of p53 in cells undergoing senescence, quiescence, and differentiation (Dulic et al., 1993; El-Diery et al., 1993; Steinman et al., 1994).

Recently, p53- and p21-like molecules have been found in cell lines from the silkworm, Spodoptera frugiperda and the fruit fly, D. melanogaster (Bae et al., 1995) in response to DNA-damaging agents. This is the first evidence for the existence of these cell cycle inhibitors in invertebrates; however attempts to clone these genes from invertebrates have been unsuccessful.
THE MOLECULAR REGULATION OF FLESH FLY DIAPAUSE

Although the fruit fly *Drosophila melanogaster* has been characterized extensively at the molecular level, it does not possess a diapause at the organismal level, merely an ovarian diapause that is difficult to induce, maintain or even recognize in the laboratory. Therefore, we feel that the flesh fly is an ideal model for the molecular characterization of insect dormancy due to its recognizable pupal diapause that is easily induced in a laboratory setting.

My research focuses on the molecular mechanism of diapause in the flesh fly, *Sarcophaga crassipalpis*, primarily on the cell cycle regulators associated with the developmental arrest observed throughout diapause. The goals of my research are:

1) To isolate diapause-upregulated and diapause-specific clones and begin the initial characterization of each clone by sequencing and expression studies.
2) To isolate diapause-downregulated clones and begin the initial characterization of each clone by sequencing and expression studies.
3) To determine if the developmental arrest seen during flesh fly diapause is the result of an underlying cell cycle arrest.
4) To determine the role(s) played by known cell cycle regulators during the diapause program in the flesh fly.
5) To evaluate the role(s) of any diapause-upregulated and diapause-downregulated genes during other environmental stresses.

REFERENCES


CHAPTER 2

DIAPAUSE-SPECIFIC GENE EXPRESSION IN PUPAE OF THE FLESH FLY.

SARCOPHAGA CRASSIPALPIS

ABSTRACT

Several cDNAs isolated from brains of diapausing pupae of the flesh fly, Sarcophaga crassipalpis, show expression patterns unique to diapause. To isolate such cDNAs a diapause pupal brain cDNA library was screened using an elimination hybridization technique, and cDNAs that did not hybridize with cDNA probes constructed from the RNA of nondiapausing pupae were selected for further screening. The 95 clones that did not hybridize in the initial library screen were selected for further characterization. These clones were then screened against diapause and nondiapause pupal poly(A)+ northern blots. The secondary screen identified 4 diapause-upregulated clones, 7 diapause-downregulated clones, 8 clones expressed equally in both diapause and nondiapause, and 75 clones without detectable expression. The diapause-upregulated and downregulated clones were further characterized by partial DNA sequencing and identity searches using Genbank. Identities between our cloned cDNAs and other genes included those linked to cell cycle progression, stress responses, and DNA repair processes. The results suggest that insect diapause is not merely a shutdown of gene expression but is a unique developmental pathway characterized by the expression of a novel set of genes.
INTRODUCTION

Most insect species have evolved a period of developmental arrest (diapause) that enables them to circumvent seasonal periods of adversity. For insects in the temperate zone, winter is the season most consistently avoided. It is not at all uncommon for a species to spend 9-10 months in diapause, with only 2-3 months of summer devoted to active development and reproduction (Saunders, 1980; Tauber et al., 1986). Within a single species the potential for diapause is usually restricted to a specific developmental stage, but embryonic, larval, pupal and adult diapauses are all well documented. Whether the insect will enter diapause is usually dictated by the daylength perceived at an earlier stage of development. Long daylengths frequently channel the insect toward uninterrupted development during late spring and early summer, while the short daylengths of late summer and autumn program the entry into diapause. Daylength thus presides over the hormonal mechanisms that direct the insect toward either diapause or nondiapause development (Denlinger, 1985).

Like hibernating mammals, insects in diapause are totally dependent on the energy reserves that have been sequestered during earlier active life cycle phases. Suppression of metabolism enables the insect to stretch its food reserves to bridge the unfavorable period. Survival during diapause may also be enhanced by the synthesis of polyols and other cryoprotective agents that reduce injury at low temperature. Thus, the diapause and nondiapause phases of the insect’s life cycle represent striking contrasts. What these differences may mean at the molecular level remains largely unknown. Whether diapause is simply a shutdown in gene expression or whether it represent a unique pattern of gene expression has long been debated. We addressed this question in the flesh fly, Sarcophaga crassipalpis, by searching for differences in gene expression in the brains of diapausing and nondiapausing pupae.
Our search focused on the brain because it is this tissue that is responsible both for receiving the environmental cues involved in inducing diapause as well as for executing the diapause program (Denlinger, 1985; Giebultowicz and Denlinger, 1986). The flesh flies used in this study enter an overwintering pupal diapause in response to cues of short daylength received during late embryonic and early larval life (Saunders, 1971; Denlinger, 1971; Gnagey and Denlinger, 1984). Hormonally, this diapause can be characterized as an ecdysteroid deficiency; the brain fails to stimulate the prothoracic gland to synthesize ecdysteroids and hence development is halted until the synthesis of ecdysteroids is again invoked at the end of diapause (Fraenkel and Hsiao, 1968; Zdarek and Denlinger, 1974; Walker and Denlinger, 1980). An examination of brain proteins synthesized during diapause (Joplin et al., 1980) demonstrated that diapause represents both a partial shutdown in gene expression (far fewer proteins were expressed in brains of diapausing pupae than in brains of nondiapausing pupae) and the expression of a unique set of genes (a cluster of 14 proteins was expressed only in brains of diapausing pupae). In this study, we isolated diapause-specific genes and evaluated the abundance of such genes among the total pool of mRNA expressed in the brain during diapause.

MATERIALS AND METHODS

Colony Maintenance. The colony of S. crassipalpis was maintained as described (Denlinger, 1972). The decision to enter pupal diapause is made during a 4-day window in late embryonic and early larval development (Denlinger, 1971). To produce nondiapause pupae, adults were maintained at 15L:9D (light:dark cycle) and 25°C. After larviposition, the larvae were reared at the same conditions as the adults for the first 4 days and were then transferred to 12L:12D, 20°C for the remainder of development. To produce diapause pupae, adults were maintained at 12L:12D, 25°C. After larviposition, the larvae were transferred immediately to 12L:12D, 20°C.
**RNA Purification.** Pupal brains were homogenized in TRIzol® reagent (Gibco BRL) and stored at -70°C. RNA was purified using the standard TRIzol® protocol (Gibco BRL). The total brain RNA was resuspended in DEPC-treated water. Total body RNA was dissolved in binding buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.3 M NaCl, 0.1% (w/v) SDS] and passed through a column of oligo dT cellulose. The column was rinsed twice with binding buffer and then eluted with elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS). The poly(A)+ RNA was quantitated, precipitated, and resuspended in formamide at 2μg/μl.

**Library Construction and Isolation of cDNAs.** The overall strategy for the isolation of diapause specific genes from the diapause cDNA library is shown in Figure 2.1. The commercial library was prepared by isolating poly(A)+ RNA from 100 diapausing pupal brains using an oligo dT-primer for first strand cDNA synthesis (Clontech). A titer of 1.8 x 10⁹ pfu/ml was obtained with 1.75 x 10⁹ (97%) being independent recombinant clones as determined by X-gal screening. The primary library screening was performed by making a complex mixture of probes from total brain RNA of nondiapause-destined pupae. Probes were constructed by performing first strand synthesis from total RNA using oligo (dT) primers and a mixture of biotin labeled dNTPs (Strategene). Library screening was performed according to the manufacturer’s instructions (Clontech). Phage that did not show hybridization were then picked as putative diapause specific clones. The phage were subjected to an in vivo excision process that resulted in the cloning of the cDNA.

**Northern Blotting.** Equivalent amounts of poly(A)+ RNA from diapausing and nondiapausuing whole pupae were loaded on a 1.2% formaldehyde (0.41M) denaturing gel and transferred to a nylon membrane via downward capillary action (Schleicher and Schuell). Prehybridization and hybridization were carried out in a 1X hybridization buffer [0.5 M NaCl, 0.1 M NaPO₄ (pH 7.0), 6 mM EDTA, 1% (w/v) SDS] at 65°C. cDNA probes were constructed using the Rad Prime system (Gibco BRL) in the presence of ³²P-
dCTP (3000 Ci/m mole). Washes consisted of a single wash in 1/4X hybridization buffer at 65°C and 2 washes in 1/4X hybridization buffer without SDS at 65°C. The membranes were then wrapped in Saran® wrap and exposed to Kodak XAR5 film at -70°C.

**DNA Sequencing.** DNA sequencing was performed on all diapause-upregulated and down-regulated genes, as well as those showing equal signal in both. All DNA sequencing was performed at the University of Georgia on an ABI 373A DNA sequencer using dye terminator chemistry according to the manufacturer's standard protocol. The BLAST® search program was used to search the Genbank sequence repository for sequence identities.

**RESULTS**

**Identification and isolation of putative diapause specific cDNAs.** Initial screening of 450 plaques yielded 355 (79%) that hybridized strongly to nondiapause pupal brain cDNAs and 95 (21%) that failed to hybridize to the RNA blots. These 95 clones were picked as putative diapause specific clones and each was given a number as, plasmid *Sarcophaga crassipalpis* diapause (pScD) 1 to pScD95.

**Secondary screen using northern blotting.** Northern blotting of total pupal poly(A)+ RNA from diapause and nondiapause pupae was used to determine if any of the 95 clones were exclusively associated with diapause. The clones were then grouped according to four hybridization patterns: 1) upregulation in diapause 2) downregulation in diapause 3) hybridization equally in diapause and nondiapause 4) no hybridization to diapause or nondiapause. Four clones (4.2%), pScD14, pScD41, pScD47 and pScD86, were diapause-upregulated (Figure 2.2B). In each of these cases, the clones were upregulated, but some hybridization was also seen to the nondiapause RNA. With a longer exposure, even pScD41 showed a weak signal in the nondiapause lane. Seven clones (7.4%) pScD9A, pScD9B, pScD24, pScD26, pScD50, pScD56 and pScD74 produced a
weaker signal to diapause RNA than to nondiapause RNA and were classified as diapause-downregulated (Figure 2.2C). Eight clones (8.4%) pScD20, pScD21, pScD22, pScD45A, pScD45B, pScD73, pScD85 and pScD93 produced a signal of equal intensity in both the diapause and nondiapause lanes. pScD85 shown in Figure 2.2A is a representative of this group. The final group consisted of the 75 pScD clones (80.0%) that did not produce a signal in either the diapause or nondiapause lanes (data not shown). Transcript sizes of the pScD clones mentioned are listed in Table 1.

Sequence identity of cDNAs. Partial sequence data were obtained for all of the diapause-upregulated and diapause-downregulated clones, and percent identity to sequences deposited in Genbank are listed in Table 1. Diapause upregulated clones pScD14 and pScD86 showed high identity to a small heat shock protein (Ingolia and Craig, 1982) and an apurinic-apyrimidinic endonuclease (Kelley et al., 1989), respectively. The other two diapause-upregulated clones showed no significant identity to any known sequence.

The diapause-downregulated clone, pScD56, has high level of identity to Proliferating Cell Nuclear Antigen (PCNA) (Ng et al., 1990). pScD9A and pScD9B appear to be the same clone; however, pScD9A contains a 70-nucleotide stretch that is not present in pScD9B. Both of the pScD9 sequences have high identity to an elastin-like protein (Li et al., 1995). The similarity of the two clones raises the possibility that transcripts are alternatively spliced. The remaining diapause-downregulated clones showed no identity to known sequences.

We also have partially sequenced pScD20, pScD21, and pScD85, all of which hybridized equally to diapause and nondiapause RNA. pScD20 and pScD21 have high identity to an apurinic-apyrimidinic endonuclease (Kelley et al., 1989), while pScD85 has high identity to the heat shock 70 cognate (Perkins et al., 1990).
Construction of cDNA library from the brains of diapausing pupae

Primary screening of cDNA library with probes from the brains of nondiapausing pupae

Removal of negative clones

Selection of negative plaques that do not show common genes

Plasmid rescue of inserts from negative clones

Secondary screen of negative clones using poly(A)+ Northern blots

Initial characterization of diapause-specific and diapause upregulated clones

FIGURE 2.1. Schematic flow chart for isolation of diapause-associated clones.
FIGURE 2.2. Hybridization of cDNA probes to Northern blots.

Four micrograms of diapause (D) and nondiapause (ND) pupal poly(A)+ RNA were hybridized with $^{32}$P-labeled probes from the indicated clones. (A) pScD85 is a representative of clones that hybridized equally to diapause and nondiapause pupal RNA; (B) pScD14, pScD41, pScD47 and pScD86 clones are diapause-upregulated; (C) pScD9A, pScD9B, pScD24, pScD26, pScD50, pScD56 and pScD74 clones are diapause-downregulated. Ethidium bromide staining was used to ensure equivalent loading of RNA.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Transcript Size (kb)</th>
<th>Protein</th>
<th>% Identity</th>
</tr>
</thead>
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<tr>
<td><strong>Diapause upregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pScD14</td>
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<td>small heat shock protein 23</td>
<td>85</td>
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<tr>
<td>pScD86</td>
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<td>apurinic-apyrimidinic endonuclease</td>
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<tr>
<td><strong>Diapause downregulated</strong></td>
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<td><strong>Expressed equally in diapause and nondiapause</strong></td>
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TABLE 2.1. Sequence identities and transcript sizes of diapause-upregulated clones, diapause-downregulated clones and clones that are expressed equally in diapause and nondiapause individuals.
DISCUSSION

The evidence we present here, combined with previous work on brain proteins (Joplin et al., 1990), suggests that diapause should be regarded as a unique developmental pathway rather than a simple shutdown of gene expression. Although certain genes are clearly downregulated during diapause, others are strongly upregulated. From the sample of 95 clones initially isolated, 4.2% proved to be diapause-upregulated. This percentage is remarkably similar to the incidence of diapause-specific brain proteins (9%) previously reported for *S. crassipalpis* based on two-dimensional electrophoresis of $^{35}$S-pulse labeled proteins (Joplin et al., 1990). A similar estimate of diapause-specific gene activity in this species was obtained by differential display of mRNA (Joplin and Denlinger, unpublished observations): a sample of 332 well-defined transcripts from diapause brains included 35 (10.5%) that appeared to be diapause-specific. The fact that this technique generates many false positives suggests that the 10.5% incidence is likely to be a high estimate. Yet, all three techniques (library screening and northern analysis, two-dimensional electrophoresis of brain proteins, differential display) indicate the presence of a discrete set of genes that are expressed in association with insect diapause. Our estimate suggests that diapause-specific and/or diapause-upregulated genes represent 4-10% of the genes being expressed during diapause in the pupal brains of the flesh fly.

Several previous studies have documented the presence of diapause associated proteins from the fat body and hemolymph of diapausing insects (Brown and Chippendale, 1978; Brown, 1980; Perferoen et al., 1982; Salama and Miller, 1992). These have proven to be storage proteins that are synthesized prior to the onset of diapause and are then utilized when development resumes at the termination of diapause (Levenbrook, 1985).
Also, a unique midgut protein is synthesized during diapause in the gypsy moth, *Lymantria dispar* (Lee and Denlinger, 1996). Although all such proteins are potentially important for maintenance of diapause, their site of synthesis, the fat body or midgut, suggests they represent events that are downstream from the site of diapause regulation. The brain, as the site of diapause regulation, is the most likely site to seek regulatory genes, yet we can assume that many diapause-specific genes expressed in the brain will also prove to be involved in coordinating downstream events and maintenance of diapause.

Only two of the four diapause upregulated clones we have isolated show high identity to known genes. pScD14, the clone that shows high identity (%) to a *D. melanogaster* small heat shock protein, could be involved in the cell cycle arrest that occurs during diapause (Tammariello and Denlinger, 1998). Yeast and human β lymphocytes express certain small heat shock proteins during cell cycle arrest (Kurtz et al., 1986; Rossi and Lindquist, 1989; Spector et al., 1992). Cold shock also elicits the expression of small heat shock proteins in *S. crassipalpis* (Joplin et al., 1990) and in the gypsy moth *L. dispar* (Yocum et al., 1991). At the onset of diapause in *S. crassipalpis*, cold tolerance increases dramatically (Lee and Denlinger, 1985), a response that is consistent with the expression of the pScD14 transcript we observed in this study. Subsequently, full-length cloning and sequencing of pScD14 have been accomplished (GenBank accession number U96099) and this gene is consistently upregulated during the entire course of diapause. pScD86, the second diapause-upregulated clone with a high identity with a known gene, appears to be an apurinic-apyrimidinic endonuclease. This enzyme is critical to DNA repair processes and acts at apurinic-apyrimidinic sites on DNA to initiate nucleotide excision repair (Kelley, 1989). Possibly such repair is critical for an insect that remains exposed to harsh environmental conditions for such a prolonged duration.

The identity of diapause-downregulated clones is of equal importance. The identity of pScD56 as the proliferating cell nuclear antigen (PCNA) is of particular interest due to
the role of PCNA in regulating development and cell cycle status (Fukuda et al., 1995). During diapause, the cells of the flesh fly brain are in a G0/G1 cell cycle arrest (Tammariello and Denlinger, 1998). Full-length cloning and sequencing of PCNA have been accomplished (GenBank accession number AF020427) and its expression during diapause is currently under investigation. The down regulation of PCNA during diapause and the rapid onset of its expression at diapause termination, when the cells again begin to cycle, suggest a potential role for PCNA in regulating the cell cycle arrest associated with diapause.

The physiological roles and identities for most of the upregulated and downregulated clones remain to be defined, and many additional clones of interest are yet to be isolated and characterized. Transcripts from many clones within the brain are likely to be expressed at very low levels that would not have been detected with our procedures. Of the 95 clones isolated, 75 did not detect homologous RNA sequences in either diapause or nondiapause individuals when used as probes.

The isolation of diapause regulated clones should enable us to assess the features of diapause regulation in flesh flies that are shared with other organisms with a diapause stage of their life cycles. Diapause is widespread in insects and, depending on the species, may occur in embryonic stages, larvae, pupae or adults. Diapause-like states are also common in other arthropods and in a wide range of invertebrates. Perhaps the best known diapause-like state in other invertebrates is the dauer larval stage of the nematode, *Caenorhabditis elegans*, a non-feeding, non-growing larval stage that is initiated in response to starvation and crowding. The use of mutants of the dauer formation (*daf*) genes has allowed investigators to order these genes in a developmental hierarchy (Georgi et al., 1990; Estevez et al., 1993). Of special importance is *daf-2*, a member of the insulin receptor family, that dramatically extends longevity (Wood and Johnson, 1994; Kimura et al., 1997). From the work on *C. elegans* it is apparent that the dauer state represents a unique
developmental pathway (Riddle et al. 1981; Gottlieb and Ruvkun, 1994), a conclusion that now also appears to be appropriate for insect diapause. It is not yet clear whether the diverse manifestations of diapause and related forms of dormancy share a common regulatory basis or have attained a similar developmental stasis by alternative regulatory mechanisms.

REFERENCES


CHAPTER 3

G0/G1 CELL CYCLE ARREST IN THE BRAIN OF SARCOPHAGA CRASSIPALPIS DURING PUPAL DIAPAUSE AND THE EXPRESSION PATTERN OF THE CELL CYCLE REGULATOR, PROLIFERATING CELL NUCLEAR ANTIGEN

ABSTRACT

During pupal diapause in the flesh fly, Sarcophaga crassipalpis, the cells of the brain are arrested in the G0/G1 phase of the cell cycle. When diapause is terminated with a topical application of hexane, cell cycling is evident within 12 hours. Four G1 and S phase regulatory genes were examined by Northern blot analysis to evaluate their expression patterns in relation to this cell cycle arrest. A distinction between diapausing and nondiapausing individuals was noted only for Proliferating Cell Nuclear Antigen (PCNA). PCNA was highly expressed after diapause was terminated but not during diapause. In contrast, cyclin E, p21, and p53 were expressed equally at all times. In situ hybridization using PCNA probes further indicated a correlation between PCNA transcription (expression) in the brain and cell cycling. Our evidence thus suggests a potential role for PCNA as an important regulator of cell cycle arrest during diapause.
INTRODUCTION

Insect diapause poses an interesting situation for probing cell cycling and cell cycle arrest. At the level of the whole organism progressive development is halted, but is this halt also evident at the cellular level in the form of a cell cycle arrest? If so, in what phase of the cell cycle does the arrest occur, and is the cell cycle arrested at the same point in different species of diapausing insects? These questions have received little attention, although experiments with diapausing embryos of the silkmoth, *Bombyx mori*, suggest a G2 cell cycle arrest in that species (Nakagaki et al., 1991).

In *Drosophila melanogaster*, the G1/S phase transition of the cell cycle is regulated by the expression of at least four genes: cyclin E, p21, p53, and proliferating cell nuclear antigen (PCNA). Low expression of cyclin E and/or PCNA causes cell cycle arrest during early development in *D. melanogaster*, while high expression of p21-like and p53-like proteins is associated with DNA damaging conditions such as exposure to irradiation, prior to the arrest necessary for DNA repair (Bae et al., 1995, Richardson et al., 1995).

Here we describe a G0/G1 cell cycle arrest that occurs in brain cells from the flesh fly, *Sarcophaga crassipalpis*, during pupal diapause. We also investigate the expression of G1/S phase regulatory genes during diapause to define the possible molecular mechanisms controlling the developmental stasis. We focus on the proliferation enhancers, *cyclin E* and *PCNA*, as well as cell cycle inhibitor genes, *p21* and *p53*.

MATERIALS AND METHODS

Insects

Nondiapausing flesh flies, *Sarcophaga crassipalpis* Macquart, were reared throughout development at 15L:9D (long daylengths) and 25°C. Induction of pupal diapause was accomplished by exposing adult flies to short day conditions (12L:12D) and 25°C until larviposition (Denlinger, 1972). Thereafter, larvae and pupae were maintained
in an environmental chamber at 12L:12D and 20°C. Diapause was terminated by directly applying 5 μl of hexane to the heads of the pupae, a technique that prompts immediate development (Denlinger et al., 1980).

Flow cytometric analysis

To establish distribution of nuclear DNA content, 20 brains each from prediapausing, third instar (wandering) larvae, 10 day diapausing pupae, 20 day diapausing pupae, and three day postdiapausing pharate adults were isolated in cold fly saline (Ephrussi and Beadle, 1936) under a dissection microscope. The brains were forced through a 100μl syringe, which was covered with fine cheesecloth, to produce a single cell suspension. The cells were ruptured by the addition of 0.1% Triton X-100 detergent. Nuclear fractions were stained with propidium iodide and allowed to incubate for three hours at 25°C. The nuclear fractions were then subjected to flow cytometric analysis at the Ohio State University Comprehensive Cancer Center using the Coulter EPICS Elite model cytometer (Hialeah, FL) equipped with a 488nm air-cooled argon laser. The percentage of nuclei in each phase of the cell cycle was determined from total counts of at least 15,000 gated nuclei per brain. Cells were gated on size (forward scatter) versus granularity (side scatter), as well as on DNA area vs. DNA peak signal using Multicycle (Phoenix Software, San Diego, CA) for DNA cell cycle analysis. DNA contents were standardized using haploid sperm from S. crassipalpis to ensure ploidy of cells and cell phase demarcation (data not shown).

RNA isolation and Northern blot analysis

Total RNA was extracted by homogenization of whole pupae in TRIzol® reagent using the standard protocol (GibcoBRL). RNA samples were taken from nondiapausing pupae, 20-day old diapausing pupae, and from individuals 12, 24, 36, 48, and 72 hours
after diapause termination. As described above, diapause was terminated by topical application of 5μl hexane to the pupal heads. Poly (A)+ RNA was isolated from each total RNA sample using the Oligotex mRNA midi® kit (Qiagen). Approximately 4μg of each poly (A)+ sample was transferred to membrane (Micron Separations, Inc.). The blots were screened using 32P-CTP labeled DNA probes produced from four full length G1/S phase transition clones, PCNA, cyclin E, p21, and p53, using the RTS RadPrime DNA labeling system (GibcoBRL). PCNA was isolated from S. crassipalpis (Flannagan et al., 1997), while we used a D. melanogaster cyclin E probe (kindly provided by Dr. Helena Richardson, University of Adelaide), and human probes for both p53 and p21 (kindly provided by Dr. Bert Vogelstein, Johns Hopkins University). All blots were prehybridized at 65°C for three hours in standard hybridization buffer (0.5M NaCl, 0.1M NaPO₄, 6mM EDTA) + 1% SDS. Hybridization was performed overnight in the same buffer, along with the probe, at 65°C. The blots were washed using one-quarter strength hybridization solution with SDS for 45 minutes at 65°C, followed by a wash using one-quarter hybridization solution without SDS for 1 hour. Finally, blots were wrapped in Saran® wrap and exposed to x-ray film (Fuji) at -70°C.

In situ hybridization

In situ hybridizations were performed on brains from diapausing and 24 hour post hexane-treated (postdiapausing) flesh flies using digoxigenin-labeled, single-stranded RNA probes made to ScPCNA. All brains were fixed in 4% formaldehyde and cell membranes were perforated by immersion in 0.1% Tween-20. Bound probe was visualized with a colorimetric alkaline phosphatase reaction as standard procedure (O’Neill and Bier, 1994). Brains were dehydrated and mounted in Aquapolymount (Polysciences Inc.) for photography.
RESULTS

Flow cytometric analysis

Flow cytometric analysis performed on brain cells from prediapausing, wandering larvae showed a population of cycling cells (Fig. 3.1a, 3.2). Each stage of the cell cycle was represented by at least 10% of the total population. But, the status of the cells changed drastically upon entry into diapause. While the G0/G1 fraction represented 50% of the cell population in the brain prior to diapause, the proportion increased to 95% in the brains of diapausing pupae 10 days after pupariation (Fig. 3.1b, 3.2). Concomitantly, the S-phase fraction decreased to <2%, compared to 20% in wandering larvae. This 10 fold decrease in the S-phase fraction between prediapause larvae and 10 day diapausing pupae implies that very few cells are entering the S-phase of the cell cycle, suggesting a population of arrested cells. Results from 20 day diapausing pupal brains also indicate a G0/G1 arrest (Fig. 3.1c, 3.2); by this time the G0/G1 fraction increased to >97%, and the S-phase fraction dropped to 1% of the population. Two days after diapause was terminated by hexane, a small population of cells began to break out of the G0/G1 arrest (Fig. 3.2), as indicated by a decrease in the fraction of G0/G1 cells to less than 80%, coupled with an increase in the S-phase fraction to 12%. Three days after hexane application an even larger population of brain cells began to cycle (Fig 3.1d, 3.2); by this time <75% of the cell population was in G0/G1 phase and over 15% comprised the S-phase fraction.

To further verify that cells were entering S-phase, brains from diapausing pupae and individuals that broke diapause were labeled with bromodeoxyuridine (Gratzner et al., 1983). A very faint signal was observed in brains from diapausing flies but a strong signal was visualized in brains from flies that had broken diapause, suggesting that cells in the brain were progressing into S-phase (data not shown).
Northern blot analysis

The levels of *ScPCNA*, *cyclin E-like*, *p21-like*, and *p53-like* transcript expression from whole body preparations were determined by Northern blot analyses (Fig 3.3). No discernible differences between diapause and nondiapause RNA were detected by probing with *Dm cyclin E*, *hp21*, or *hp53*. However, *ScPCNA* expression varied between diapause and nondiapause mRNA. Low expression levels were present in mRNA from diapausing flies, while high expression was observed in nondiapause mRNA.

To further characterize expression, *ScPCNA* transcript levels were detected in the flies at multiple time points from diapause (0 hr) to 72 hours postdiapause. Very low levels of expression were seen in poly (A)+ RNA extracted from diapausing pupae, but within 12 hours after diapause termination, a dramatic increase of *ScPCNA* expression level occurred, with expression peaking 48-72 hours after hexane application (Fig. 3.4).

In situ hybridization

Pupal brains were hybridized with a single-stranded RNA probe produced from the entire 1.5 kb reading frame of *ScPCNA*. In brains from diapausing pupae, *ScPCNA* transcript was expressed in only a few individual cells (Fig. 3.5a), in a small section of the distal region of the brain. However, extensive *ScPCNA* expression was evident in brains from postdiapausing (24 hours after hexane application) flies (Fig. 3.5b,c). Unlike the expression pattern observed in diapause brains, *ScPCNA* transcript was not restricted to cells in the distal areas of the brain. Three distinct regions of *ScPCNA* expression were observed: the developing eye, a portion of the eye lobes, and the central region containing the protocerebrum. At the distal region of the eye lobe, expression was limited to a crescent-shaped rim of cells. The crescent is evident in Fig. 3.5c, and can also be seen faintly on the opposite surface of the brain shown in Fig. 3.5b. By their size and location, these appear to be surface glial cells, possibly affiliated with the developing optic sheath. If
so, these will likely form the connection between the adult ommatidia and the eye lobe of the brain (Strausfeld, 1976).
FIGURE 3.1. Flow cytometric analyses from brains of prediapause, diapause and postdiapause flesh flies.

Representative flow cytometric diagrams showing percentage of cells in G0/G1, S, and G2/M from (A) prediapausing (third instar) larvae, (B) 10 day diapausing pupae, (C) 20 day diapausing pupae, (D) and postdiapause pharate adults three days after diapause was terminated by application of hexane. All were measured according to nuclear DNA content using a Coulter elite flow cytometer. Results shown are based on at least 15,000 gated nuclei per brain for each developmental stage.
FIGURE 3.2. Flow cytometric analyses conclusion graph.

Percentage of cells in G0/G1 (♦), S (□), and G2/M (■) phases of the cell cycle before, during, and after diapause, calculated from the data in Fig. 1.
FIGURE 3.3. Northern analysis of whole body diapause (D) and nondiapause (ND) pupal mRNA probed with $^{32}$P-labelled *S. crassipalpis* PCNA, *D. melanogaster* cyclin E, human p21, and human p53 (left to right).

All northerns were carried out using 4 µg of diapause and nondiapause poly (A)+ RNA and were exposed to x-ray film at -70°C for 24 hours.
FIGURE 3.4. ScPCNA expression at diapause termination.

Expression of ScPCNA mRNA during diapause and following diapause termination. $^{32}$P-labelled ScPCNA was hybridized to a northern blot of whole body poly (A)$^+$ RNA. RNA was isolated from diapausing pupae (0) and 12, 24, 48, and 72 hours following diapause termination by hexane application. The same blot was also hybridized with a $^{32}$P-labelled probe generated to a control gene, $S$. crassipalpis heat shock 70 cognate (hsc70), which is expressed at constant levels in all samples. Blots were exposed to x-ray film at -70°C for 24 hours.
FIGURE 3.5. Localization of ScPCNA transcript in brains of diapausers and broken diapausers by in situ hybridization.

Expression of ScPCNA transcripts in brain tissue from diapausing pupae and pharate adults 24-hour postdiapause. Brains were hybridized with a digoxigenin-labelled ScPCNA RNA probe. A. Anterior view of brain from diapausing pupa exhibits weak expression in the eye lobe and no expression in the protocerebrum. B. Anterior view of postdiapausing brain, 24 hours after hexane treatment. Expression of ScPCNA is localized in the developing eye, the distal region of the eye lobe, and the protocerebrum (arrows, from left to right). C. Posterior view of postdiapausing S. crassipalpis brain, 24 hours after hexane treatment, exhibits high expression in three regions, the developing eye, the eye lobe (in a characteristic crescent), and in the protocerebrum (arrows, from left to right). Distance from the midpoint of the brain to the periphery of the eye lobe is approximately 1.5 mm.
FIGURE 3.5. Localization of ScPCNA transcript in brains of diapausers and broken diapausers by in situ hybridization.
DISCUSSION

A G0/G1 cell cycle arrest is evident in brain cells of the flesh fly, *S. crassipalpis*, within 10 days after the onset of diapause. At this time, only a small percentage of cells are in the S or G2/M phases of the cell cycle. Twenty days after the onset of diapause, an even greater disparity can be observed between the percentage of cells in G0/G1 as compared to those in S or G2/M. Once diapause is terminated, a noticeable population of cells breaks the cell cycle arrest and begins proliferation. This data suggests that a cell cycle arrest is a critical aspect of the initiation and maintenance of flesh fly diapause. A diapause cell cycle arrest has also been reported in the silkworm, *Bombyx mori* (Nakagaki et al., 1991), where cells from whole embryos are arrested during diapause in the G2 phase of the cell cycle. This result differs from ours, perhaps because diapause in the silkworm occurs during an early embryonic stage of development while diapause in flesh flies occurs in the pupal stage. Throughout embryonic development, *D. melanogaster* fails to exhibit a G1 phase of the cell cycle, progressing directly from the S phase to the G2 phase (Edgar and Lehner, 1996). If this is also true for embryonic development in Lepidoptera, it may explain why a G2 cell cycle arrest is observed during the embryonic diapause of *B. mori*, but not in the pupal diapause of *S. crassipalpis*.

Though cells can be arrested at several points throughout the cell cycle, the majority of research has focused on arrests at the G1/S and G2/M transitions (Pardee, 1989, Elledge, 1996). In *D. melanogaster*, as well as other eukaryotic organisms, the G1/S phase transition has been studied extensively at the molecular level. Several gene products that regulate this transition have been isolated and characterized (Edgar and Lehrer, 1996).

Since a G0/G1 arrest was observed in diapausing flesh fly brains, expression levels of G1 and G1/S phase transition cell cycle regulation genes were tested using Northern blot hybridization. Two genes necessary for cell proliferation (*PCNA* and *cyclin E*) and two cell cycle inhibitor genes (*p53* and *p21*) were used in the study.
Expression levels of \( p53 \)-like and \( p21 \)-like transcripts were equivalent during diapause and nondiapause development. This suggests that neither plays an important role in the regulation of diapause in \( S. \) crassipalpis. The gene products of \( p53 \) and \( p21 \) arrest the cell cycle by direct inhibition of cyclin/cyclin dependent kinase function following DNA damage (El-Diery et al., 1993, Harper et al., 1993, Levine et al., 1994). Expression of these proteins overrides any other cell cycle progression pathway, resulting in an immediate arrest of the cell population.

Cyclin \( E \)-like transcripts were also detected in both diapause and non-diapause flesh flies, with no appreciable increase in expression visible in either lane. This result suggests that the down-regulation of cyclin \( E \) expression is also not necessary for the cell cycle arrest during \( S. \) crassipalpis diapause. Other investigators have reported that cyclin \( E \) expression is required for progression into the S phase during \( D. \) melanogaster embryogenesis (Knobloch et al., 1994). Further, in \( D. \) melanogaster cyclin \( E \) expression is upstream to that of PCNA and has been shown to provoke premature PCNA expression following ectopic expression of cyclin E (Duronio and O'Farrell 1994, Richardson et al 1995).

PCNA plays a vital role in developmental progression and cell cycle status. It is an essential accessory factor to \( \delta \) polymerase, an enzyme necessary for leading strand DNA replication and DNA repair (Prelich et al. 1987a,b, Shivji et al., 1992). PCNA expression during cell cycle progression is so vital that exposure of cell lines to antisense oligodeoxyneucleotides made to the first 36 nucleotides of the \( \text{DmPCNA} \) sequence causes inhibition of cellular proliferation (Jaskulski et al., 1988). PCNA is localized in the nucleus at sites of active DNA replication, thus expression is limited to just prior to, and during, the S-phase of the cell cycle.

Low expression of the \( \text{ScPCNA} \) transcript occurs in cells from whole body preparations of diapausing flesh flies, with a distinct increase in \( \text{ScPCNA} \) levels evident
twelve hours after hexane has been applied to terminate diapause. Expression of ScPCNA increases gradually until maximum levels are observed 48-72 hours after hexane application. When Northern blot analysis data are compared to flow cytometric data, a strong correlation can be seen between ScPCNA levels and the cell cycle status of the fly. When ScPCNA expression levels are low, the cell population is arrested, resulting in a halt in DNA replication and proliferation. When ScPCNA transcript expression is high, cells initiate DNA replication, ultimately leading to cell proliferation and adult development.

This points to ScPCNA playing a key role in coordinating the cellular events associated with diapause and the resumption of postdiapause development.

During diapause, expression of the ScPCNA transcript is only evident at the very distal region of the eye lobes, suggesting that few cells are undergoing DNA replication or proliferation. However, 24 hours after diapause termination, following hexane treatment, extensive ScPCNA expression is observed throughout the brain. High levels are detected in the developing eye, the eye lobe (characteristic crescent), and throughout the protocerebrum. How and why these cells are the first to break the arrest may be important for understanding the mechanism controlling diapause termination.

Though PCNA is vital in the control of cell proliferation, low levels are also detected in senescent and quiescent cells (Leibovici et al., 1990). This is consistent with our observation of whole body mRNA from diapausing pupae, where minute amounts of ScPCNA are being transcribed. The onset of DNA replication and/or proliferation after diapause termination may depend on a critical amount of ScPCNA being produced in the cell population.

ScPCNA is one of several genes that appears to be down-regulated during pupal diapause in flesh flies. When protein synthesis in the brain is examined it is evident that far fewer proteins are synthesized during diapause (Joplin et al., 1990), and we have now isolated seven clones, including ScPCNA, that are down-regulated or are nondiapause
specific (Flannagan et al., 1997). Thus far, none of the other down-regulated or nondiapause specific clones have been fully sequenced. Flesh fly diapause is also characterized by synthesis of several diapause-specific brain proteins (Joplin et al., 1990), and four clones that are diapause specific or diapause up-regulated have been isolated from a brain cDNA library (Flannagan et al., 1997). One of the diapause up-regulated genes is a small heat shock protein, but the others have not yet been identified.

That ScPCNA is down-regulated during diapause is consistent with its known physiological functions (Fukuda et al., 1995). Our results indicate that low ScPCNA expression correlates with cell cycle arrest during diapause, while an elevation in expression correlates with the termination of diapause and the release of the cells from arrest. Arrest of the cell cycle is likely to be a key factor regulating the developmental stasis observed during pupal diapause.
REFERENCES


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CHAPTER 4

CLONING AND SEQUENCING OF PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) FROM THE FLESH FLY, SARCOPHAGA CRASSIPALPIS, AND ITS EXPRESSION IN RESPONSE TO COLD SHOCK AND HEAT SHOCK

ABSTRACT

We report the isolation and sequencing of a 1326 base pair (bp) cDNA fragment encoding the cell cycle proliferation protein ScPCNA from the flesh fly, Sarcophaga crassipalpis. The amino acid (aa) sequence shows 91% and 79% identity to Drosophila melanogaster and Bombyx mori proliferating cell nuclear antigen (PCNA), respectively. The coding sequence is interrupted by a single intron of 60 bp, resulting in a deduced aa sequence of 260 residues. The gene is transcribed as a single mRNA (approx. 1.2kb) as determined by northern blot hybridization. Following a cold shock at -10°C for 1 hr expression of ScPCNA decreased in S. crassipalpis whole body mRNA, suggesting a possible cell cycle arrest in response to a cold shock. One hour after removal from cold shock, ScPCNA transcript levels were restored to the control level. By contrast, a 1 hr heat shock at 45°C did not alter expression of ScPCNA.
INTRODUCTION

The nuclear protein proliferating cell nuclear antigen (PCNA) is essential for multiple cell cycle pathways including DNA replication, DNA elongation (leading strand synthesis), and DNA excision repair (Madsen and Celis, 1985; Prelich and Stillman, 1988; Waseem et al., 1992; Zeng et al., 1994). It is also utilized in cell cycle control through direct interaction with cyclin/cdk complexes. allowing progression through the G1/ S boundary of the cell cycle (Zhang et al., 1993). PCNA functions by forming a homotrimeric ring-shaped structure producing a clamp to which the DNA pol (delta), DNA template, and replication factor-C (RF-C) will form a processive DNA polymerase complex (Prelich et al., 1987; Bravo et al., 1987; Fukuda et al., 1995).

Previous studies show that PCNA expression is downregulated during the cell cycle arrest observed throughout pupal diapause in the flesh fly, Sarcophaga crassipalpis (Tammariello and Denlinger, 1998). As adult development resumes following diapause termination ScPCNA, the PCNA gene from S. crassipalpis (Flannagan et al., 1998), transcript level increases dramatically in association with cell proliferation. This suggests that ScPCNA is an important regulatory cell cycle control protein used by the flesh fly.

We recently cloned the entire open reading frame, the internal intron and much of the 3’ and 5’-untranslated regions (UTR) of ScPCNA. In this study we describe the sequence analysis of ScPCNA, a gene with high identity to both the Drosophila melanogaster and Bombyx mori PCNA genes. Further, we studied the effects of temperature stress on ScPCNA expression to test the possibility of a correlation between ScPCNA expression and DNA damaging events.
EXPERIMENTAL AND DISCUSSION

Fly rearing and colony maintenance

The colony of *S. crassipalpis* Marquart was maintained as described (Denlinger, 1972). Flies were reared throughout their life cycle in a daily 15hr light- 9 hr dark cycle at 25°C.

Cloning and sequencing of ScPCNA

*ScPCNA* was isolated and cloned through an elimination hybridization technique in which a diapause pupal brain cDNA library was screened using a complex mixture of cDNA probes constructed from brain mRNA of nondiapausing flesh fly pupae (Flannagan et al., 1998). From this initial screen, we isolated 95 clones that failed to hybridize to the cDNA library. Secondary screening of these clones was accomplished by northern blot hybridization using diapause and nondiapause poly (A)+ RNA blots. The clone ultimately corresponding to *ScPCNA* (clone 56) exhibited a single, strong band of approximately 1.2kb in nondiapause RNA but little to no signal in the diapause RNA; this clone was then considered to be diapause downregulated. Clone 56 was subsequently subjected to an *in vivo* excision and cloned into pBluescriptKS(+) vector for sequencing.

Clone 56 was sequenced on both strands using the pBluescriptKS(+) vector’s primer sites for initial sequencing from the 3' and 5' distal regions of the clone, followed by construction of internal primers and primer walking as sequence data became known (Fig. 4.1). Sequence analyses were performed at the University of Georgia molecular genetics facility on an ABI 373A DNA sequencer using dye termination chemistry according to the manufacturer's protocol. The BLAST® search program was used to search the Genbank sequence repository for verification of sequence data and identities.

The total length of the sequence from clone 56 was 1326 bp, consisting of an open reading frame of 780 bases and a 60 bp intron that will be spliced out at the consensus intron-exon boundaries prior to translation (Fig. 4.2). The deduced aa sequence from the
nucleotide sequence analysis of clone 56 revealed a high degree of aa residue conservation to *D. melanogaster* PCNA (*DmPCNA*) (Yamaguchi et al., 1990) and *Bombyx mori* PCNA (*BmPCNA*) (Takahashi, 1997 unpublished). BLAST searches verified the identity of clone 56 as a PCNA-like gene that had a 91% identity (235/260 residues) to *DmPCNA* and 79% identity to *BmPCNA* (206/260 residues) at the aa level (Fig. 4.3). Among the 25 aa residue differences between *ScPCNA* and *DmPCNA*, all but two residues contained chemically equivalent aa substitutions.

**Structure of ScPCNA**

The aa sequence of PCNA is highly conserved throughout eukaryotes, with multiple regions necessary for proper function. A monomer of PCNA contains four (alpha) helices and 18 beta sheets. Deletion experiments suggest that these regions, as well as the C- and N- termini, are vital for forming the proper homotrimeric, native structure essential for the binding of PCNA to its target proteins (Fukuda et al., 1995; Fein and Stillman, 1992). Each of the four alpha helices contains two or three highly charged residues conserved from human to yeast. Nine residues (Lys 13, Lys 14, Lys 20, Lys 77, Lys 80, Lys 146, Arg 149, Arg 210 and Lys 217) must be present in the internal alpha helices of each PCNA monomer to stimulate DNA pol (delta) activity (Fukuda et al., 1995). All nine residues are conserved within the structure of ScPCNA, implying functional homology to other eukaryotic PCNAs. Similarly, all 14 beta sheet regions are conserved between *ScPCNA* and *DmPCNA*.

The largest divergent regions are at the N- and C- termini. The first six aa residues are conserved between *ScPCNA* and *DmPCNA*, followed by two non-equivalent aa substitutions, the result of two base changes (A to G at 17, A to C at 22). The C-terminus exhibits slightly higher divergence with two of the final three aa residues different between the two species (*Dm*- NET; *Sc*- DES). Once again, single base substitutions cause these residue changes (A to G at 1111, C to G at 1116). Though two of the three are different
aa's, both are chemically equivalent substitutions, suggesting that functions of the N- and C-terminal regions are consistent with DmPCNA.

Expression of ScPCNA

PCNA expression is normally associated with cycling or proliferating cell populations (Bravo et al., 1981). The localization of PCNA in the nucleus coincides with sites of ongoing DNA replication (Madsen and Celis, 1985; Bravo and MacDonald-Bravo, 1985). To investigate expression specificity of flesh fly PCNA, northern blot analyses were carried out on RNA from pharate adult flesh flies that had either been cold shocked (-10°C) or heat shocked (45°C) for 1 h. Samples were taken at multiple time points (0, 1, 24 hours) following removal of the flies from the cold or heat. Total RNA was extracted by homogenization of whole pharate adult flies in TRIZol® reagent using the standard protocol (GibcoBRL). Poly (A)+ RNA was isolated from each total RNA sample using the Oligotex mRNA midi® kit (Qiagen). Approximately 4µg of each poly (A)+ sample was transferred to membrane (Micron Separations, Inc.) by gravity transfer for one hour. The blots were screened using 32P-CTP labeled DNA probes produced from ScPCNA using the RTS RadPrime® DNA labeling system (GibcoBRL). All blots were prehybridized at 65°C for three hours in standard hybridization buffer (0.5M NaCl, 0.1M NaPO₄, 6mM EDTA) + 1% SDS. Hybridization was performed overnight in the same buffer, along with the probe, at 65°C. The blots were washed using one-quarter strength hybridization solution with SDS for 45 minutes at 65°C, followed by a wash using one-quarter hybridization solution without SDS for 1 hour. Finally, blots were wrapped in Saran® wrap and exposed to x-ray film (Fuji) at -70°C.

All northern blot analyses of poly (A)+ RNA from control, cold and heat shocked pharate adult flesh flies, probed with full-length ScPCNA, resulted in a single transcript of approximately 1.2 kb (Fig. 4.4). The amount of the ScPCNA mRNA was relatively high in control flies kept at 25°C. Although transcript levels were relatively abundant for a
number of genes directly after removal from cold (data not shown), a noticeable decrease in 
ScPCNA expression was observed immediately following removal from a cold shock at -
10°C for 1 h. By one hour after removal from cold, ScPCNA levels were once again 
restored to control levels. In contrast, exposure to heat for 1 hr (45°C) did not alter the 
expression level of ScPCNA. These results are consistent with other reports in the 
literature suggesting that certain aspects of the response strategies to heat and cold stress are 
independent (Yocum et al., 1991). ScPCNA thus appears to be downregulated during 
diapause (Tammariallo and Denlinger, 1998) as well as in response to cold shock, two 
conditions that the fly is likely to encounter during the winter.

CONCLUSIONS

(1) The nucleotide (nt) sequence of Sarcophaga crassipalpis PCNA (ScPCNA) containing 
the entire ORF, an internal intron and much of the 3’ and 5’ UTR was determined.

(2) A single transcript of approximately 1.2kb was observed in whole body RNA by 
northern analysis, suggesting the presence of a single ScPCNA mRNA species.

(3) The deduced amino acid sequence shows 91% identity to Drosophila melanogaster 
PCNA and 79% identity to Bombyx mori PCNA.

(4) 1 hr of cold shock (-10°C) decreased the expression of ScPCNA but 1 hr of heat shock 
(45°C) did not alter ScPCNA expression levels.
FIGURE 4.1. Sequencing strategy for primer walking S. crassipalpis PCNA (ScPCNA).

Horizontal arrows represent initial and subsequent primer construction and sequenced region, and the vertical arrow indicates the intron. The insert was cloned into pBluescriptKS(+) with initial sequencing using plasmid T7 and T3 primer sites. All sequencing was done on an ABI 373A sequencer at the University of Georgia molecular genetics facility.
FIGURE 4.2. DNA sequence of ScPCNA cDNA isolated from a brain cDNA library (GenBank accession number AF020427).

The 5'-UTR (nt -273 to -1) and 3'-UTR (nt 844 to 1021) are shown in lower case and the coding region (nt 1 to 843) is underlined. The start codon (AUG) is at nt positions 1 to 3 and the stop codon (TAA) is at nt positions 841 to 843. The intron sequence which will be spliced out prior to translation is italicized. The consensus exon-intron boundaries are indicated (•).
**FIGURE 4.3.** Alignment of the deduced aa sequences of the putative *Sarcophaga crassipalpis*, *Drosophila melanogaster* (Yamaguchi et al, 1990) and *Bombyx mori* PCNA (Takahashi, 1997).

Conserved aa’s (*), chemically equivalent substitutions (•), and non-equivalent aa substitutions (no mark), are demarcated.
FIGURE 4.4. Expression of the *S. crassipalpis* PCNA gene following cold shock (-10°C) and heat shock (45°C) from whole body mRNA.

Northern blot containing 4 μg poly (A)+ RNA from control flies (reared and maintained at 25°C) as well as flies that were cold shocked (-10°C) and heat shocked (45°C) for 1 h. All RNA samples were run on a 1.5% agarose gel, transferred to membrane by gravity, and hybridized at 65°C for 12 h. The blot was then exposed to x-ray film overnight at -70°C.
REFERENCES


Takahashi, Y. (1997) Involvement of the DNA replication-related element (DRE) and DRE-binding g factor (DREF) in transcriptional regulation of the Bombyx mori gene. GenBank accession number AB002264.


CHAPTER 5

DESICCATION ELICITS HEAT SHOCK TRANSCRIPTION IN THE FLESH FLY, SARCOPHAGA CRASSIPALPIS, BUT DOES NOT ENHANCE TOLERANCE TO HIGH AND LOW TEMPERATURES

ABSTRACT

Although hsp production has been observed in response to multiple environmental stresses, no link has been established between desiccation and hsp production. Following a nonlethal desiccation at 0% relative humidity (R.H.) for up to 48 hours, two heat shock protein transcripts, hsp23 and hsp70, are highly upregulated in pupae of the flesh fly, Sarcophaga crassipalpis. Levels are almost nonexistent in control pupae, but within 24 hours after being placed at 0% R.H. peak transcript expression is visualized. This suggests that desiccation protection may be linked to the general survival strategy employed by flesh flies against environmental stress. Adaptive cross-tolerance to cold shock and heat shock following a hsp inducing desiccation pretreatment were also tested. Although two hsp trans are upregulated in response to desiccation, we detect their presence is insufficient to induce cross-tolerance to high and low temperatures.
INTRODUCTION

All organisms express a particular set of proteins in response to such stresses as temperature extremes, insecticide application, anoxia, high levels of growth hormone, and many other environmental insults (Ashburner and Bonner, 1979; Lindquist, 1986; Hiromi et al., 1986). These proteins, commonly referred to as heat shock proteins (hsp), are normally divided into three families, the 90 kDa, 70 kDa, and small heat shock proteins (Parsell and Lindquist, 1993). The proteins of the 90 kDa hsp family are moderately conserved in eukaryotic organisms and are usually expressed following exposure to temperature extremes, but appear nonessential (Lindquist, 1988). Members of the hsp 70 family are highly conserved in all eukaryotes and prokaryotes that have been investigated and seem to be the dominant protein expressed following most environmental insults. The small hsps are the least conserved among the eukaryotes, and may be involved in microfilament dynamics following stress (Parsell and Lindquist, 1993).

Although hsp expression has been documented in response to several environmental stimuli, an important stress facing terrestrial arthropods, namely desiccation, has not been tested for hsp production. Biochemical analyses in response to desiccation have been initiated, and a 28 kDa protein from the hemolymph of the mealworm, Tenebrio molitor has been isolated and characterized (Graham et al., 1996a). This protein does not appear to have structural similarity to any known hsps, and its role has not been established in the cellular response to desiccation.

Preconditioning cells or organisms can facilitate increased survival under subsequent stress conditions (Lindquist and Craig, 1988). Such enhancement of S. crassipalpis tolerance has been best demonstrated for high and low temperatures, but Hoffmann (1991) has reported a similar result in Drosophila melanogaster, whereby a short desiccation pretreatment elicits an increase in desiccation resistance.
Cross-tolerance is a form of pretreatment that links a response to one form of stress with protection against a different form of stress. For example, pretreating flesh flies at a high, nonlethal temperature (approximately 36°C) confers protection against a normally lethal cold shock at -10°C (Chen et al., 1991). Hoffmann (1991) reported that a nonlethal cold shock pretreatment increases desiccation resistance in D. melanogaster. Cross tolerance is also observed between nonlethal temperature pretreatment and insecticide resistance in the mosquitoes, Anopheles stephensi and Aedes aegypti (Patil et al., 1996).

In this study we examine the relationship between osmotic stress and hsp expression in the flesh fly, Sarcophaga crassipalpis and demonstrate that certain hsp transcripts are expressed in response to desiccation. Since hsp70 is thought to be a key protein involved in thermotolerance we explored the possibility that stimulating hsp expression by a pretreatment of desiccation, i.e. is the production of hsps elicited by desiccation sufficient to protect flesh flies against a normally lethal thermal injury? We studied the expression patterns of three heat shock transcripts from pupae of S. crassipalpis in response to sublethal desiccation at 0% relative humidity (R.H.) for up to 48 hours. Further, we tested the ability of desiccation to induce cross-tolerance to high (45°C) and low (-5°C and -10°C) temperatures.

MATERIALS AND METHODS

Insect rearing

All experimental and control animals were from a laboratory colony of the flesh fly, Sarcophaga crassipalpis Macquart. To produce non-diapause individuals, the flies were reared throughout their life cycles under longday conditions (15:9; L:D) at 25°C, as described by Denlinger (1972).
Desiccation

Triplicate sets of twenty pupae (4d after pupariation at 25°C) were desiccated on Drierite® for 12, 24, 48, or 96 h at 25°C. Control pupae were not placed on Drierite® at 25°C. Survival was based on the success of adult eclosion. Only those flies that completely extricated themselves from the puparium were considered survivors.

RNA isolation and Northern blot hybridization

Total RNA was extracted from nondesiccated and desiccated pupal flesh flies by homogenization in TRIzol® reagent using the standard protocol (GibcoBRL). RNA samples were taken from control pupae, as well as from pupae that had been desiccated on Drierite® for 12, 24, and 48 hours. Approximately 20μg of each total RNA sample was transferred to Magnacharge+ nylon membrane (Micron Separations, Inc.). The blots were screened using 32P-CTP labeled DNA probes produced from two hsp genes cloned from S. crassipalpis (hsp 70 and 23) using the RTS RadPrime DNA labeling system (GibcoBRL). The probe produced from a partial hsp70 clone corresponded to amino acids (a.a.) 501-613 (Rinehart and Denlinger, 1998), and the hsp23 probe was produced from the region corresponding to a.a residues 1-205, which spans the entire ORF and much of the 3' and 5' untranslated regions (Yocum and Denlinger, 1998).

All blots were prehybridized at 65°C for three hours in standard hybridization buffer (0.5M NaCl, 0.1M NaPO₄, 6mM EDTA) + 1% SDS. Hybridization was performed overnight in the same buffer along with the probe, at 65°C. The blots were washed using one-quarter strength hybridization solution with sodium dodecyl sulfate (SDS) for 45 minutes at 65°C, followed by a wash using one-quarter hybridization solution without SDS for 1 hour. Finally, blots were wrapped in Saran wrap and exposed to x-ray film (Fuji) overnight at -70°C.
Cross tolerance to thermal stress

Crosstolerance to thermal stress was tested using a desiccation pretreatment of 4d pupae for 24 h at 25°C. Following desiccation, pupae from all experimental and control groups were either cold shocked at -10°C or heat shocked at 45°C for 60, 90 or 120 min. We also tested the ability of desiccation pretreated flies to withstand a less lethal temperature (-5°C) for longer duration. Experimental (24 h desiccation pretreatment) and nondesiccated control flies were subjected to -5°C. After 12, 24 and 48 hours a set of pupae was removed from cold or heat shock and evaluated for eclosion success. Three replicates of twenty pupae were used for all nondesiccated control and predesiccated experimental groups.

Statistics

All data were normalized by arcsin transformation (Sokal and Rohlf, 1981). Data represented in figures as curves were analyzed by ANOVA without replication to verify that there was no significant variance within groups. If variance within groups was insignificant, then the possible differences between individual curves was demonstrated by ANOVA with replication for equal sample size, followed by the Tukey multiple comparison procedure.

RESULTS

Response to desiccation

To determine the effect of osmotic stress on survival of flesh flies, pupae (4d) were subjected to desiccation on Drierite® for 12, 24, or 48 hours and scored for success of adult eclosion (data not shown). No significant difference was observed between the lengths of desiccation tested and adult emergence. From this data we established that a desiccation treatment up to 48 hours was a nonlethal stress on the flies.
Expression of hsp transcripts

After establishing that 48 hours of desiccation was nonlethal to both wandering larvae and 4d pupae, we tested the possibility that desiccation elicited a cellular stress response that could be detected by expression of stress protein transcripts. Levels of two hsp transcripts were measured following non-lethal desiccation of 4d pupae. Using northern blot hybridization, a comparison was made between RNA samples from control 4d pupae and pupae that had been desiccated for 12, 24 and 48 hours.

A single band (approx. 2900 bp) was observable at extremely low levels in 4d pupal control RNA using *S. crassipalpis hsp70* (Fig. 5.1). *Hsp70* transcript levels in 4d pupae peaked after 24 hours of desiccation, but was again lower in flies that had been desiccated for 48 hours.

The expression pattern of *hsp23* differed somewhat from the pattern of *hsp70* (Fig 5.2). The lowest level of expression was observed in the control pupae that had not been desiccated (0 hr. in Fig. 5.2). As observed with the *hsp70* transcript, the *hsp23* transcript was highly expressed following 24 h of desiccation, but in contrast to *hsp70*, expression of *hsp23* also remained high after 48 hours of desiccation.

Cross protection between desiccation and temperature stress

To test whether the elevated levels of the two hsps in response to desiccation could confer cross tolerance to temperature extremes in flesh flies, pupae were desiccated for 24 hours, then subjected to either a cold shock (-10°C) or heat shock (45°C) for 30, 60, 90 or 120 min, or subjected to a less extreme cold temperature (-5°C) for 24, 48, or 72 hours. Both -10°C and -5°C are above the supercooling point (SCP), but usually cause high mortality if flies are exposed for more than 2h. Non-desiccated control pupae were also cold or heat shocked at the same temperatures for the same durations.

Tolerance to -10°C was not increased in 4d pupae following a 24 hour desiccation stress (Fig. 5.3). In fact, the desiccation pretreatment reduced tolerance to -10°C. The
greatest disparity in survival between the control and experimental groups was observed in response to a 90 min. exposure to -10°C, and statistical significance was observed only at this time point (ANOVA: \( P=0.070 \)).

To test whether -10°C was too low a temperature for detecting tolerance in response to a desiccation pretreatment, we desiccated 4d pupae for 24 hours, and subsequently placed them at -5°C, a much less stringent temperature to flesh flies. The results mirrored those found at -10°C. After 24 hours at -5°C, 42% successful adult emergence was observed compared to 6% adult eclosion following a pretreatment of desiccation for 24 h (data not shown). Statistical analyses indicated a significant difference between control and experimental flesh flies after 24 h at -5°C (ANOVA; \( P<0.001 \)).

Exposures to -5°C for 48 or 72 hours were lethal to both the controls and the experimental group (data not shown).

A similar result was observed following exposure of desiccated and non-desiccated pupae to 45°C. Following a heat shock at 45°C for 30 or 60 min., eclosion success was much higher for the non-desiccated individuals than for those that had previously been desiccated. >40% of the non-desiccated flies emerged, while only 17% of the desiccated flies successfully did so (Fig 5.4). Neither control or pre-desiccated pupae could tolerate 90 minutes or longer at 45°C. The differences at 30 and 60 minutes are statistically significant (ANOVA: \( P<0.001 \) and \( P=0.001 \), respectively).
FIGURE 5.1. Expression of hsp70 in response to desiccation.

Numbers above figure represent hours of desiccation.
FIGURE 5.2. Expression of \textit{hsp23} in response to desiccation.

Numbers above figure represent hours of desiccation.
FIGURE 5.3. Survival of *S. crassipalpis* to -10°C by adaptive crosstolerance following a desiccation pretreatment.
FIGURE 5.4. Survival of *S. crassipalpis* to 45°C by adaptive cross tolerance following a desiccation pretreatment.
DISCUSSION

The phenomenon of heat shock protein (hsp) production after environmental stress has been widely reported (reviewed by Parsell and Lindquist, 1993). Many environmental tokens have been reported to elicit hsp expression including exposure to high and low temperatures, insecticides and other chemicals, steroid hormones, and hypoxia (Landry et al., 1982; Li, 1983; Joplin et al., 1990; Yocum et al., 1991). These hsps are general homeostatic response elements thought to play important roles in cell and protein protection following periods of environmental insult. But little attention has been given to hsp production during periods of osmotic stress. Desiccation survival and tolerance in arthropods have been studied extensively in arthropods (Hoffmann, 1991; Yoder and Denlinger, 1991); these studies describe the physiological response to osmotic stress at the organismal level, but none address the underlying molecular mechanisms involved during desiccation response.

An initial characterization of the physiological response to desiccation has yielded a 28 kDa protein from the hemolymph of the yellow mealworm beetle, *Tenebrio molitor*, that is desiccation induced (Graham et al., 1996b). Sequence analysis suggests that it contains multiple juvenile hormone binding domains, but it shows no similarity to any known gene or protein sequence. Further, it does not seem to contribute to the freezing point depression observed in *Tenebrio* hemolymph. This protein represents the majority of the molecular data known following osmotic stress in arthropods. With this in mind, we tested the transcription levels of two hsps following varying periods of desiccation at 0% R.H. in the hopes of establishing a hsp response from a nonlethal desiccation stress.

*S. crassipalpis* pupae (4d) were subjected to 0% relative humidity (R.H.) on Drierite® for up to 48 hours in order to establish a survival curve to osmotic stress. No significant difference in percent successful eclosion was observed after 48 hours or less of desiccation and non-desiccated controls. These results are consistent with earlier findings
published on desiccation survival of *S. crassipalpis*, which suggests that over 24.5% of their original body mass must be lost to result in eclosion failure (Yoder and Denlinger, 1991). As long as the puparium is kept intact the fly never loses this much water; in fact flesh flies can survive while living on Drierite® continuously from the wandering larval stage throughout adult eclosion.

Following the establishment of a nonlethal desiccation treatment of 48 hours or less on Drierite®, transcript upregulation of two hsps (70 and 23) is observed in RNA from pupae of the flesh fly, *S. crassipalpis*. This suggests that the desiccation response may be linked to the general survival mechanism observed following environmental insult in *S. crassipalpis*. Both *hsp23* and *hsp70* expression have been reported in response to high and low temperatures, and *hsp23* is also developmentally regulated with its expression observable throughout diapause in *S. crassipalpis* (Yocum et al., 1998; Joplin et al., 1990).

A difference does exist in the time of desiccation necessary for peak production of hsps among different life stages of *S. crassipalpis*. 48 hours of desiccation will cause the highest expression of *hsp* s in larval RNA (data not shown), while 24 hours of desiccation produced the peak transcription levels of *hsp* s in RNA samples from 4d pupae. Though both wandering larvae and 4d pupae exhibit upregulation of hsps in response to desiccation, the response is more striking in the RNA samples from flesh fly pupae. Very little transcript is noticeable in the pupal controls, but by 24 hours of desiccation transcript levels increase dramatically. Since a non-lethal stress resulting in the upregulation of two hsps was established, *S. crassipalpis* pupae (4d) were tested for signs of desiccation-induced crosstolerance to temperature extremes.

The phenomenon of adaptive crosstolerance has been widely reported in the last decade. Pre-exposure to high but sublethal temperatures confers adaptive cross-tolerance to the carbamate insecticide propoxur in the mosquitoes *Anopheles stephensi* and *Aedes*
aegypti (Patil et al., 1996). Furthermore, Watts et al. (1987) demonstrated that mosquitoes reared in warmer insectaries could tolerate much higher virus loads.

Following 24 hours of desiccation (peak hsp expression) no increase in tolerance to high (45°C) or low (-5°C, -10°C) lethal temperatures was observed in pupal flesh flies. In fact, pre-treatment (24 hours of desiccation) seems to decrease flesh fly tolerance to low and high temperatures, well below control flies. Thus desiccation-induced expression of hsp does not seem to be sufficient to increase cold tolerance and thermotolerance in the flesh fly, S. crassipalpis.

These data are in contrast to many models offered explaining how organisms overcome adverse cold temperatures. The common model presented is that cold tolerance and desiccation work in conjunction to prevent cellular freezing, preventing death by ice crystal formation. Data have been presented that suggest a thermal stress placed on certain plants, tardigrades, and earthworms can induce desiccation tolerance in that organism (Anandarajah et al., 1991; Holmstrup, 1993; Somme, 1996). But these organisms have the ability to protect themselves by cuticular structural adaptations. Tardigrades enter a stage of anhydrobiosis in a tun and the lumbricid earthworm, Dendrbaena octaedra, can produce a cocoon-like structure around the developing worms. Tardigrades producing tuns are actually in a state of anhydrobiosis; therefore barring innoculative freezing, they can withstand extreme low temperatures. Although nondiapausing flesh fly pupae are encased in a puparium, they are incapable of surviving a period of anhydrobiosis as they can not afford losing more than approximately 25% of their body water before dying (Yoder and Denlinger, 1991).

Although the preceding examples were from organisms physiologically different from the flesh fly, similar results have been reported Diptera related to the flesh fly. The flies D. melanogaster and D. simulans also become more desiccation tolerant after exposure to thermal stress (Hoffmann, 1991; and Hoffmann and Parsons, 1993). This response is
greatest following a low temperature nonlethal shock, but also functions after high
temperature exposure.

A connection between cold hardiness and desiccation has also been observed in
research done on the arctic collembolon, *Onychiurus arcticus* Tullberg (Holmstrup and
Somme, 1998). Results from this study show that this collembolon undergoes dehydration
when exposed to subzero temperatures, lowering the body fluid melting point and the
supercooling point of the insect, thus avoiding body freezing.

With this body of research the assumption could easily be made that the reverse
scenario would be functional as well; namely that a desiccation pretreatment should confer
thermotolerance in eukaryotic organisms. But is desiccation truly sufficient for protection
against cold temperatures?

The Antarctic nematode, *Panagrolaimus davidi*, does not appear to protect itself
from freezing by desiccation. Hyperosmotic and hypoosmotic stress adversely affect the
nematodes' ability to survive freezing (Wharton and To, 1996). Experimental evidence
observed from the American dog tick, *Dermacentor variabilis*, also suggests that
dehydration may not be used by all organisms to survive cold temperatures. These ticks
actually raise their body water content throughout winter survival (R. Stewart, personal
comm.). These two organisms are quite physiologically different than *S. crassipalpis*, but
their cold weather survival mechanisms suggest that cold survival ability is not always
linked to desiccation. We know that flesh flies do not decrease body water throughout
diapause, in fact one of the main functions of the puparium is to retard water loss
throughout diapause. Therefore, we propose that a desiccation stress placed on a flesh fly
pupae acts as a synergist to increase the effect of subsequent temperature stress placed on
the fly.
REFERENCES


CHAPTER 6

PARTIAL CLONING OF THE CYCLIN-DEPENDENT KINASE INHIBITOR P21
FROM THE FLESH FLY, SARCOPHAGA CRASSIPALPIS

ABSTRACT

Progression through the cell cycle is catalyzed by cyclin-dependent kinases (CDKs)
and is negatively controlled by CDK inhibitors (CKIs) (Sherr, 1996). The CKI p21^{waf1/cip1}
has been suggested to mediate multiple growth arrest scenarios, such as differentiation,
senescence and apoptosis (Elledge, 1996). We have cloned a portion of a p21-like cDNA
from the flesh fly, Sarcophaga crassipalpis. DNA primers were constructed to known p21
gene sequences from human (hp21) and mouse (mp21) using polymerase chain reaction
(PCR). A band of approximately 360 bp was cloned and sequenced for identification.
Southern blot analysis using human p21 (wafl) revealed a strong hybridization signal,
while screening with mouse p21 (CIP1) revealed no signal. The PCR product was
sequenced and its deduced protein has 95% identity to hp21 and 75% identity to mp21 at
the amino acid level.
INTRODUCTION

The cyclin-dependent kinase (cdk) inhibitor p21 has been suggested to mediate growth arrest periods triggered by DNA damage (El-Diery et al., 1993; Dulic et al., 1994). This protein binds directly with cyclin-cdk complexes which inhibits the phosphorylation of the retinoblastoma (Rb) protein, thus arresting cell proliferation at the G1-S boundary. p21 also functions to remove cells from the cell cycle during differentiation, senescence, quiescence and following DNA damage (Steinman et al., 1994).

Presently p21 has only been cloned from mammalian species (human, cat, rat and mouse), with no record of a clone from any invertebrate species (Harper, personal comm.). Therefore using reverse-transcription polymerase chain reaction (RT-PCR), we attempted to clone p21 from the flesh fly, *Sarcophaga crassipalpis*. This would represent the first example of a cell cycle inhibitor to be isolated and cloned from an invertebrate species.

MATERIALS AND METHODS

Reverse-Transcription PCR

Two units of RNAse-free DNAse (GibcoBRL) were added to 2 μg total RNA from diapausing pupae and incubated at 37°C for 20 minutes and then 70°C for 20 minutes. The reverse transcription reaction contained 1mM of all 4 dNTPs, 10mM DTT, 50mM Tris-HCl, 75mM KCl, 3mM MgCl₂, 2.5 pg oligo-dT₁₇ tail primer, 0.5 units RNAse inhibitor and 2.0μg DNAse treated total RNA. The entire reaction was incubated at 42°C for 10 minutes, then 200 units Superscript RNAse H- reverse transcriptase (GibcoBRL) was added. The samples were incubated at 42°C for 1 hour and then 70°C for 15 minutes to complete construction of the cDNA pool.
Degenerative oligonucleotide primers were designed to conserved regions of the human and mouse \( p2l \) genes that encode almost the entire open reading frame (ORF) of the protein. Both primers were 20 nucleotides in length and had the following sequences: primer one \( TG(T/C)GA(T/C)GC(C/G)CT(A/T/C/G)ATGGC(C/G)GG \) and primer two \( ATGAC(A/T/C/G)GA(T/C)TTTTA(T/C)CA(T/C)AG \) (both 5' to 3'). PCR reactions contained 200 nM each primer, 200nM of all four dNTP's, 1.5 mM \( \text{MgCl}_2 \), 16.5 mM Tris-acetate (pH 7.9), 33 mM sodium acetate, 5mM magnesium acetate, 50\( \mu \)g/ml BSA, 250 nm DTT, 2.5 units Taq DNA polymerase (GibcoBRL) and 2.0 \( \mu \)l cDNA from the RT reaction. PCR reaction parameters were 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 60°C for 45 seconds and 72°C for 30 seconds. All PCR reactions were performed on a thermal cycler (Perkin Elmer).

Positive control PCR reactions were also run using human \( p2l \) as a template, to ensure that the primers were designed correctly. Both control and experimental PCR amplification products were analyzed on 2% agarose gels alongside a 100 bp DNA ladder (Fig. 6.1).

**Southern blot hybridization**

PCR products were run on a 2% agarose gel, then transferred to membrane as described previously (Chapter 2- Northern blotting protocol). Once transferred, the blot was screened using a probe constructed to human \( p2l \) using a Radprime probe kit (GibcoBRL)(Chapter 3- DNA probe construction).

**DNA sequencing**

The PCR products were sequenced at the University of Georgia Molecular Genetics Facility on an ABI 373A DNA sequencer using dye terminator chemistry according to the manufacturer’s protocol. Sequence comparisons and conceptual translations were performed using the CLUSTAL V computer program.

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RESULTS

Isolation and sequencing of a p21-like PCR product

As the initial step to clone *S. crassipalpis* p21, we used RT-PCR amplification using degenerative primers designed to know mammalian p21 sequences. PCR amplification resulted in a single band, approximately 360 bp in length (Fig. 6.1). When this PCR product was probed using a radioactive human p21 probe, a strong band was observed (Fig. 6.2). Finally, DNA sequence data (Fig. 6.3) revealed that this product had high identity to both human p21 (95%) and mouse p21 (75%) at the amino acid level (Fig. 6.4).
Figure 6.1. Cloning of *S. crassipalpis* p21 by RT-PCR.

Products were run on a 2% acrylamide gel and stained with ethidium bromide for visualization.
Figure 6.2. Southern bolt hybridization of RT-PCR product probed with human p21.

The probe was constructed using the Radprime® primer production kit (GibcoBRL). Hybridization was for 14 h at 60°C.
Figure 6.3. DNA sequence of isolated RT-PCR product.
Figure 6.4. Alignment of deduced amino acid sequence of ScP21 vs. human and mouse p21 using the SeqApp computer program.
DISCUSSION

Using RT-PCR we were successful in cloning a 380 bp PCR product that has high identity to known p21 genes. Identity to human p21 was considerably higher than the identity to mouse p21, in fact enough to raise suspicion of the true identity of our PCR product. Fortunately, we know that mouse p21 DNA probes fail to hybridize to RNA blots made from D. melanogaster RNA (J. W. Harper, personal comm.), but human p21 probes do hybridize to D. melanogaster RNA blots (Bae et al., 1995). This would at least explain why S. crassipalpis p21 has higher identity to human p21 than to mouse p21. Recently, the p21 gene from the cat has also been published. It has 80% identity to Sarcophaga p21 at the amino acid level, but attempts to hybridize a cat p21 probe to flesh fly nucleic acids have not been initiated.

This initial cloning step may be important for future experiments on the development of the flesh fly, S. crassipalpis since p21-like transcripts have been observed in D. melanogaster (Bae et al., 1995) and S. crassipalpis (Tammariello and Denlinger, 1998) using human probes. Isolation of the flesh fly clone might allow higher specificity for developmental and physiological studies that the cannot be completed using the human clone.
REFERENCES


CHAPTER 7

CONCLUSIONS

Insect diapause is an excellent model to study differential gene expression. Diapause is an alternate developmental pathway that insects commonly utilize to circumvent periods of environmental adversity and is characterized by decreased metabolism and developmental stasis. As early as the mid 1960's reports were published proposing that diapausing insects are biochemically distinct from their nondiapausing counterparts. Although these initial reports were thorough in describing the physiological differences between diapausers and nondiapausers, they were merely descriptive and failed to provide an adequate model for diapause regulation and maintenance. Even well into the 1990's researchers were successful at describing the characteristics of diapause, but failed to address the underlying molecular mechanism(s) that control insect diapause. Therefore, we attempted to define insect diapause at the molecular level by isolating diapause-upregulated and diapause-downregulated genes and characterizing the temporal and spatial expression of their gene products in association with flesh fly diapause.

Using a subtractive library technique followed by northern blot hybridization, we were successful in isolating four diapause-upregulated clones, seven diapause-downregulated clones and eight clones that were expressed at equivalent levels in both diapause and nondiapause. Partial sequence analysis revealed that two of the diapause-upregulated clones, pScD14 and pScD86, have high identity to a small heat shock protein 23 and an apurinic-apyrimidinic endonuclease, respectively. The remaining two diapause-
upregulated clones (pScD41 and pScD47) show no identity to any known protein sequences. These two clones are quite intriguing as they may represent novel diapause regulatory genes, and therefore it will be of vital importance to further characterize them. Three diapause-downregulated clones also show high identity to known protein sequences. pScD9A and pScD9B exhibit high identity with elastin-like proteins, pScD74 has identity to a protein kinase and pScD56 shows identity to the proliferating cell nuclear antigen (PCNA). The remaining diapause-downregulated clones show no identity with any known proteins.

This project represents the first large-scale screening for diapause-associated clones, the initial and vital step to unlocking the mystery of diapause regulation. The primary goal of this research proposal, the isolation and characterization of diapause-associated clones, was fully realized.

CELL CYCLE REGULATION DURING DIAPAUSE

The molecular regulation underlying the developmental arrest associated with flesh fly diapause is another area lacking data. Diapause-destined larvae develop quite normally until pupation at which time the developmental arrest commences. But what is underlying this developmental arrest that can last up to several months in flesh fly pupae? One possible scenario is that the cells themselves are arresting, leading to an organismal developmental arrest. Data have been published suggesting that senescent, quiescent and differentiating cell lines are all removed from the proliferation cycle in a cell cycle arrest. With this in mind we tested the proliferation status of brain cells before, during and after diapause in the flesh fly using flow cytometry. Our results show that cells from prediapausing and postdiapausing fly brains are cycling normally, but the cells from diapausing brains are arrested in the G0/G1 phase of the cell cycle. Thus, it appears likely that a cell cycle arrest is regulating the developmental arrest associated with flesh fly diapause.
We then tested the expression of four G1-S phase regulatory genes to elucidate a possible role that any play during diapause. Three of the genes (cyclinE, p21 and p53) showed no differential transcript expression between diapause and nondiapause, but one (PCNA) was found to be diapause-downregulated.

The diapause-downregulated clone (pScD56) was sequenced and its regulation was studied during and after diapause. This clone (ScPCNA) shows 91% and 79% identity to Drosophila melanogaster and Bombyx mori proliferating cell nuclear antigen (PCNA), respectively. ScPCNA expression seems to be linked to periods of cell cycling during development in the flesh fly and may be necessary for cell proliferation. During diapause ScPCNA is expressed at very low levels in whole body RNA, but following diapause termination its expression increases until peaking between 48 and 72 hours. In situ hybridization studies show that ScPCNA expression is lacking in diapausing pupal brains, but at diapause termination expression is observable in many brain cells including cells in the developing eye, the optic lobe and the protocerebrum.

From the cell cycle data and the expression studies of ScPCNA, a rudimentary model can be constructed explaining how the diapause-associated developmental arrest might be regulated. This represents the first model to be proposed regarding the molecular mechanism that may control diapause maintenance. We have established that the brain (and probably most of the body) cells are undergoing a G0/G1 cell cycle arrest during diapause, and we believe this arrest is the underlying mechanism controlling the developmental stasis observed during diapause. We have further noticed a direct correlation between expression of the cell cycle regulatory gene ScPCNA and cell cycle status; when ScPCNA levels are high cells are cycling, when ScPCNA levels are low cells are arrested. Thus our model proposes that the downregulation of ScPCNA may regulate the developmental stasis observed during flesh fly diapause. One method to verify this model would require the production of transgenic flesh flies that would incorporate our ScPCNA gene under the
influence of a controllable promoter. If the flies terminated diapause from overexpression of ScPCNA, we could conclude that ScPCNA expression controls nondiapause status.

To further define the cell cycle arrest during diapause, we attempted to clone the cyclin-kinase inhibitor gene p21 from our flesh flies using RT-PCR. We were successful in isolating a band from RT-PCR that may represent a portion of fly p21. Sequence analysis suggests that this PCR product has very high identity (95%) to human p21 at the amino acid level, higher than we initially expected. To ensure that this product is the fly p21, we would have to try a genomic Southern to pull out the actual gene sequence. If this product is fly p21, it would represent the first time this gene was cloned outside of mammals.

EXPRESSION OF DIAPAUSE-ASSOCIATED GENES IN RESPONSE TO OTHER ENVIRONMENTAL STRESSES

We have isolated diapause-upregulated and diapause-downregulated genes from the flesh fly, but are these genes only utilized in the diapause program or are they general function genes that play a role(s) in multiple environmental stress conditions? To test this, we studied the expression of the diapause-downregulated gene ScPCNA in response to temperature stress and the diapause-upregulated heat shock genes (hsp 23 and hsp70) in response to desiccation stress.

Following a cold shock at -10°C for 1 hr, expression of ScPCNA decreased in S. crassipalpis whole body mRNA, suggesting a possible cell cycle arrest in response to a cold shock. One hour after removal from cold shock, ScPCNA transcript levels were restored to the control level. By contrast, a 1 hr heat shock at 45°C did not alter expression of ScPCNA.

Although hsp production had been observed in response to multiple environmental stresses, no link had been established between desiccation and hsp production. Following
a nonlethal desiccation at 0% relative humidity (R.H.) for up to 48 hours, two heat shock protein transcripts, *hsp23* and *hsp70*, are highly upregulated in pupae of the flesh fly, *Sarcophaga crassipalpis*. Levels are almost nonexistent in control pupae, but within 24 hours after being placed at 0% R.H. peak transcript expression is visualized. This suggests that desiccation protection may be linked to the general survival strategy employed by flesh flies against environmental stress. Adaptive cross-tolerance to cold shock and heat shock following a hsp inducing desiccation pretreatment were also tested. Although two hsp us are upregulated in response to desiccation, we detect that their presence is insufficient to induce cross-tolerance to high and low temperatures.
BIBLIOGRAPHY


Takahashi, Y. (1997) Involvement of the DNA replication-related element (DRE) and DRE-binding g factor (DREF) in transcriptional regulation of the Bombyx mori gene. GenBank accession number AB002264.


