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RICE UNIVERSITY

The *Drosophila melanogaster* Mutation, *bemused*, is a new Allele of *pumilio*: Insights into Understanding the Role of Translational Regulation in the Maintenance of Proper Neuronal Excitability

by

Brett Alan Schweers

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ABSTRACT

The *Drosophila melanogaster* gene, *bemused*, is a new allele of *pumilio*: Insights into understanding the role played by translational regulation in the maintenance of proper neuron excitability

by

Brett Schweers

Maintenance of proper neuronal excitability is vital to proper nervous system function. Genes involved in regulation of neuronal excitability can be isolated with mutant screens to identify flies exhibiting behavioral abnormalities. A subset of these behavioral mutants exhibits defective motor neuron excitability, which can be monitored with electrophysiological methods. Application of this approach has allowed us to identify a P-element insertion mutant, called *bemused (bem)*, which exhibits female sterility, sluggishness, and increased motor neuron excitability. The *bem* P-element is located in the large intron of the previously characterized translational repressor gene *pumilio (pum)*. Here, by several criteria, I show that *bem* is a new allele of *pum*. First, ovary specific expression of *pum* partially rescues *bem*
female sterility. Second, *pum* null mutations fail to complement *bem* female sterility, behavioral defects, and neuronal hyper-excitability.

Third, heads from *bem* flies exhibit greatly reduced levels of Pum protein and the absence of two of the *pum* transcripts. I also show that two previously identified *pum* mutants exhibit neuronal hyper-excitability, and that over-expression of *pum* in the nervous system reduces neuronal excitability which is the opposite phenotype to *pum* loss of function.

Furthermore, I report that *bem* hyper-excitability is suppressed by reduced gene dosage *hunchback (hb)* suggesting that translational repression of *hb* by *pum* may be important to the maintenance of neuronal excitability. Collectively, these findings describe a new role of *pum* in the regulation of neuronal excitability and may afford the opportunity to study the role of translational regulation in the maintenance of proper neuronal excitability.
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<td>bemused</td>
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<tr>
<td>pum</td>
<td>pumilio</td>
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<tr>
<td>Na⁺</td>
<td>sodium</td>
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<tr>
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<td>frequenin</td>
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<td>hb</td>
<td>hunchback</td>
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<tr>
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<td>nanos response element</td>
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<tr>
<td>nos</td>
<td>nanos</td>
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<td>upstream activating sequence</td>
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<td>elav</td>
<td>embryonic lethal abnormal vision</td>
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<td>ejp</td>
<td>excitatory junctional potential</td>
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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Overview

Neuronal excitability is regulated by the balance and activation of functional Na\(^+\) and K\(^+\) channels expressed in these neurons. Mutations that cause an increase in the ratio of Na\(^+\) channel activity to K\(^+\) channel activity cause neurons to become hyper-excitabale, whereas mutations that decrease this ratio result in neurons with reduced excitability. Past research efforts have resulted in the generation and characterization of numerous such excitability mutants. For example, duplication of the Na\(^+\) channel gene paralytic (para) or loss-of-function mutations in K\(^+\) channel genes such as Shaker (Sh) and Hyperkinetic (Hk) each increase the ratio of Na\(^+\) channels to K\(^+\) channels and thereby result in hyper-excitabale motor neurons (Jan et al., 1977; Kamb et al., 1988; Loughney et al., 1989; Stern and Ganetzky, 1989; Stern et al., 1990). Also, application of the K\(^+\) channel blocking drug quinidine, which inactivates the delayed rectifier K\(^+\) channel and thus increases the ratio of functional Na\(^+\) channels to functional K\(^+\) channels,
results in hyper-excitable neurons as well (Stern and Ganetzky, 1992; Wu et al., 1989). Alternatively, loss of function mutations in *para* cause a decrease in the ratio of functional Na\(^+\) channels to functional K\(^+\) channels thereby leading to a decrease in motor neuron excitability (Ganetzky and Wu, 1982; Loughney et al., 1989; Stern et al., 1990; Suzuki et al., 1971).

At one synapse, the Drosophila larval neuromuscular junction (nmj), the most readily observable effect of increased neuronal excitability is an increased rate of onset of a phenomenon termed either long-term facilitation (LTF) or augmentation (Jan and Jan, 1978; Wang et al., 1994). LTF at the nmj is a phenomenon in which repetitive nerve stimulation at a sufficient frequency and duration causes subsequent nerve stimulation to evoke a prolonged release of neurotransmitter. This prolonged neurotransmitter release results from prolonged Ca\(^{2+}\) sensitivity of the pre-synaptic nerve terminal, and causes a correspondingly significant increase in the amplitude and duration of the response of the muscle cell (Jan and Jan, 1978). At the Drosophila nmj, mutations that increase neuronal excitability often cause an increase in the LTF onset rate. For example, over-expressing the *para* Na\(^+\)
channel or the guanylate cyclase activator frequentin (frq), and loss of function mutations of the K+ channel gene Hk each increase neuronal excitability and the rate of LTF onset at the nmj (Mallart, 1991; Rivosecchi et al., 1994; Stern and Ganetzky, 1989; Stern et al., 1990).

Previously, a mutant called bemused (bem) was isolated in the Stern lab (Stern et al., 1995). This mutant exhibits the pleiotropic phenotypes of female sterility, uncoordination, the inability to fly, and hyper-excitable neurons characterized by an increased rate of LTF onset at the nmj (Stern et al., 1995). The bem mutation is caused by insertion of a single P-lacW element into region 85D1, 2 of the polytene chromosome (Bier et al., 1989; Stern et al., 1995). This region also contains the pumilio (pum) transcription unit and more precise analysis (described herein) revealed that the bem P-element is located within the pum transcription unit. The pum locus has been studied in great detail and elucidation of the molecular mechanism by which pum functions in Drosophila embryogenesis has been well characterized (Barker et al., 1992; Macdonald, 1992). Pum protein binds directly to specific sequences in the 3’ untranslated region (UTR) of
maternally supplied hunchback (hb) mRNA (known as nanos-response elements or NREs) and then recruits at least two other proteins Nanos, (Nos) and Brain Tumor (Brat), to the mRNA (Murata and Wharton, 1995; Sonoda and Wharton, 2001; Sonoda and Wharton, 1999). The resulting complex results in repression of hb translation via de-adenylation of the hb message (Wreden et al., 1997). nos translation is regulated in a complex manner involving at least eight other genes (Gavis and Lehmann, 1994). This regulation results in nos translation only in the posterior pole of the developing oocyte and production of a Nos protein gradient emanating from the posterior end of the developing oocyte (Gavis and Lehmann, 1994).

Therefore the entire quaternary complex is formed only in the posterior of the oocyte, thus restricting hb repression to the posterior (Barker et al., 1992). This repression is important for proper embryogenesis because hb encodes a transcription factor that mediates development of anterior features, and improper expression of hb in the posterior of the embryo leads to abrogation of posterior structures such as abdominal segments (Barker et
al., 1992). A schematic cartoon showing the molecular mechanism by which *pum* functions in posterior pattern formation is shown in figure 1.

Whereas the mechanism by which *pum* functions in embryogenesis is the best understood, *pum* has been shown to affect other cellular systems as well. For example, members of the *ovarette (ovt)* class of *pum* mutant alleles exhibit defects in germ-line stem cell development and maintenance (Forbes and Lehmann, 1998; Lin and Spradling, 1997). These studies have also shown that *pum* plays a role in larval ovary development and that somatic expression of *pum* is important to the process of oviposition (Parisi and Lin, 1999). Furthermore, several findings that suggest a neuronal role for *pum* have been reported. Experiments were performed on flies which were expressing a *nos*<sup>+</sup> transgene under the transcriptional control of an eye specific promoter (Wharton et al., 1998). This eye specific expression of *nos* resulted in ablation of the eye (Wharton et al., 1998). The eye ablation is not present in *pum* mutants suggesting that Pum and Nos, when both present in the eye, cause improper translational regulation of eye mRNAs and subsequent ablation of the eye (Wharton et al., 1998). Also, Pum was shown
In the anterior of developing embryos: 
\( hb \) mRNA is translated

\[
\begin{array}{ccc}
hb & \text{NRE} & \text{poly-a} \\
\end{array}\]

Pum

In the posterior of developing embryos: 
\( hb \) mRNA translation is repressed

\[
\begin{array}{ccc}
hb & \text{NRE} & \text{poly-a} \\
\end{array}\]

Pum

Nos

Brat

**Figure 1.** Mechanism of Pum function in the process of posterior pattern formation. In the anterior of developing embryos, Pum binds directly to sequences present in the \( hb \) 3' UTR known as nanos response elements (NREs) but this binding has no effect on \( hb \) translation. In the posterior of developing embryos, Nos and Brat are recruited to the Pum \( hb \) complex and the resulting quaternary complex results in repression of \( hb \) translation. The exact mechanism by which the translational repression is achieved is unknown but it is thought to involve deacylation of the \( hb \) message. This repression is vital to proper posterior pattern formation and in its absence development of posterior structures such as abdominal segments is abrogated.
to be able to repress eye translation of an adenovirus transgene containing a NRE in its 3'UTR thereby preventing eye ablation (Wharton et al., 1998).

These experiments show that the Pum protein is expressed in, and able to repress translation in, the neuronal tissue of the Drosophila eye (Wharton et al., 1998). However, these experiments did not identify any neuronal target mRNAs of Pum nor did they address the mechanism by which the neuronal translational repression occurs. Also, another pum allele known as pumuckel (pkl) has been shown to be defective in the process of optic nerve path-finding (Schmucker et al., 1997). These experiments once again did not identify any Pum neuronal targets nor did they address the mechanism of Pum function in the process of nerve path-finding.

Here we expand on the body of knowledge surrounding pum. We show that bem is a new allele of pum, which demonstrates that pum is involved in maintaining proper neuronal excitability. We also show that previously isolated and characterized pum alleles exhibit the same neuronal hyper-excitability observed in bem mutants and that over-expression of pum in the nervous system of otherwise wild type third instar larvae leads to a
decrease in neuronal excitability. We suggest that the regulation of neuronal excitability by *pum* occurs by regulating the expression of Na⁺ or K⁺ channels directly, or by effects on an upstream component of the pathway that controls expression of these channels. Furthermore, our observation that *bem* hyper-excitability is partially suppressed by reduction in *hb* gene dosage suggests that translational regulation of *hb* by *pum* may also play a role in maintaining proper neuronal excitability.

### 1.2 Action potential propagation and synaptic transmission

Signals are propagated through the nervous system by a complex process involving action potential propagation and synaptic transmission. This process is described in detail in “Nerve, Muscle, and Synapse” (Katz, 1966). Briefly, neurons, the functional unit of the nervous system, maintain a negative membrane potential as a consequence of ionic gradients and differential membrane permeability. In nerve cells, Na⁺ concentration is low and K⁺ concentration is high. This results in a situation where Na⁺ diffuses into the cell while K⁺ diffuses out, both down their respective concentration gradients. This results in the establishment of an electrical potential across
the membrane that opposes the diffusion of Na\(^+\) and K\(^+\) ions. This opposition of diffusion by the electrical potential is not sufficient to stop Na\(^+\) and K\(^+\) ion diffusion leading to a situation whereby the negative resting potential would be lost upon the achievement of concentration equilibrium. This situation is avoided due to the action of the sodium-potassium ATPase that uses metabolic energy to pump Na\(^+\) ions out of and K\(^+\) ions into the nerve cell against their concentration gradients. The passive diffusion of Na\(^+\) and K\(^+\) ions is exactly balanced by the action of the sodium-potassium ATPase which pumps three Na\(^+\) ions out for every two K\(^+\) in. Overall, the resting membrane potential is dependent upon the equilibrium potentials of Na\(^+\) and K\(^+\), the permeability of the membrane relative to these two ions and the function of the sodium-potassium ATPase.

When a neuron receives a stimulus, a small depolarization results that causes the opening of voltage gated Na\(^+\) channels. Na\(^+\) ions enter the neuron, thus depolarizing the membrane. This depolarization propagates itself via the opening of adjacent voltage gated Na\(^+\) channels. In the wake of the action potential, voltage gated K\(^+\) channels are opened allowing the
efflux of $K^+$ ions and the subsequent re-polarization of the membrane potential. When the action potential reaches a synapse, voltage gated $Ca^{2+}$ channels are opened and $Ca^{2+}$ enters the nerve terminal. This $Ca^{2+}$ influx induces the fusion of vesicles carrying neurotransmitter to the neuronal membrane thus releasing neurotransmitter into the synapse. The neurotransmitter then diffuses across the synapse and binds to receptors on the post-synaptic neuron initiating the generation of a new action potential. Since this signal propagation process is dependent on $Na^+$ and $K^+$ channels, excitability of the neuron is directly affected by the ratio of $Na^+$ to $K^+$ channels. If the ratio of functional $Na^+$ channels to functional $K^+$ channels is high, the neuron will be more excitable than if this ratio is low.

1.2.1 Neurotransmission in Drosophila

One way to assess the excitability of a Drosophila neuron is to measure the rate of LTF onset at the neuromuscular junction (nmj). LTF at the Drosophila nmj is characterized by a prolonged release of neurotransmitter due to a prolonged $Ca^{2+}$ sensitivity of a repetitively stimulated pre-synaptic nerve terminal (Jan and Jan, 1977). It is still
unknown how the Na\(^+\) build up leads to the prolonged sensitivity of the pre-synaptic nerve terminal to Ca\(^{2+}\), but the prolonged neurotransmitter release causes a significant increase in the amplitude and duration of the response of the corresponding muscle cell (Jan and Jan, 1978). A sample trace showing LTF onset at the nmj is shown in Figure 2.

Several Drosophila mutants that exhibit hyper-excitable motor neurons have been identified, and these mutants are found to exhibit an increased rate of onset of LTF. Mutation of Hyperkinetic (\textit{Hk}) causes disruption of a K\(^+\) channel β-subunit and subsequently increases both the ratio of functional Na\(^+\) channels to functional K\(^+\) channels and the excitability of the motor neuron (Stern and Ganetzky, 1989). Also, the over-expression of the guanylate cyclase activator \textit{frequenin} (\textit{frq}) results in increased motor neuron excitability (Rivosecchi et al., 1994). Furthermore, over-expression of the Na\(^+\) channel gene \textit{paralytic} (\textit{para}) increases the excitability of the motor neuron in Drosophila (Stern et al., 1990). In addition to exhibiting hyper-excitability at the nmj, mutation of \textit{Hk}, and the over-expression of \textit{frq} and \textit{para} all result in an increased rate of onset of
Figure 2. Long-term facilitation onset at the Drosophila neuromuscular junction. Shown is a low [Ca\textsuperscript{2+}] (0.15 mM) electrophysiological trace from a nerve that is being stimulated at 10 Hz. The horizontal black line represents the voltage of the muscle cell from which data is being recorded. Vertical black lines represent stimulation artifacts that correspond to each nerve stimulus. The small bumps following each of the first three stimuli are excitatory junctional potentials (eJPs). The eJPs are small in amplitude because small amounts of neurotransmitter are released following each stimulus. However, after repetitive stimulation for a sufficient amount of time (four stimuli in this example) a larger amount of neurotransmitter is released following each stimulus and the muscle cell responds with a much larger amplitude of response. This is referred to as long-term facilitation (black arrowhead).
LTF (Rivosecchi et al., 1994; Stern and Ganetzky, 1989; Stern et al., 1990).

These examples allow us to infer that mutants that exhibit an increased rate of onset of LTF, exhibit increased excitability of the motor neuron as well.

1.3 The *bem* mutation affects female fertility and neuronal excitability

1.3.1 The role of *bem* in motor neuron excitability

In order to identify novel genes involved in nervous system function in Drosophila, Karina Walters and Dr. Mike Stern performed a P-element insertion mutagenesis (Bier et al., 1989). The insertion lines were initially screened for behavioral abnormalities such as poor coordination, slow climbing times, and the inability to fly. Insertion lines that exhibited abnormal behavior patterns were further scrutinized via electrophysiological means to determine if these flies exhibited abnormal neuronal function as well. The insertion line that was chosen for further study was named *bemused (bem)*. When compared to parental controls, *bem* flies exhibit female sterility, lack of coordination, difficulty climbing, and an increased rate of LTF onset.
The electrophysiological experiments revealed that *bem* flies exhibited facilitation after 1.5 seconds of repetitive stimulation at 10 Hz, while the parental controls did not exhibit facilitation until after 3.5 seconds of repetitive stimulation (Stern et al., 1995). When the potassium channel blocking drug, quinidine, was applied to the nerve, the increased rate of onset of facilitation was even more pronounced. The onset of long-term facilitation occurs after about 0.5 seconds of 10 Hz stimuli in *bem* larvae in the presence of quinidine, while the onset of long-term facilitation in parental control lines occurs after about 2.5 seconds of 10 Hz stimuli in the presence of quinidine (Figure 1) (Stern et al., 1995). Voltage-clamping experiments verified that the increased rate of onset of facilitation was due to a defect in the neuron and not the muscle (Stern et al., 1995). As mentioned, this phenotype has been observed previously in hyper-excitible *Drosophila* nervous system mutants and leads to the suggestion that the *bem* mutation also causes motor neuron hyper-excitability.
1.3.2 The role of bem in female fertility and oogenesis

In Drosophila, oogenesis is an extremely well characterized and complex process. Briefly, oogenesis begins with the asymmetric division of a germ line stem cell to produce a daughter stem cell and a cystoblast (Spradling, 1993). The cystoblast then undergoes a series of incomplete divisions resulting in 15 nurse cells and one oocyte which are connected by cytoskeletal pores called ring canals (Spradling, 1993). Finally, the cytoplasm from the nurse cells is dumped into the oocyte during a late stage of development and the fully developed oocyte is ready for fertilization and deposition (Spradling, 1993).

To fully characterize the bem female sterility phenotype, Karina Walters performed a detailed analysis of oogenesis in bem females. While bem ring canals are present and localized properly and nurse cell cytoplasm appears to be transported to the oocyte properly, bem egg chambers exhibit a wide variety of defects (Walters, 1997). These defects include weak cortical actin boundaries between the nurse cells and oocyte, improperly localized nurse cell nuclei, and absence of or improper localization of the oocyte
(Walters, 1997). These defects in oogenesis result in \textit{bem} female flies that lay a reduced number of eggs, which fail to properly develop into larvae (Walters, 1997). Therefore, \textit{bem} flies must be maintained as a heterozygous stock.

1.3.3 Location of the \textit{bem} P-element and initial attempts to clone the affected gene

To verify the presence of only one P-element insertion and to determine the location of insertion, \textit{in situ} hybridizations were performed on squashed polytene chromosomes. A single P-element insertion was found to be present in region 85D1, 2 of chromosome 3 (Stern et al., 1995). Free recombination revealed that the P-element always segregated with the mutant phenotype, and excision of the P-element was seen to restore the wild type phenotype (Walters, 1997). These experiments proved that the P-element insertion was causing the mutant phenotype.

Initial attempts to clone the \textit{bem} gene originated from the P-element insertion. Karina Walters used the plasmid rescue technique in order to isolate a 6-kb fragment of DNA flanking the P-element. This 6-kb fragment
was then used to probe a lambda-phage genomic library. Clones isolated from this screen cover over 18.9 kb of DNA and 14.4 kb of this DNA has been sequenced. Unfortunately, no open reading frames (ORFs) are present (Walters, 1997). Also, analysis of the sequence with the GRAIL program suggests that this DNA is not transcribed (Xu et al., 1994). Dr. Walters also performed a cDNA library screen and several putative cDNAs were isolated. However, sequencing of the putative cDNAs revealed no ORFs or poly-A tails, and therefore these cDNAs are suspected to actually be genomic contaminants or unprocessed transcripts. These cloning attempts revealed that the P-element is inserted into a non-transcribed regulatory or intronic region that is affecting the bem gene. More thorough analysis made possible by the completion of the Drosophila genome has shown that the bem P-element is in the 120-kb intron between pum exons eight and nine. Also, other P-elements found in this same intron have been shown to cause their phenotypes due to disruption of Pum function (Lin and Spradling, 1997). These mutations constitute the pumilio<sub>ovarette</sub> class alleles and will be discussed in more detail subsequently.
1.4 The role of *pum* in posterior pattern formation in the developing Drosophila embryo

1.4.1 Production and characterization of the mutant

The *pum* gene is a member of the posterior pattern formation group of Drosophila genes. This group of genes is necessary for proper development of posterior features in the Drosophila embryo. Ruth Lehman and Christiane Nüsslein-Volhard identified the first *pum* allele in a screen for maternal effect mutations, and subsequently identified twelve other *pum* alleles based on non-complementation of the maternal effect phenotype (Nüsslein-Volhard et al., 1987) (see *pum*-plets in Table 1). Embryos from *pum* mutant females develop at most, two of the normal complement of eight abdominal segments (Nüsslein-Volhard et al., 1987). This phenotype was initially thought to be due to the improper transport of an abdominal signal in the developing embryo (Nüsslein-Volhard et al., 1987). Subsequent experiments, discussed below, uncovered the molecular mechanism by which *pum* functions in the developing embryo.
Table 1. List and description of *pum* alleles

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Allele Class</th>
<th>Phenotype</th>
<th>Mutagen</th>
<th>Molecular information</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pum</em>¹</td>
<td>Loss of function</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>²</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>³</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>⁴</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>⁵</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>⁶</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>⁷</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>A3890T; premature stop codon; No RNA binding domain</td>
</tr>
<tr>
<td><em>pum</em>⁸</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>⁹</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>Deletion from 4224-4498; No RNA binding domain</td>
</tr>
<tr>
<td><em>pum</em>¹⁰</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>¹¹</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>¹²</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>------</td>
</tr>
<tr>
<td><em>pum</em>¹³</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>EMS</td>
<td>G1330D; Mutation in RNA binding domain</td>
</tr>
<tr>
<td><em>pum</em>¹⁶⁸⁸</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>P-element</td>
<td>Intron between exons 3&amp;4</td>
</tr>
<tr>
<td><em>pum</em>¹⁰⁰³</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>P-element</td>
<td>Intron between exons 8&amp;9</td>
</tr>
<tr>
<td><em>pum</em>¹²⁰³</td>
<td>hypomorph</td>
<td>lethal</td>
<td>P-element</td>
<td>Intron between exons 8&amp;9</td>
</tr>
<tr>
<td><em>pum</em>¹²⁷⁷</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>P-element</td>
<td>Intron between exons 8&amp;9</td>
</tr>
<tr>
<td><em>pum</em>¹⁴⁸⁰⁶</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>P-element</td>
<td>Intron between exons 8&amp;9</td>
</tr>
<tr>
<td><em>pum</em>¹⁶⁸⁹⁷</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>P-element</td>
<td>Intron between exons 8&amp;9</td>
</tr>
<tr>
<td><em>pum</em>¹⁷⁰⁹⁸</td>
<td>hypomorph</td>
<td>Female sterile</td>
<td>P-element</td>
<td>Intron between exons 8&amp;9</td>
</tr>
</tbody>
</table>

----- indicates that no molecular information is available

(adapted from Fly-base, http://flybase.bio.indiana.edu )
1.4.2 Cloning and molecular characterization of *pum*

The cloning and molecular characterization of the gene responsible for the *pum* mutation was accomplished by two groups in 1992 (Barker et al., 1992; Macdonald, 1992). The gene structure was found to be unusual in that it spans at least 160 kb, contains thirteen exons, and has one large intron of approximately 120 kb (Barker et al., 1992; Macdonald, 1992). The *pum* gene is located at cytological position 85D1, 2 and was predicted to encode a 160-kDa cytoplasmic protein (Barker et al., 1992; Lehmann and Nusslein-Volhard, 1991; Macdonald, 1992). More recent western analysis revealed that *pum* encodes three different protein isoforms corresponding to the molecular sizes of 156-, 130-, and 98-kDa (Parisi and Lin, 1999). The Pum protein consists of regions enriched in single amino acids as well as eight tandem repeats of a 36 amino acid unit (Macdonald, 1992). Several additional RNA binding proteins which contain a conserved series of tandem repeats like the ones present in the Pum protein have been discovered.

Initially, the *C. elegans* FBF protein which exhibits conservation of eight tandem repeats that mediate FBF binding to the 3’ UTR of *fem-3* mRNA
was identified (Zhang et al., 1997). Thereby, these two proteins became the first two members of the PUF domain (Pum and Ebf) containing protein family. More recent studies have shown that proteins containing a PUF domain are members of an evolutionarily conserved family of RNA binding proteins found in organisms from yeast to humans (Coglievina et al., 1995; Kraemer et al., 1999; Miosga and Zimmermann, 1996; Purnelle and Goffeau, 1997; Tadauchi et al., 2001; Zamore et al., 1997). Also, a new PUF family member that is homologous to pum has recently been identified in Xenopus (Nakahata et al., 2001). Whereas orthologs of pum have been isolated in several organisms, the molecular mechanism by which pum functions has been characterized in most detail in the process of posterior pattern formation during Drosophila embryogenesis.

1.4.3 Elucidation of the molecular mechanism by which pum functions

Subsequent to cloning of the pum gene, extensive characterization of the molecular mechanism by which it functions was performed. Initially, the idea that pum was responsible for the distribution of the posterior determinant nanos (nos) mRNA was investigated (Barker et al., 1992). This
idea was found to be false when embryo in situ hybridizations showed that

*nos* mRNA and protein were expressed in an identical manner in *pum*

mutant embryos as in wild type embryos (Barker et al., 1992). The Nos

protein was seen to be present in a concentration gradient from posterior to

anterior in both cases (Barker et al., 1992). However, it was found that the

anterior pattern formation gene, *hunchback (hb)*, was expressed in an

aberrant fashion in the posterior of *pum* mutant embryos (Barker et al.,

1992). The Hb protein is present in a concentration gradient from anterior to

posterior in wild type embryos while it is present uniformly throughout *pum*

mutant embryos (Barker et al., 1992).

The Nos protein had previously been shown to block translation of

maternal *hb* transcripts (Tautz, 1988). This translational block was achieved

through sites present in *hb* mRNA 3' UTR known as *nanos* response

elements (NREs) (Wharton and Struhl, 1991). However, there was no

evidence of a direct interaction between Nos protein and *hb* mRNA (Murata

and Wharton, 1995). This led to the hypothesis that Nos binds to and

inhibits translation of *hb* mRNA through a mechanism involving the Pum
protein. This possibility was tested by in vitro binding assays which proved that Pum binds directly to *hb* mRNA through the NREs (Murata and Wharton, 1995). Also, transgenic flies that were carrying an altered *hb* gene (shown to be incapable of binding Pum in *in vitro* binding assays) produced embryos with fewer than the normal number of abdominal segments (Murata and Wharton, 1995). These experiments showed that the *in vivo* function of *pum* is dependent on its ability to bind directly to the NREs present in the 3'UTR of *hb* mRNA (Murata and Wharton, 1995). It was shown that Pum protein binds directly to *hb* mRNA NREs throughout the embryo but only inhibits translation by recruitment of Nos protein, which is only present in the posterior of the embryo (Sonoda and Wharton, 1999). Recently, it was discovered that another protein, Brain Tumor (Brat), in addition to Nos is recruited to the Pum *hb* mRNA complex (Sonoda and Wharton, 2001). Brat protein must be present in the complex in order for *hb* translation to be repressed in the posterior of the developing embryo, but the spatial information is still thought to be entirely supplied by Nos since Brat is expressed throughout the embryo (Sonoda and Wharton, 2001).
The mechanism by which *pum* and *nos* inhibit translation was then investigated. It was found that *hb* mRNAs were adenylated to a far greater extent in the posterior of *pum* and *nos* mutant embryos than in the posterior of wild type embryos (Wreden et al., 1997). This evidence showed that the inhibition of *hb* mRNA translation in the posterior of Drosophila embryos was correlated with de-adenylation of the *hb* message by a Pum/Nos complex (Wreden et al., 1997). This finding fits nicely into the emerging view that translation is directly related to the extent of poly-adenylation present on the 3'UTR of the mRNA (Wickens et al., 1997).

The RNA-binding domain of the Pum protein has been characterized (Wharton et al., 1998; Zamore et al., 1997). Production of various truncated Pum proteins and subsequent in vitro binding studies have shown that the eight tandem repeats with a small C-terminal extension define the minimal RNA binding domain of the Pum protein (Wharton et al., 1998; Zamore et al., 1997). Interestingly, this minimal Pum RNA-binding domain is sufficient to rescue *pum* mutant embryos from two abdominal segments to eight (Wharton et al., 1998). This observation shows that the minimal Pum
RNA-binding domain serves a dual function. It is not only responsible for the binding of *hb* mRNA but it also functions as a translational repressor in the absence of the remainder of the protein (Wharton et al., 1998).

**1.4.4 Pum structure determination**

Recently, in order to understand the structural basis of Pum RNA binding, Nos and Brat recruitment, and *hb* translational repression, the Pum RNA binding domain (PUF domain) has been crystallized and its structure solved (Edwards et al., 2001; Edwards et al., 2000). The crystal structure revealed that the PUF domain contains eight tandem repeats, each of which is composed of three α-helices, arranged in an arch (Edwards et al., 2001). The PUF domain is a member of an α-helix repeat protein super-family which has members such as the Armadillo (ARM) repeats present in β-catenin, the nuclear import protein Karyopherin, and HEAT repeats in protein phosphatase 2A (Conti et al., 1998; Edwards et al., 2001; Groves et al., 1999). The previously identified family members utilize their α-helical repeats to bind other proteins, whereas Pum utilizes its PUF domain to bind RNA (Edwards et al., 2001; Murata and Wharton, 1995). This finding
suggests that certain RNA binding proteins may implement similar features
to bind RNA as some proteins implement to bind peptides and other proteins
(Edwards et al., 2001). These structural studies, in combination with
mutational analysis of the Pum RNA binding domain, have begun to identify
which regions of the Pum RNA binding domain are involved in contacts
with *hb* mRNA, Nos, and Brat (Edwards et al., 2001). Specifically, the
concave portion of the arch is suggested to be the RNA interaction surface
while repeats seven, eight and nine are thought to be the portions of the PUF
domain that interact with Nos and Brat (Edwards et al., 2001). These studies
have provided the first structural insight on the Pum RNA binding domain
(PUF domain) and should begin to elucidate the complex structural basis for
translational repression by a protein complex (Edwards et al., 2001).

1.5 Other roles of *pum*

While *pum* was initially identified and characterized as a maternal
effect gene that functions in Drosophila embryo posterior pattern formation
through translational regulation, later research has revealed that *pum*’s
function in the fruit fly extends far beyond posterior pattern formation.
While the mechanism by which \textit{pum} functions in posterior pattern formation is characterized in the most detail, several examples of additional \textit{pum} roles will be discussed below.

1.5.1 The \textit{ovarette (ovt) class of pum mutations}

Germ line stem cells play an important role in a wide variety of organisms by providing a steady source of germ cells that can then be used for gamete production (Lin and Spradling, 1997). The germ line stem cells present in the most apical portion of the Drosophila ovariole provide an excellent model for the study of stem cell division and development (Lin and Spradling, 1997). Identification of a new protein structure called the spectrosome allowed Lin and Spradling to perform an in depth investigation of the vital yet poorly understood role of germ line stem cells in Drosophila gametogenesis (Lin and Spradling, 1997). The spectrosome is a structure rich in membrane skeletal proteins that segregates asymmetrically during cystoblast formation (Lin and Spradling, 1995). Antibody staining of spectrosome specific components and subsequent confocal and electron
microscopy allowed the asymmetric division of germ line stem cells to be observed (Lin and Spradling, 1997).

The two to three germ cells were observed to undergo striking asymmetry in spectrosome behavior during the process of mitosis (Lin and Spradling, 1997). This observation supported the previous hypothesis that Drosophila germ line stem cells are located in the most apical portion of the ovariole. This asymmetric division results in production of a daughter stem cell and a distally located cystoblast (Lin and Spradling, 1997). Subsequently, the cystoblast differentiates into an oocyte with nurse cells while the daughter stem cell continues asymmetric divisions producing many new cystoblasts.

In order to identify genes involved in regulating germ line stem cell asymmetric division in Drosophila, Lin and Spradling performed a screen of single P-element enhancer-trap female sterile mutants (Lin and Spradling, 1997). These mutants were analyzed by immunostaining and electron microscopy (Lin and Spradling, 1997). Mutants which underwent aberrant germ line stem cell division were expected to have small ovaries and two to
three germ line stem cells which had differentiated into egg chambers (Lin and Spradling, 1997). Many mutants, that fit these criteria were identified and found to belong to two different complementation groups (Lin and Spradling, 1997). One of the complementation groups was located in region 85C-D and the other was located in 34A-F of the polytene chromosome. All previously mapped mutants in the 34A-F region complemented the germ line stem cell asymmetric division mutants identified in the screen (Lin and Spradling, 1997). This group of mutants is known as *piwi* and will not be discussed further.

Conversely, the eight P-element mutants from region 85C-D (named *ovt*) showed failure to complement with the previously mapped *pum* mutants (Lin and Spradling, 1997). The trans-heterozygous flies produced in complementation tests between the eight new alleles and four old alleles of *pum* fell into four different phenotypic categories (Lin and Spradling, 1997). The first category was lethal, meaning that no trans-heterozygous offspring survived (Lin and Spradling, 1997). The second category exhibited defective oogenesis, and these trans-heterozygous females laid no eggs (Lin
and Spradling, 1997). The third category was termed maternal effect lethal and these trans-heterozygous females laid eggs that failed to hatch (Lin and Spradling, 1997). The final category of trans-heterozygous females were termed weak maternal effect lethal and were able to lay normal eggs of which only a few developed into adulthood (Lin and Spradling, 1997). These eight mutants define a new group of \textit{pum} alleles with different phenotypes than had been previously described for \textit{pum} mutants and are therefore referred to as \textit{pum}^{ovarette} mutations (Lin and Spradling, 1997) (see \textit{pum}^{1688}\textit{pum}^{7098} in Table 1).

Comparison of the fertility defects present in \textit{bem} and \textit{pum}^{ovr} females reveals some interesting similarities. Beyond the reduced fertility observed in both \textit{bem} and \textit{pum}^{ovr} females, similar defects in oogenesis seem to be present. This is evidenced by improperly developed egg chambers that are present in both \textit{bem} and \textit{pum}^{ovr} females (Parisi and Lin, 1999; Walters, 1997). In fact, some of the defects seen in \textit{bem} egg chambers are nearly identical to the defects seen in \textit{pum}^{ovr} egg chambers. For instance, females from both genotypes possess egg chambers with improper nurse cell nuclei
number (Parisi and Lin, 1999; Walters, 1997). The most strikingly similarity is that females from both genotypes have egg chambers with mis-localized nurse cell nuclei (Parisi and Lin, 1999; Walters, 1997). In both genotypes, mis-localized nurse cell nuclei are sometimes present throughout the developing egg chamber instead of being localized to the opposite end of the egg chamber from the oocyte (Parisi and Lin, 1999; Walters, 1997).

Since pum acts by repressing translation of hb mRNA and thus allows formation of abdominal segments, it was initially hypothesized that it may act by the same mechanism in germ line stem cell maintenance (Lin and Spradling, 1997). The working model was that the Pum protein may repress translation of germ line specific mRNA in stem cells until the protein product is needed at subsequent developmental times, (Lin and Spradling, 1997). Therefore, in pum mutants, inappropriate expression of these genes at a premature developmental time may lead to germ cells that assume a cystoblastic fate or proceed down an improper developmental pathway (Lin and Spradling, 1997). Identification of the germ line target mRNA was elusive, but recent studies have shown that pum and nos act together to
inhibit pole cell (germline progenitors) division by binding sequences
similar to NREs present in the 3' UTR of cyclin B (a cell cycle control gene)
mRNA thereby repressing its translation (Asaoka-Taguchi et al., 1999). An
as of yet unidentified pole cell specific factor (possibly similar to brat) is
also implicated by the observation that pum and nos do not affect cyclin B
translation in somatic cells (Richter and Theurkauf, 2001).

1.5.2 Neuronal phenotypes of pum

Mutation of the pum gene has also been shown to affect the process of
optic nerve projection in Drosophila larvae (Schmucker et al., 1997). The
larval optic nerve projection from the eye into the brain is a three-phase
process (Schmucker et al., 1997). The nerve changes directions at two
distinct signaling centers, P1 and P2 (Schmucker et al., 1997). After
reaching the second signaling center, P2, the nerve turns and enters the
central brain (Schmucker et al., 1997). In 1997, Schmucker et al. performed
a genetic dissection of this process in which EMS and P-element mutants
were screened, via immunostaining and microscopy, for defects in the
outgrowth of the larval optic nerve. Thirteen different genes that affect optic nerve projection at various stages were identified (Schmucker et al., 1997).

One of the mutants generated exhibits proper nerve growth and fasciculation until reaching P2, at which time the nerve undergoes terminal sprouting instead of taking the proper turn into the central brain (Schmucker et al., 1997). This leads to the production of several nerve branches that remain on the surface of the brain (Schmucker et al., 1997). This mutant, known as pumuckel (pkl), is caused by a P-element insertion in region 85D1.2 of the polytene chromosome, and was shown to be an allele of pum through complementation analysis (Schmucker et al., 1997). Further molecular characterization of this pum allele has not yet been performed; however it does illustrate another non-posterior pattern formation role of the pum gene. Moreover, this allele shows that pum function is important to the process of optic nerve pathfinding.

The Pum protein is also expressed and able to repress transcription in the neuronal tissue of the Drosophila eye (Wharton et al., 1998). This was discovered through the use of transgenic flies that express nos and an
adenovirus gene \((E1A)\) specifically in the eye (Wharton et al., 1998). When \(nos\) alone is expressed ectopically in the eye, ablation of the eye structure is observed (Wharton et al., 1998). The origin of this eye structure deformation is unknown, but it is dependent on the endogenous expression of \(pum\) as well as the ectopic expression of \(nos\) (Wharton et al., 1998). This is demonstrated by the fact that ectopic expression of \(nos\) in the eye of a \(pum\) mutant fly causes no aberrant eye structure (Wharton et al., 1998).

Presumably, \(nos\) and \(pum\) are acting in conjunction to inappropriately repress translation of an eye specific mRNA, when they are both present in the eye. Eye expression of an \(E1A\) transgene causes gross deformation of the eye (Wharton et al., 1998). However, if an NRE is present in the 3’ UTR of the eye expressed \(E1A\) transgene and \(nos\) is expressed ectopically in the eye of flies that are wild type for the \(pum\) gene, no deformation of the eye occurs (Wharton et al., 1998). Furthermore, if an NRE is present in the 3’ UTR of the eye expressed \(E1A\) transgene and \(nos\) is expressed ectopically in the eye of \(pum\) mutants, eye ablation occurs (Wharton et al., 1998). These experiments show that \(pum\) is endogenously expressed in the Drosophila eye
and is able to repress translation in a nos dependent manner, through the binding of NREs (Wharton et al., 1998). Interestingly, these experiments do not identify any neuronal targets of Pum nor do they give insight into the molecular details of Pum function in the eye. In the eye, pum may act alone or in conjunction with another protein to repress the translation of eye specific mRNAs. It is also possible that pum has no function in the eye of wild type flies that are not expressing nos. Nonetheless, these experiments leave open the possibility that pum functions to repress translation in the eye as well as in the developing embryo.

Recent studies have begun to directly address the expression and function of pum in the nervous system. One group has reported that pum mRNA and protein are expressed in the midline central nervous system, lateral central nervous system, and in the lateral bipolar dendritic neuron of the peripheral nervous system (Menon and Zinn, 2001). They have shown that pum loss of function mutants exhibit abnormal axon guidance phenotypes in the transverse and inter-segmental nerves (Menon and Zinn, 2001). Furthermore, they have shown that pum over-expression also causes
aberrant axon path finding in the inter-segmental nerve b motor pathway (Menon and Zinn, 2001). While these research efforts have not identified a neuronal target of *pum* translational repression or a mechanism for *pum* neuronal function, they do show that *pum* is important to the development of the neuromuscular system in Drosophila.

The examples described above along with the fact that certain strong *pum* alleles are lethal mutations (see *pum*4203 and in table 2) illustrate that there are a number of non-posterior pattern formation roles for *pum* in Drosophila.

1.6 Translational regulation by 3'UTR binding proteins

The Pum protein is not unique in its ability to regulate mRNA translation by binding to the 3' UTR of the targeted mRNA. Many proteins that regulate translation do so by binding the 3' UTR of the targeted mRNA, and this mechanism is emerging as a common translational regulation motif (Wickens et al., 1997). One such example is the murine seizure related gene, *PTZ-17* (Kajiwara et al., 1997). Injection of the *PTZ-17* transcript into Xenopus oocytes causes the appearance of large inward calcium currents
and an intracellular calcium concentration increase (Kajiwara et al., 1997).

Alterations of specific 3’ UTR sequences of the PTZ-17 gene caused either
the increase or disappearance of this calcium entry and these same 3’ UTR
sequences demonstrated interactions with 60- and 47-kDa proteins
(Kajiwara et al., 1997). These findings suggest that a particular region
within the 3’ UTR of the PTZ-17 gene is involved in PTZ-induced calcium
entry through specific interactions between the mRNA and RNA-binding
proteins (Kajiwara et al., 1997).

Another example of this translational regulation motif is the silencing
of LOX mRNA in erythroid differentiation (Ostareck et al., 1997). Although
LOX mRNA is present in early stages of erythroid differentiation, a control
element present in its 3’UTR confers translational inhibition until late stages
of erythropoiesis (Ostareck et al., 1997). This translation inhibition has
been shown to be due to the binding of hnRNP-K and hnRNP-E1 proteins to
this region of the 3’ UTR (Ostareck et al., 1997). The resulting mRNA
complex suppresses LOX translation until the protein is needed (Ostareck et
al., 1997).
1.7 The role of translational regulation in the nervous system

In the neuron, under default conditions, mRNAs are translated in the cell body, but certain mRNAs are transported to either the dendrites or the axon terminal. Local protein synthesis occurring in these locations is important to proper control of long term facilitation in Aplysia and rats (Steward, 1997; Steward et al., 1996). These observations give insight into how *pum* may function in the nervous system and account for the behavioral and electrophysiological phenotypes of *hem* flies.

1.7.1 Translational machinery and mRNA in dendrites

Initial identification of translational machinery in the dendrites of central nervous system (CNS) neurons led to the hypothesis that a certain group of mRNAs would be localized in the dendrites in order to allow certain proteins to be synthesized locally in post-synaptic sites (Steward, 1997). The identification of many neuronal genes and subsequent in situ hybridizations probed with these genes showed that certain mRNAs are present in dendrites and the hypothesis was therefore confirmed (Steward, 1997). Also, translation of these dendritic mRNAs has been shown to be of
importance to proper neuronal function (Kang and Schuman, 1996). This was shown by the fact that neurotrophic factor induction of long term facilitation in rat hippocampus was abolished by the localized application of translation inhibiting drugs to dendrites (Kang and Schuman, 1996). The disruption of translation was found to occur specifically in the dendrites of the post-synaptic neuron (Kang and Schuman, 1996).

1.7.2 Translational machinery and mRNA in axon nerve terminals

A certain subset of neuronal mRNAs has been shown to be present in the axon terminus of CNS neurons by in situ hybridizations as well (Steward, 1997). Initially, the significance of these axon terminal mRNAs was not clear because translational machinery had not been identified in the axon terminal (Steward, 1997). However, it has been shown that long term facilitation in Aplysia neurons can be synapse specific as well as being a result of local protein synthesis (Martin et al., 1997). This was illustrated by the fact that the application of serotonin (which induces long term facilitation in Aplysia neurons) to a single axon of a bifurcated neuron results in long term facilitation only in the axon treated with serotonin.
(Martin et al., 1997). This branch specific long term facilitation was seen to be due to local protein synthesis in the pre-synaptic axon terminal (Martin et al., 1997).

1.7.3 Translational regulation in the Drosophila nervous system

Whereas initial efforts to investigate the role played by translation in the nervous system centered on identifying translational machinery within dendrites and axon terminals, more recent efforts have focused on determining the mechanism by which this machinery elicits effects on development, anatomy, and physiology. For example, the translational initiation factor (eIF4E) and the poly(A)-binding protein (PABP) have been identified in subsynaptic compartments (post-synaptic compartments on the muscle surface just beneath the neve terminal) of the larval nmj (Gallie, 1998; Sigrist et al., 2000; Sonenberg and Gingras, 1998). These aggregates have recently been shown to be the centers of concentrated synaptic translation (Sigrist et al., 2000). Also, the well characterized hyper-excitabile ether-a-gogo Shaker double mutant and the cAMP phosphodiesterase mutant dunce both exhibited an increase in eIF4E/PABP aggregates (Budnik
et al., 1990; Davis and Kiger, 1981; Sigrist et al., 2000). These findings show that there is a relationship between neuronal activity and subsynaptic translation levels and are consistent with the emerging view that synaptic activity can control translation (Balling et al., 1987; Sigrist et al., 2000; Weiler et al., 1997; Wu and Bag, 1998). It is also important to note that this increase in synaptic translational aggregates is accompanied by alterations in the levels of the synaptic junctional protein Fascilin II, a cell adhesion molecule, and DGlur-IIA, a post-synaptic glutamate receptor subunit (Schuster et al., 1996; Sigrist et al., 2000). Furthermore, it was observed that mutants which exhibited an increased level of translational aggregates had significantly larger nmjs (Sigrist et al., 2000). Overall, these results suggest that translation is a common method of achieving long term changes in neuronal anatomy and physiology (Sigrist et al., 2000).

Translational regulation has also been shown to be of great importance to neuronal development in Drosophila (Okabe et al., 2001). During mechanosensory bristle development, asymmetric cell division directs the establishment of non-neuronal and neuronal cells (Guo et al.,
1995; Reddy and Rodrigues, 1999). The asymmetry between neuronal and non-neuronal cell lineage is controlled by Notch signaling (Jan and Jan, 1998). Downstream of Notch, a zinc-finger transcriptional repressor, tramtrack69 (ttk69), serves as the non-neuronal determinant (Giesen et al., 1997; Guo et al., 1995). It has recently been shown that the regulation of Ttk69 protein expression is achieved at the translational level (Okabe et al., 2001). Also, this translational repression is achieved by direct interactions between the RNA-binding protein Musashi to the 3’UTR of ttk69 mRNA (Nakamura et al., 1994; Okabe et al., 2001). Interestingly, this mechanism is strikingly similar to the one employed to maintain germ line stem cells through Pum repression of cyclinB mRNA (Asaoka-Taguchi et al., 1999).

Repression of ttk69 expression results in a neuronal cell fate, whereas ttk69 expression results in a non-neuronal cell fate (Giesen et al., 1997; Guo et al., 1995). Furthermore, vertebrate homologs of Notch and musashi are expressed in the developing vertebrate nervous system suggesting that translational regulation is an evolutionarily conserved mechanism for
directing nervous system development (Gaiano et al., 2000; Johansson et al., 1999; Sakakibara et al., 1996).

Additional research on the role of translational regulation in Drosophila nervous system has begun to give some insight into the molecular basis for human mental retardation (Zhang et al., 2001). The fragile X mental retardation gene (FMRI) encodes an RNA-binding translational repressor, FMRP (Ashley et al., 1993; Brown et al., 1998; Verkerk et al., 1991). However, the mRNA target/s of FMRP are unknown. Recent studies with the Drosophila FMRI homolog, dfxr, have given insight into the molecular function of this gene (Zhang et al., 2001). Dfxr loss-of-function mutants exhibit synaptic terminals that are larger than wild-type terminals whereas dfxr over-expression results in fewer and larger synaptic boutons (Zhang et al., 2001). The abnormal neuronal structure is accompanied by altered neuronal physiology (Zhang et al., 2001). Most notably, the dfxr loss-of-function mutants exhibit a significantly smaller response to photoreceptor depolarization (Zhang et al., 2001). The anatomical and physiological consequences associated with dfxr loss of
function are similar to the phenotype seen in *futsch*, a gene encoding a microtubule associated protein (Hummel et al., 2000; Roos et al., 2000). It was also shown that dFxr associates with *futsch* mRNA and that *dfxr futsch* double mutants regain normal synaptic structure and physiology (Zhang et al., 2001). These findings suggest that Dfxr translationally represses *futsch* mRNA and thereby regulates microtubule based neuronal growth and function (Zhang et al., 2001). These studies may eventually prove to be vital in understanding the molecular basis for human Fragile X syndrome, the most common inherited disorder that causes mental retardation (Verheij et al., 1993; Verkerk et al., 1991).

Overall, these recent Drosophila studies serve to provide insight into the role played by translational regulation in neuronal development and function and to show that proper translational regulation is vital to many aspects of proper invertebrate nervous system function and development.

### 1.7.4 Translational regulation in the vertebrate nervous system

While the mechanistic details of translational regulation have been deciphered to a greater extent in invertebrates, there is evidence that similar
mechanisms are important to vertebrate nervous system development and function (Boyl et al., 2001; Gao and Keene, 1996; Miyashiro et al., 1994). Earlier studies found that RNA-binding proteins, Hel-N1 and Hel-N2, are associated with poly(A)-mRNA in medulloblastoma cells and that this association is important for neuronal differentiation (Gao and Keene, 1996). Also, differential mRNA distribution was implicated in being important to neuronal function in rat hippocampi (Miyashiro et al., 1994). More recently, specific mRNA translational regulation has been shown to be of great importance to mouse brain development (Boyl et al., 2001). Specifically, Otx-2, which plays an important role in brain development by specifying the neuroectoderm fated to become the for-mid and rostral hindbrain, must be translationally controlled in the epiblast and neural progenitor cells in order for proper brain development to occur (Boyl et al., 2001). The translational regulation of Otx-2 is mediated by sequences present in its 3' UTR (Boyl et al., 2001). This conclusion was drawn from experiments on mice that had the 3' UTR of Otx-2 perturbed by small insertions of λ phage DNA and therefore exhibit aberrant brain development (Boyl et al., 2001). Whereas
specific RNA binding proteins that interact with Otx-2 mRNA have not been identified it was observed that Otx-2 mRNA with perturbed 3' UTR sequences exhibited difficulty forming polyribosome complexes (Boyl et al., 2001).

Overall, these studies provide experimental evidence that translational regulation mediated by 3' UTR sequences are important in vertebrate brain development. Furthermore, these results taken in conjunction with the invertebrate experiments implicate translational regulation as an evolutionarily conserved mechanism for achieving proper neuronal development, anatomy, and physiology (Boyl et al., 2001).

1.8 The role of hb in the developing Drosophila nervous system

Beyond the well defined role of hb in embryogenesis, hb is also expressed in early neuroblasts where Hb functions as a transcription factor (Brody and Odenwald, 2000). In the cellular blastoderm stage hb regulates the expression of another transcription factor, encoded by nubbin (nub), which functions in nervous system and wing development (Brody and Odenwald, 2000). Expression of hb is limited to the early stages of nervous
system development where *nub* transcription is repressed (Brody and Odenwald, 2000). The absence of *hb* and the presence of *nub* mRNA accumulation characterize intermediate stages of nervous system development (Brody and Odenwald, 2000). During late stages of nervous system development, a third transcription factor gene, *castor (cas)*, is expressed (Brody and Odenwald, 2000). The *cas* expression once again represses *nub* transcription (Brody and Odenwald, 2000). The temporal expression patterns of *hb* and *cas* are therefore needed to silence *nub* transcription in early and late nervous system development. This is important due to the fact that the particular sub-lineage taken by a neuroblast is dependent on which of the three transcription factors it is expressing (Brody and Odenwald, 2000). The mechanisms by which *hb* expression is regulated in the developing nervous system are not yet understood, but it is clear that improper *hb* expression would result in improper neuroblast sub-lineage determination and subsequently improper nervous system development.
CHAPTER 2

MATERIALS AND METHODS

2.1 Fly stocks and crosses

2.1.1 Husbandry

All fly stocks were maintained on standard cornmeal/agar Drosophila media at room temperature (20-22°C). Bottle stocks were transferred to fresh media once per month and vial stocks were transferred every two to three weeks.

2.1.2 Genetic strains

The wild-type strain from which bem was produced is named S880. Isogenized second and third chromosomes and w on the X-chromosome characterize S880. The bem mutation is caused by a single P-element (P-lacw) insertion on the third chromosome of S880.

The pumovt and pumrevertant lines (Lin and Spradling, 1997) were kindly provided by the lab of Dr. Haifan Lin (Duke University, Durham, NC). The nos-pum rescue construct (Barker et al., 1992) and pum+ parental control lines were kindly provided by the lab of Dr. Ruth Lehmann (New York
University, New York, NY). The Bloomington Stock Center provided \textit{pum}^{3}, \textit{pum}^{13}, \textit{hb}^{4}, and \textit{hb}^{12} and the Umea Stock Center provided \textit{pum}^{7} and \textit{pum}^{9}.

The \textit{pum}^{ovt} mutants are all caused by P-element insertions (\textit{PZ} vector) within the \textit{pum} transcription unit (Lin and Spradling, 1997). The \textit{pum}^{1688} P-element is located in the \textit{pum} intron between exons 3 and 4 whereas all of the other \textit{pum}^{ovt} alleles are caused by a P-element inserted into the large intron between \textit{pum} exons eight and nine (Parisi and Lin, 1999). The \textit{pum}^{revertant} is a \textit{pum}^{1688} line produced by precise excision of the P-element.

The \textit{pum}^{7} allele is an ethyl methane sulfonate (EMS)-induced A to T point mutation at nucleotide 3890, which causes a premature stop codon at amino acid 949 and encodes a Pum protein product without an RNA binding domain (Forbes and Lehmann, 1998; Tearle and Nusslein-Volhard, 1987).

Similarly, \textit{pum}^{9} is an EMS-induced deletion of nucleotides 4224 to 4498 resulting in production of a Pum protein product that lacks the RNA binding domain (Forbes and Lehmann, 1998; Tearle and Nusslein-Volhard, 1987).

An EMS-induced point mutation within the Pum RNA binding domain that results in a single amino acid substitution, G1330D, characterizes \textit{pum}^{13} and
produces a protein product which is able to bind \( hb \) mRNA but is unable to repress translation (Tearle and Nusslein-Volhard, 1987; Wharton et al., 1998). There has been no molecular information reported on \( pum^d \).

Mutations and balancers not described here can be found in *The Genome of Drosophila melanogaster* (Lindsley and Zimm, 1992).

### 2.1.3 Electrophysiological balancer

For electrophysiological experiments on third instar larvae, the *pum* and *bem* mutations were balanced over a *TM6* balancer chromosome marked with the dominant *Tubby* (*Tb*) marker so that homozygous and heterozygous larvae could be distinguished. The *Tb* mutation causes larvae to be shorter and fatter than wild type; this allows homozygous mutants to be identified by their normal length and girth.

### 2.1.4 Construction of \( hb \) *bem* double mutant and its \( hb^+ \) *bem* control

Since *pum* and *hb* are less than one map unit apart on the third chromosome, the following method was used to produce the \( hb^+bem^+/hb^+bem^+ \) double mutant and \( hb^+bem^+/hb^+bem^+ \) control lines. Third chromosomes containing the *bem* P-element, the *scarlet* (*st*) marker gene,
and $hb^{12}$ mutation were allowed to recombine in females. Recombinants that were mutant for the $st$ marker gene and were expressing the $white$ ($w$) gene product from the $bem$ P-element were selected by eye color. The recombinant chromosomes were then crossed to the amorphic $hb^t$ and $hb^{12}$ lines and the resulting trans-heterozygotes were tested for lethality. A $hb^t$ $bem^t$ double mutant chromosome that was lethal when combined with $hb^t$ and $hb^{12}$ as well as several $hb^t$ $bem^t$ control chromosomes that were viable when combined with $hb^t$ and $hb^{12}$ were obtained, maintained over $TbTM6$, and crossed to $bem/TbTM6$ for generation of third instar larvae for experimentation.

2.2 Molecular techniques

2.2.1 Plasmid DNA preparation

Small scale ($\leq 20 \mu g$ of high copy plasmid DNA) DNA preparations from three ml $E. coli$ overnight cultures in Luria-Bertani (LB) media were performed using the QIAgen QIAprep Spin Plasmid Kit in accordance with the manufacturer’s instructions.
Large scale (≤ 500 μg of high copy plasmid DNA) DNA preparations from 100 ml *E. coli* overnight cultures in Luria-Bertani (LB) media were performed using the QIAgen QIAfilter Maxi Kit in accordance with manufacturer’s instructions.

### 2.2.2 Sub-cloning

All sub-cloning techniques including but not limited to DNA transformations into *E.coli*, restriction digests, and ligation were performed as described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989).

### 2.2.3 Polymerase chain reaction protocols

Primers that serve to amplify the desired portion of template DNA were designed to have approximately 50% cytosine and guanine content, to end in a cytosine or guanine especially at the 3’ end, and to lack any obvious homologies (hairpins or primer dimers).

The polymerase chain reactions (PCR) consisted of template DNA, 1X Promega PCR buffer with MgCl₂, 10 picomoles of each primer, 50 mM dNTPs, and two units of Promega Taq polymerase in a total volume of 50
μl. The reaction was initially incubated at 95°C for five minutes in order to
denature the DNA. Then 35 cycles of a standard three temperature PCR
protocol (95°C for 30 seconds, 55°C for 30 seconds, and 72°C for one
minute per kilobase of template to be amplified) was performed. A final
five-minute incubation at 72°C was then used to allow complete
incorporation of dNTPs into the newly formed product. The thermal-cycler
used to perform all reactions was a Perkin-Elmer Cetus 9600 Gene Amp
PCR system.

When necessary, the PCR product was purified to remove remaining
buffers, enzymes, and unincorporated dNTPs using the QIAGen QIAquick
PCR Purification Kit in accordance with manufacturer’s instructions.

The *pum* Northern probe was a PCR product produced in this manner
using primers that amplify a product that corresponds to *pum* cDNA
nucleotides 3600 to 4400 and a template vector containing the entire *pum*
cDNA (pNB40/R7-1) from the lab of Ruth Lehmann (Barker *et al.* 1992).
2.2.4 Bacteriophage library screen

The Tamkun et al. *D. melanogaster* genomic bacteriophage library (1992) was screened using the bacteriophage growth, purification, and recombinant isolation technique described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989). Over 50,000 genomic plaques were screened to ensure a greater than 99% chance of isolating single copy sequences. LE392 plating bacteria were used and recombinant DNA was harvested from the phage as described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989).

2.2.5 Probe radio labeling reaction

Probes used in Northern and Southern blots were radio labeled in a manner similar to that described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989). Briefly, 20 to 50 nanograms of the probe, 2 μl of bovine serum albumin (BSA, 10mg/ml), 5 μl of fresh $^{32}$P-dCTP (specific activity > 3000 Ci/m mole), 5 units of the *E. coli* DNA polymerase I Klenow fragment, and 10 μl of 5X oligonucleotide labeling buffer (250 mM Tris-Cl, pH 8.0; 25 mM MgCl$_2$; 5 mM β-mercaptoethanol; 2 mM each of
dATP, dGTP, dTTP; 1M Hepes, pH 6.6; 1 mg/ml random primer oligonucleotide) in a total volume of 50 µl were incubated together overnight at 37°C.

The radio-labeled probe was then purified away from residual oligonucleotides, buffers, and unincorporated dNTPs by spinning through a size exclusion Sephadex G-50 column and stored at -20°C until used for membrane hybridization. This storage time was kept to a minimum (< 24 hours) because radioactivity increases the degradation rate of the probe.

2.2.6 Southern blot technique

Southern blots were performed in a manner very similar to that described in Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989). Briefly, the DNA to be analyzed was separated by size on a one-percent agarose gel. The gel was then soaked in 1.5 M NaCl, 0.5 N NaOH for 45 minutes in order to denature the DNA. Next, the gel was neutralized by soaking in 1.5 M NaCl, 1.0 M Tris (pH 7.8) for 30 minutes. The DNA was then transferred to a nylon membrane with a pore size of 0.45 µm by capillary action. The DNA was then cross-linked to the membrane with
ultraviolet irradiation and the membrane was incubated at 42°C for at least
two hours in 6X SSC, 50% formamide, 0.5% SDS, and 100 μg/ml salmon
sperm DNA. The radio labeled probe was then added to the incubating
membrane and the probe allowed to hybridize to the membrane at 42°C
overnight. The membrane was then washed at 60°C with gentle agitation in
0.1% SSC, 0.1% SDS until the level of radiation remaining on the
membrane was suitable for exposure to film/imaging plate (between 20 to 50
counts per second). Finally, the membrane was dabbed dry, wrapped in
plastic wrap, and exposed to either film with an intensifying screen
overnight or to a phosphor imaging plate for 30 minutes to three hours.
Results were visualized by either film development in an AFP Imaging X-
ray Film Processor or by using Fuji MAC-BAS imaging software (imaging
plate). The membrane was then stripped in boiling wash buffer so that the
same membrane could be used in subsequent hybridizations if desired.

2.2.7 mRNA isolation

When appropriate, flies were decapitated by freezing in liquid
nitrogen and subsequent vigorous shaking through an U.S.A. Standard Sieve
#25, which has an opening size of 710 μm. mRNA was extracted from 40 flies or 800 heads. Total RNA was obtained by a Trizol : chloroform extraction. Briefly, one ml of Trizol reagent (Gibco BRL) was added to the Drosophila tissue immediately upon harvesting. The tissue was then homogenized in the Trizol by grinding with a pellet pestle. Next, 200 μl of chloroform was added and the tubes shaken vigorously by hand for 15 seconds. The samples were then spun at 4°C and 12,000 g for 15 minutes. After spinning, the aqueous (top) phase was removed and incubated for 10 minutes at room temperature with 500 μl of isopropyl alcohol to precipitate the total RNA. The samples were spun again at 4°C and 12,000 g to pellet the total RNA. The pellet was then washed once by vortexing in one ml of 75% ethanol (DEPC treated). The pellet was then air dried and re-suspended in 50 μl of DEPC-treated H₂O.

Selection for mRNA was performed using an oligo-dT resin from the QIAgen Oligotex mRNA Kit in accordance with manufacturer’s instructions. The mRNA was then stored at -80°C until it was needed for further experimentation.
2.2.8 Northern blot technique

Northern blots were performed in a manner similar to that described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989).

Briefly, the mRNA to be analyzed was run on a 1%
agarose/formaldehyde/MOPS gel. Next, the gel was soaked in five volumes of H₂O in order to remove the formaldehyde and the mRNA was then transferred to a nylon membrane by capillary action. The mRNA was then cross-linked to the membrane by ultraviolet irradiation. The mRNA was then visualized on the membrane, to ensure that the samples have not degraded, by staining the membrane in methylene blue (0.02 % methylene blue, 0.5 M NaAc, pH 5.2) and de-staining in 20 % ethanol until the bands were visible. The stained membrane was then photographed prior to stripping the methylene blue from the membrane by washing in 0.2 SSPE, 1.0 % SDS for 15 minutes. Next, the membrane was incubated in 5X SSPE, 50% formamide, 5X Denhardt’s, 1.0 % SDS and 100 μg/ml salmon sperm DNA at 42°C for two to four hours. Then, the radio labeled probe was added to the incubation and the probe was hybridized to the membrane at
42°C overnight. The membrane was washed in 2X SSPE, 2.0 % SDS at 65°C with gentle agitation until the radiation level of the membrane was suitable for exposure to film or an imaging plate (20 to 50 counts per second). The membrane was then dabbed dry, wrapped in plastic wrap, and exposed to either film with an intensifying screen overnight or to a phosphor imaging plate for 30 minutes to three hours. Results were visualized by development in an AFP X-ray Film Developer or by Fuji MAC-BAS imaging software (imaging plate). The probe was then stripped from the membrane by incubation with boiling wash buffer so that the membrane could be used in future hybridizations if desired.

In order to decrease degradation of the mRNA, all buffers used in the Northern were treated with DEPC (1 ml per liter) overnight and autoclaved prior to use and all lab equipment used was first soaked in 0.5 M NaOH, rinsed with copious amounts of Millipore purified H₂O, and autoclaved when possible.
2.2.9 Protein isolation

Frozen heads were shaken through a sieve in the same manner described for mRNA isolation. Protein was extracted from the heads of 40 flies by freezing the tissue on dry ice, grinding the frozen tissue to a powder with a pellet pestle, homogenization of the powder in 2X SDS extraction buffer (0.1 M Tris-Cl, pH 6.8; 4.0% SDS; 20% glycerol), and two cycles of freezing for 10 minutes followed by boiling for 10 minutes. The samples were then spun at top speed in a table top centrifuge (13,000 rpm), the supernatant removed, and the remainder of the tissue discarded. The supernatant was then stored at -80°C until it was needed for further experimentation.

Prior to running the protein samples on a gel, dithiothreitol (DTT) and protease inhibitor was added and the final volume adjusted to give an SDS concentration of 2% (approximately a two-fold dilution).

2.2.10 Western blot technique

Western blots were performed in a manner similar to that described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989). Briefly,
the protein to be analyzed was run on a 6% denaturing SDS/acrylamide resolving gel with a 5% stacking gel. The protein was then transferred from the gel to a nylon membrane with a pore size of 0.45 μm using a Bio-Rad Trans Blot SD semi-dry transfer cell. The membrane was then blocked in 1X blotto (1X TBS, 3% powdered milk, 0.2% Tween 20) for one hour. Next, the membrane was incubated overnight at 4°C with gentle rocking with the primary antibody diluted in 1X blotto. The diluted and used primary antibody was saved and re-used repeatedly. The membrane was then washed three times for 10 minutes each in 1X blotto at room temperature with gentle agitation and then the secondary antibody diluted in 1X blotto was added and allowed to incubate with the membrane for one hour at room temperature. Three final 10 minute room temperature washes in 1X blotto followed by one 5 minute wash with 1X PBS were then performed prior to the color reaction. Results were visualized using a Pierce Bioluminescent 3,3′-diaminobenzidine (DAB) Substrate Kit in accordance with manufacturer’s instructions and subsequent exposure of the membrane
to film for one to 30 minutes. The film was then developed using an AFP Imaging X-ray Film Processor.

The anti-Pum antibody used was a rabbit polyclonal called anti-PUM-I that was produced from the internal portion of the Pum protein (Zamore et al. 1997). The anti-PUM-I antibody was kindly provided by the lab of Dr. Ruth Lehmann and was used at a 1:3000 dilution as directed by Lehmann lab protocol (Forbes and Lehmann 1998). Anti-PUM-I was used in conjunction with a 1:2000 dilution of sheep-anti-rabbit IgG conjugated to horseradish peroxidase from Boehringer Mannheim used in accordance with manufacturer’s instructions.

2.3 Behavioral and fertility analyses

2.3.1 Negative gravitaxic analysis

Negative gravitaxis experiments were performed by placing single six-day-old males of the appropriate genotype in an empty vial. The fly was then banged to the bottom of the vial and the time required for the fly to right itself and climb five centimeters up the side of the vial was recorded.
2.3.2 Flight analysis

Flight experiments were performed by emptying single flies onto a flat and clean tabletop. When necessary, flies were encouraged to fly by prodding with a paintbrush. Any flies that were unable to achieve flight after five seconds of prodding were deemed non-fliers.

2.3.3 Fertility analysis

Single females of the appropriate genotypes were crossed to three wild-type males. The parental flies were removed from the vial 10 days after initiation of the cross. Offspring were counted and cleared from the vials as they eclosed. Counting was terminated and offspring number totaled once adult F1 offspring ceased eclosing.

2.4 Electrophysiological experiments

2.4.1 Third instar larval dissection

Larvae were pinned down in filter sterilized 1X Jans Ringers solution containing 0.128 M NaCl, 2.0 mM KCl, 4.0 mM MgCl₂, 0.34 M sucrose and 5.0 mM Hepes at a pH of 7.1 (Jan and Jan, 1976). Next, the larvae were cut open down the dorsal midline, the entrails removed, and the motor nerves
severed from the ventral ganglia. Finally, the pinned down and dissected pelts were washed three times with 1X Jans Ringers.

2.4.2 LTF onset rate determination

Individual nerves were pulled into a suction electrode and stimulated at various frequencies using a World Precision Instruments A310 Accupulser and a Dagan Cornerstone S-910 stimulus isolator. The response of the corresponding muscle was recorded and digitized with a Tektronix 2201 Digital Storage oscilloscope and a MacIIci with GW Instruments Superscope II software in order to determine the number of stimuli needed to elicit the onset of LTF. LTF onset rates were determined in the presence of 0.1 mM quinidine and at an external Ca$^{2+}$ concentration of 0.15 mM.

2.4.3 Ejp failure rate determination

Failure rates were determined using third instar larvae that were dissected in the same manner and using the same electrophysiological instruments as described above. The nerves were then stimulated for 10 seconds at a frequency of one Hz. The number of stimuli per 10-second stimulation train that failed to evoke any muscular response was recorded.
Failure rate analysis was performed in the absence of quinidine and at an
external Ca$^{2+}$ concentration of either 0.15 mM or 0.10 mM.

2.5 Production of UAS-*pum* transgenic flies

The *pum* coding sequence was removed from the pNB40/R7-1
plasmid (Barker et al., 1992), which contains the entire *pum* coding
sequence as well as *pum* 5' and 3' UTR, (kindly provided by the lab of Dr.
Ruth Lehmann) via digestion with *NheI* and *XbaI*. The *pum* sequence was
then cloned into the *XbaI* site of pUAST (Brand and Perrimon, 1993) in
order to create pUAS-*pum*. Transgenic flies were then produced using the
standard embryo pole plasm injection technique. Briefly, a mixture of
pUAS-*pum*, Δ2-3 (a helper vector which encodes a transposase), glycerol,
and green food coloring were injected into the posterior pole plasm of zero
to 1.5 hour old dechorionated embryos from yw parents. Transformants
were identified by their yellow to orange eye color, which results from the
*w* $^+$ gene that is present on the pUAS-*pum* plasmid. The transformant line
chosen for further experimentation had the integration site on the second
chromosome and this stock was named UAS-*pumII*. 
2.6 Immunocytological studies

2.6.1 Embryo collection and fixation

The embryos to be analyzed were collected from egg lay bottles on grape plates smeared with yeast paste and subsequently fixed. Briefly, the embryos were rinsed with H$_2$O, dechorionated in 100% bleach for two minutes, washed with 0.02% Triton, and then rinsed again with copious amounts of H$_2$O. The embryos were then fixed in heptane and Cohen fix buffer (120 mM Hepes; 2.67 mM MgSO$_4$; 1.33 mM EGTA, pH 6.9). Next the embryos were devitellinized in heptane and methanol (devitellinized embryos fall to the bottom) and washed two times in methanol. Finally, the embryos were washed three times in ethanol and stored in ethanol at -20°C until staining was performed.

2.6.2 Embryo antibody staining

Initially, embryos were equilibrated by washing for five minutes in 50% ethanol, 50% BBT (0.14 M NaCl, 140 mM Na$_2$HPO$_4$, 60 mM KH$_2$HPO$_4$, 0.1% Tween 20, 0.1% BSA) followed by three five minute washes in BBT. The BBT diluted primary antibody was then added for an
overnight incubation at 4°C with gentle shaking. The primary antibody was
saved and reused at least four times as long as no bacterial contamination
had occurred. The embryos were then washed twice for 10 minutes in BBT
in order to remove residual primary antibody. The BBT diluted secondary
antibody was then added for a two-hour incubation at room temperature with
gentle shaking. Residual secondary antibody was then washed away with
three 10 minute washes in PBT (0.14 M NaCl, 140 mM Na₂HPO₄, 60 mM
KH₂HPO₄, 0.1% Tween 20). Next, the color reaction was performed using a
Vector DAB Peroxidase Substrate Kit in accordance with manufacturer’s
instructions. Once the desired stain appeared the embryos were washed five
times in PBT. The stained embryos were then dehydrated with an ethanol
series and mounted on a glass slide under a cover slip in Permount.

The rat polyclonal anti-Pum antibody used for embryo staining
experiments was provided by the lab of Dr. Paul MacDonald and it was
applied in a 1:250 dilution. The anti-Pum antibody was used in conjunction
with a sheep anti rat horseradish peroxidase conjugated secondary antibody
from Boehringer Mannheim that was used at a 1:2500 dilution in accordance with manufacturer's instructions.
CHAPTER 3

EXPERIMENTAL RESULTS

3.1 Isolation of genomic DNA flanking the *bem* P-element

Initial attempts to clone the *bem* gene originated from the P-element insertion. Prior to my arrival in the lab, the plasmid rescue technique was used to isolate a 6-kb fragment of DNA flanking the P-element. This 6-kb fragment was then used to probe phage genomic and cDNA libraries. Unfortunately, clones isolated from the genomic library screen contained no open reading frames (ORFs) and the putative cDNAs were determined to be genomic contaminants which also contained no ORFs. A more detailed description of the initial attempts to clone the gene disrupted by the *bem* P-element is present in chapter one of this thesis.

My initial efforts were focused on cloning the *bem* gene and to these ends the same genomic library (Tamkun et al., 1992) that was screened previously was screened again with new probes in an effort to isolate more DNA flanking the P-element. The new probes were PCR products derived from the most distal ends of the previously isolated P-element flanking
genomic DNA. After the primary screen twelve plaques were isolated, however secondary and tertiary screens revealed that five of the positives from the primary screen were false. DNA from the remaining seven plaques was amplified and extracted. Southern blots of DNA from the positive plaques were independently probed with radio-labeled probes spanning the previously sequenced P-element flanking genomic DNA in order to determine the direction and degree of extension of the newly identified clones.

Unfortunately, all of the new clones were found to span the majority of the previously cloned and sequenced region flanking the P-element and thereby not to extend significantly beyond this region. Therefore, no further characterization of these clones was performed. This result was not entirely surprising since Karina Walters obtained similar results when she attempted a final step in the chromosome walk using the same genomic library and probes from similar regions.

The initial cloning attempts suggested that the *bem* P-element is inserted into a large non-transcribed regulatory or intronic region that is
affecting the *bem* gene. This finding suggested that the use of additional library screens may not be the most effective manner in which to continue the attempts towards cloning the *bem* gene. Instead, an approach utilizing the recently completed *Drosophila melanogaster* genome sequence was initiated.

### 3.2 Analysis of the genetic interaction between *bem* and *pum*

#### 3.2.1 The *bem* P-element is located within the *pum* transcription unit

DNA flanking the *bem* P-element was compared to the entire *Drosophila* genome database using the Berkeley *Drosophila* Genome Project Blast program. This search revealed that the *bem* P-element is inserted into a large intron (≈ 120 kb) between exons eight and nine of the *pum* transcription unit (Figure 3). More precise examination revealed that the insertion site is approximately 54 kb from *pum* exon eight and 75 kb from *pum* exon nine. Interestingly, many of the previously identified *pum*\(^{onr}\) mutants are caused by P-element insertions into the same region of this large intron (Figure 3, Parisi and Lin 1999). Also, there is a predicted gene (CG11997) located in the large *pum* intron and although the *bem* P-element
Figure 3. The *bem P-lacW* element is inserted into the large intron between *pum* exons eight and nine. Map of the *pum* transcription unit and intron exon boundaries. The map is adapted from Barker *et al.* (1992) and Parisi and Lin (1999). Transcription occurs from right to left, distal to proximal on chromosome 3. Open boxes represent alternatively spliced exons that correspond to the 5' UTR, while all other exons, representing the ORF, are indicated by black boxes. Intron and exon sizes are approximate. The location of the *bem P-lacW* element is designated by the black flag and the locations of previously characterized *pum* alleles, caused by insertion of the PZ-element, are designated by the white flags. The locations of the pertinent EMS alleles (*pum* /, *pum* /, and *pum* /) are indicated. Also, the location of the only predicted gene (CG11997), which is located within the *pum* transcription unit, is shown.
is not located within its transcription unit the possibility that the \textit{bem} P-element is affecting regulation of CG11997 must be considered. This possibility will be addressed in more detail subsequently.

Overall, the finding that the \textit{bem} P-element is located within the \textit{pum} transcription unit suggested the possibility that improper regulation or aberrant splicing of the \textit{pum} transcript causes \textit{bem} mutant phenotypes. Future experiments designed to investigate this possibility were performed.

3.2.2 Certain $\textit{pum}^{\text{ovr}}$ alleles fail to complement \textit{bem} female sterility

To begin investigating the relationship between \textit{bem} and \textit{pum}, a series of genetic experiments were performed. First, $\textit{pum}^{\text{ovr}}$ mutants were tested for their ability to complement the \textit{bem} female sterility defect. These alleles were tested first because of the similar defects in fertility and in egg chamber morphology that are present in females form both \textit{bem} and \textit{pum}^{\text{ovr}} females. Production of the appropriate trans-heterozygous females and subsequent fertility tests with these females revealed that $\textit{pum}^{1203}$ fails to complement the \textit{bem} female fertility defect as demonstrated by the greatly reduced fertility exhibited by the trans-heterozygous females (Table 2). The fact
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of offspring per female</th>
</tr>
</thead>
<tbody>
<tr>
<td>$bem^+/bem^+$ (n = 7)</td>
<td>$60 \pm 10.6$</td>
</tr>
<tr>
<td>$bem/bem^+$ (n = 7)</td>
<td>$74 \pm 9.3$</td>
</tr>
<tr>
<td>$bem/bem^-$ (n = 6)</td>
<td>$1 \pm 0.4^*$</td>
</tr>
<tr>
<td>$pum^{1688}/bem^+$ (n = 10)</td>
<td>$74.2 \pm 5.3$</td>
</tr>
<tr>
<td>$pum^{1688}/bem$ (n = 9)</td>
<td>$51.6 \pm 7.0$</td>
</tr>
<tr>
<td>$pum^{1201}/bem^+$ (n = 10)</td>
<td>$116 \pm 6.3$</td>
</tr>
<tr>
<td>$pum^{1203}/bem$ (n = 10)</td>
<td>$5 \pm 2.2^*$</td>
</tr>
<tr>
<td>$pum^+/bem^+$ (n = 9)</td>
<td>$109 \pm 4.9$</td>
</tr>
<tr>
<td>$pum^+/bem^-$ (n = 10)</td>
<td>$9 \pm 3.4^*$</td>
</tr>
<tr>
<td>$pum^+/bem^+$ (n = 9)</td>
<td>$108 \pm 6.2$</td>
</tr>
<tr>
<td>$pum^+/bem^-$ (n = 10)</td>
<td>$1 \pm 0.4^*$</td>
</tr>
<tr>
<td>$pum^{+/}/bem^+$ (n = 6)</td>
<td>$74 \pm 12.2$</td>
</tr>
<tr>
<td>$pum^{+/}/bem^-$ (n = 18)</td>
<td>$6 \pm 1.0^*$</td>
</tr>
<tr>
<td>$pum^{+/}/bem^+$ (n = 9)</td>
<td>$77 \pm 3.6$</td>
</tr>
<tr>
<td>$pum^+/bem^+$ (n = 10)</td>
<td>$98 \pm 9.5$</td>
</tr>
<tr>
<td>$pum^+/bem^+$ (n = 5)</td>
<td>$109 \pm 7.3$</td>
</tr>
</tbody>
</table>

Means and standard errors (unpaired t-test) of offspring number from the indicated genotypes are presented. The following pairwise combinations had statistically significant differences in number of offspring per female: $bem^+/bem^+$ vs. $bem^+/bem^-$, $pum^{1201}/bem^+$ vs. $pum^{1201}/bem^-$, $pum^+/bem^+$ vs. $pum^+/bem^-$, $pum^+/bem^+$ vs. $pum^+/bem^-$, $pum^+/bem^+$ vs. $pum^+/bem^+$ (*, $P < 0.0001$).
that *bem* female sterility is not complemented by *pum*\textsuperscript{3203} is in agreement with the hypothesis that *bem* female sterility is due to disruption of Pum protein function. However, another *pum*\textsuperscript{ort} allele, *pum*\textsuperscript{1688}, was found to be able to complement *bem* female sterility (Table 2). This result suggests a different molecular nature for *pum*\textsuperscript{1688} and this result is not entirely unexpected because the *pum*\textsuperscript{1688} P-element is inserted into an entirely different region of the *pum* transcription unit. The *pum*\textsuperscript{1688} P-element is inserted in the intron between *pum* exons three and four while the *pum*\textsuperscript{3203} and *bem* P-elements are both in the large intron between exons eight and nine (Figure 3). Therefore, *pum*\textsuperscript{1688} likely affects *pum* regulation or splicing in a manner that is distinct from the manner in which *bem* affects *pum*. The possibility that the two P-elements in turn affect specific and unique *pum* functions may account for their ability to complement one another. Also, since all three of these alleles are hypomorphic in nature, it is possible that *pum*\textsuperscript{3203} has a more significant effect on *pum* function than *pum*\textsuperscript{1688} and this could account for the inability of *pum*\textsuperscript{3203} to complement *bem* female sterility
while pum\textsuperscript{1688} complements this same defect. These possibilities will be addressed in greater detail later in this thesis.

During preliminary experiments it was also noticed that two other $pum^{mut}$ mutant lines, $pum^{1203}$ and $pum^{4806}$, are also able to complement $bem$ female sterility. Due to the preliminary nature of these experiments the average number of offspring per female was not calculated, therefore these values are absent from Table 2. These two alleles are caused by P-elements inserted into the same large intron of the $pum$ transcription unit as the $bem$ P-element. These two $pum$ alleles are also hypomorphic and their ability to complement $bem$ sterility may result from a less severe effect on $pum$ function than the effect caused by the non-complementing $pum^{1203}$.

Due to the fact that $pum^{1203}$ failed to complement $bem$ female sterility whereas other $pum^{mut}$ alleles complemented this defect, further experimentation was performed in order to determine whether disruption of $pum$ function is responsible for $bem$ female sterility.
3.2.3 Certain EMS induced *pum* alleles fail to complement *bem* female sterility

In preliminary experiments with EMS-induced *pum* alleles, *pum*³ was found to complement *bem* female sterility. Due to the preliminary nature of these results the average number of offspring per female was not calculated and therefore this value is not present in Table2. There is no information regarding the molecular nature of *pum*³ available in the literature and therefore the ability of *pum*³ to complement *bem* female sterility is not understood. But, the possibility that *pum*³ and *bem* affect specific and unique functions of *pum* with respect to fertility may account for their ability to complement one another. These results were less than satisfying and further experiments investigating the ability of *bem* and *pum* to complement one another with respect to female sterility were performed.

Additional complementation experiments were performed utilizing the molecularly characterized EMS mutants *pum*⁷, *pum*⁹, and *pum*¹³ (the locations of these mutations are shown in Figure 3). Both *pum*⁷ and *pum*⁹ are EMS-generated alleles that specifically affect the *pum* transcription unit and
result in production of a truncated protein product that lacks the RNA binding domain (Forbes and Lehmann, 1998; Tearle and Nusslein-Volhard, 1987). Similarly, \textit{pum}^{13} is an EMS-generated allele that results in production of an altered Pum protein product that is able to bind to the NRE sequence but is unable to repress translation of \textit{hb} mRNA (Tearle and Nusslein-Volhard, 1987; Wharton et al., 1998). Therefore, these mutants should function as null \textit{pum} alleles with respect to any function that relies on the ability of Pum protein to bind to and repress translation of mRNA.

The appropriate trans-heterozygous females were produced and subsequent fertility analysis revealed that \textit{pum}^{7}, \textit{pum}^{9}, and \textit{pum}^{13} all fail to complement \textit{bem} female sterility (Table 2). These results show that mutations that specifically affect the ability of Pum protein to bind to and repress translation of \textit{hb} mRNA are unable to compensate for the \textit{bem} female fertility defect. This data strongly supports the conclusion that \textit{bem} female sterility is caused by improper \textit{pum} function. Also, these results show that the Pum protein function in female fertility requires the properly functioning RNA binding domain. Furthermore, these results suggest that
bem female sterility is not caused by improper regulation of the predicted
gene CG11997 since these non-complementing alleles are known to
specifically and singularly affect pum.

3.2.4 Ovarian expression of pum rescues bem female fertility

In order to show definitively that bem female sterility is caused by
improper pum function, an additional experiment was performed. If
improper pum function is responsible for bem female sterility, then
expression of a wild type pum in bem mutant females should rescue the
fertility defects and render the females fertile. In order to test this
prediction, homozygous bem mutant females carrying a nos-pum rescue
construct on their second chromosome (Barker et al., 1992) were produced
and tested for fertility. These flies express pum under the regulation of the
ovary-specific nos promoter and therefore could be used to assay pum’s
ability to compensate for bem female sterility. Female fertility was
significantly restored in homozygous bem females carrying the nos-pum
rescue construct although the level of rescue was not to wild type levels
(Figure 4). The differential in fertility seen between the rescued bem
Figure 4. Ovarian expression of pum is able to rescue bem female sterility. Single females of the appropriate genotypes were crossed to three wild type males. The parental flies were removed from the vial 10 days after initiation of the cross. Offspring were counted and cleared from the vials as they eclosed. Counting was terminated and offspring number totaled once adults ceased eclosing. The means and standard errors (unpaired t-test) of offspring number from the indicated genotypes are presented. The difference in offspring number between bem°/bem− and bem°/bem−; nos-pum is statistically significant (*, P < 0.0001). The sample sizes were as follows: bem°/bem− and bem°/bem− (n = 7), bem°/bem−; nos-pum (n = 7), bem°/bem− (n = 6).
mutants and wild-type females might be an effect of insufficient Pum protein dosage in the ovaries due to the presence of only one copy of the nos-pum rescue construct. Alternatively, the difference in fertility might reflect an additional role for pum in female fertility in a tissue in which the nos promoter is not active. These results, in addition to the complementation results described above, show that the fertility defects seen in bem mutant females are indeed caused by improper pum function. These observations led me to investigate the possibility that the behavioral and electrophysiological defects seen in bem mutants are caused by disruption of pum function as well.

3.2.5 pum fails to complement bem behavioral defects

To investigate whether the behavioral defects seen in bem flies are due to disruption of pum function, we investigated the ability of several pum alleles to complement the uncoordination and inability to achieve flight exhibited by bem mutants. Flies trans-heterozygous for the bem P-element and one of several pum alleles were produced and tested for negative gravitaxis. The pum^{1203} P-element allele, pum^{7}, and pum^{9} were each unable
to compensate for the negative gravitaxis or flight defect seen in \textit{bem} homozygotes (Figure 5A and B, data not shown). However, \textit{pum}\textsuperscript{1688} was able to complement \textit{bem} behavioral defects (Figure 5A). This result was not unexpected because \textit{pum}\textsuperscript{1688} complements \textit{bem} female sterility, is located in a distinct region of the \textit{pum} transcription unit, and is hypomorphic. This result supports the fact that \textit{pum}\textsuperscript{1688} has a different molecular effect from other \textit{pum} mutations. Nonetheless, the results from the behavioral complementation experiments with \textit{pum}\textsuperscript{2203}, \textit{pum}\textsuperscript{7}, and \textit{pum}\textsuperscript{9} show that the behavioral defects exhibited by \textit{bem} mutants are caused by disruption of \textit{pum} function. Furthermore, the results with \textit{pum}\textsuperscript{7} and \textit{pum}\textsuperscript{9} suggest that \textit{bem} behavioral defects are not caused by improper regulation of the predicted gene CG11997 since these non-complementing alleles are known to specifically and singularly affect \textit{pum}.

\subsection{3.2.6 Certain \textit{pum} alleles exhibit neuronal hyper-excitability}

Once the \textit{bem} fertility and behavioral defects were shown to be caused by improper \textit{pum} function we wanted to investigate the possibility that some of the previously characterized \textit{pum} mutants may exhibit the neuronal
Figure 5. Several pum alleles fail to complement bem behavioral defects. Negative geotaxis experiments were performed by placing single-six-day-old males of the appropriate genotype in an empty vial. The fly was then banged to the bottom of the vial and the time required for the fly to right itself and climb five centimeters up the side of the vial was recorded. The means and standard errors (unpaired t-test) of climbing times from the indicated genotypes are presented. A. Results obtained from tests with pum<sup>mut</sup> mutants. The following pairwise combinations had statistically significant differences in climbing time: bem<sup>-/-</sup> vs. bem<sup>+/+</sup> (*, P < 0.0001) and pum<sup>1203</sup>/bem<sup>-</sup> vs. pum<sup>1203</sup>/bem<sup>-</sup> (*, P = 0.0007). The sample sizes were as follows: bem<sup>-/-</sup> (n = 23), bem<sup>+/+</sup> (n = 12), pum<sup>1203</sup>/bem<sup>-</sup> (n = 33), pum<sup>1203</sup>/bem<sup>-</sup> (n = 27), pum<sup>1688</sup>/bem<sup>-</sup> (n = 28), pum<sup>1688</sup>/bem<sup>-</sup> (n = 15), pum<sup>mut</sup>/bem<sup>-</sup> (n = 34). B. Results obtained from tests with EMS-induced pum alleles. The following pairwise combinations had statistically significant differences in climbing time: bem<sup>-/-</sup> vs. bem<sup>+/+</sup>, pum<sup>1</sup>/bem<sup>-</sup> vs. pum<sup>2</sup>/bem<sup>-</sup>, and pum<sup>9</sup>/bem<sup>-</sup> (*, P < 0.0001). The sample sizes were as follows: bem<sup>-/-</sup> (n = 23), bem<sup>+/+</sup> (n = 12), pum<sup>1</sup>/bem<sup>-</sup> (n = 29), pum<sup>2</sup>/bem<sup>-</sup> (n = 27), pum<sup>9</sup>/bem<sup>-</sup> (n = 41), pum<sup>9</sup>/bem<sup>-</sup> (n = 37), bem<sup>-/-</sup> (n = 17).
hyper-excitability similar to that seen in *bem*. In order to investigate this possibility, homozygous *pum*\textsuperscript{ovl} lines were tested electrophysiologically in order to determine whether they exhibit the increased rate of LTF onset that has been described for *bem*. Sample traces showing examples of LTF onset are shown in Figure 6A and the results for *pum*\textsuperscript{ovl} onset rates are shown in Figure 6B. I found that both *pum*\textsuperscript{2003} and *pum*\textsuperscript{4806} exhibit increased neuronal excitability at the Drosophila nmj whereas the *pum*\textsuperscript{+} allele, *pum*\textsuperscript{revertant} (revertant of P-element insertion *pum*\textsuperscript{1688}), exhibits wild type excitability. In fact, the LTF onset rates of *pum*\textsuperscript{2003} and *pum*\textsuperscript{4806} are virtually indistinguishable from the rate observed in *bem* mutants. These findings support the possibility that *bem* hyper-excitability is due to improper *pum* function.

However, not all *pum* alleles tested exhibited neuronal hyper-excitability. One allele, *pum*\textsuperscript{13}, which is able to bind *hb* mRNA but is unable to repress translation, had neurons with wild-type excitability levels characterized by normal LTF onset rates (Figure 6B). This finding is discussed thoroughly in section 4.2.1 of this thesis.
Figure 6. Certain pum alleles exhibit neuronal defects. LTF onset rates were determined by experiments on dissected third instar larvae. Following larval dissection, nerves were pulled into a suction electrode and stimulated at the indicated frequencies. The response of the corresponding muscle was recorded and digitized and the number of stimuli needed to elicit the onset of LTF was determined. LTF onset rates were determined in the presence of 0.1 mM quinidine and at an external Ca²⁺ concentration of 0.15 mM. A. Representative traces of 10 Hz stimulation showing LTF onset at the nmj for the indicated genotypes. Black arrowheads indicate LTF onset. B. Means and standard errors (unpaired t-test) of LTF onset rates for the indicated genotypes at the indicated stimulation frequencies (Hz). For bem⁻/bem⁺, n = 22; for bem⁻/bem⁻, n = 12; for pum²⁰⁰³/pum²⁰⁰³, n = 8; for pum⁴⁸⁰⁶/pum⁴⁸⁰⁶, n = 6; for pum¹³/pum¹³, n = 6.
Overall, the results regarding neuronal excitability of pum<sup>ov</sup> mutant larvae support the possibility that bem hyper-excitability is due to improper pum function. Therefore, further experiments designed to address this possibility were performed.

### 3.2.7 pum fails to complement bem neuronal hyper-excitability

Because some pum alleles were unable to complement bem behavioral defects and previously characterized pum<sup>ov</sup> mutants exhibit the same hyper-excitability defects seen in bem, we predicted that the bem neuronal excitability defects were due to improper pum function. In order to test this prediction directly we investigated the ability of pum<sup>+</sup> and pum<sup>−</sup> to complement bem neuronal hyper-excitability. The appropriate trans-heterozygous third instar larvae were produced and the rate of LTF onset was determined. These experiments showed that the trans-heterozygous larvae exhibited a significantly faster rate of LTF onset than larvae heterozygous for pum, bem, and wild type control larvae (Figure 7A and B). These results allow us to conclude that bem hyper-excitability is due to improper pum function. Also, these results show that Pum protein function
Figure 7. Certain pum alleles fail to complement bem neuronal hyper-excitability. Means and standard errors (unpaired t-test) of LTF onset rates for the indicated genotypes at the indicated stimulation frequencies (Hz). A. The inability of pum$^9$ to complement bem neuronal hyper-excitability. (for bem$^-$/pum$^7$, n = 8; for bem$^-$/pum$^9$, n = 8; for bem$^+$/pum$^-$, n = 8; for bem$^-$/pum$^-$, n = 7) B. The inability of pum$^9$ to complement bem neuronal hyper-excitability. (for bem$^-$/pum$^9$, n = 6; for bem$^-$/pum$^9$, n = 5; for bem$^+$/bem$^-$, n = 8; for bem$^-$/pum$^-$, n = 7)
in maintaining proper neuronal excitability requires the presence of the RNA binding domain. Furthermore, these results suggest that \textit{bem} hyper-excitability is not caused by improper regulation of the predicted gene CG11997 since these non-complementing alleles are known to specifically and singularly affect \textit{pum}.

\textbf{3.3 \textit{pum} expression studies}

\textbf{3.3.1 \textit{pum} mRNA is expressed aberrantly in \textit{bem} and \textit{pum} mutant flies and heads}

Because the genetic experiments showed that all of the \textit{bem} phenotypes were due to improper \textit{pum} function, we decided to investigate the effect of the \textit{bem} P-element on \textit{pum} expression. To determine whether \textit{pum} is transcribed properly in \textit{bem} flies, a series of Northern blots were performed. First, a blot was performed using mRNA extracted from whole flies. We observed a modest reduction in transcript levels in \textit{bem} flies when compared to the parental wild type control (Figure 8A). In addition, the transcripts detected in \textit{bem} adults appeared to be of a smaller size than the transcripts present in the wild type control adults.
**Figure 8.** *pum* mRNA is expressed improperly in *bem* and *pum*<sup>α<sup>st</sup> mutant flies and heads. Northern blots performed using mRNA extracted from 40 flies or 800 heads of each genotype. The probe used was a PCR product produced from *pum* nucleotides 3600 to 4400 of the *pum* cDNA. Results were visualized using a phosphor imaging system. For panels B and C flies were initially decapitated by freezing in liquid nitrogen and subsequent vigorous shaking through an U.S.A. Standard Sieve #25, which has an opening size of 710 μm. A. Northern blot of mRNA extracted from 40 whole flies and probed for *pum*. A and B. The lower panel is the same membrane shown in the upper panel probed for the ribosomal protein gene L29a to serve as a loading control. B and C. Northern blot of mRNA extracted from 800 heads and probed for *pum*.
Second a blot was performed on mRNA extracted from heads. A striking difference was observed between the transcripts present in \textit{bem} and wild-type heads (Figure 8B). In particular, wild-type heads express four different \textit{pum} mRNA species corresponding to the apparent molecular sizes of 9.0 and 6.8 kb as well as a doublet at 8.1 kb, whereas the 9.0 kb transcript and the upper band of the 8.1 kb doublet are totally absent from \textit{bem} heads (Figure 8B).

A final Northern blot was performed in order to investigate \textit{pum} mRNA expression in the heads of previously generated and characterized \textit{pum} \textsuperscript{ort} mutants. One allele analyzed, \textit{pum}\textsuperscript{1196}, exhibited wild type expression of \textit{pum} head mRNA suggesting that this allele may not affect the neuronal function of \textit{pum} (Figure 8C). However, \textit{pum}\textsuperscript{1203}, which was unable to complement \textit{bem} behavioral defects, and \textit{pum}\textsuperscript{1688}, which complements these defects, are each missing the same 9.0 kb head transcript that is absent from \textit{bem} heads (Figure 8C).

The mRNA expression studies show that the \textit{bem} P-element is indeed affecting \textit{pum} expression in adult flies and especially in heads. Also, since
pum\textsuperscript{1688} is lacking the 9.0 kb transcript and is able to complement bem behavioral defects, it appears that the 9.0 kb transcript is not necessary in heads for proper behavior. However, these results do not allow definitive determination of which pum transcripts are needed for proper neuronal function.

3.3.2 Pum protein expression is greatly reduced in bem and pum\textsuperscript{ovt} mutant heads

Next, we wanted to investigate the possibility that improper pum mRNA expression results in improper Pum protein expression in bem and pum\textsuperscript{ovt} heads. A western blot was performed and the results of this experiment revealed that Pum protein is greatly reduced in abundance in heads from bem mutants as well as some pum\textsuperscript{ovt} mutants, when compared to bem parental and pum\textsuperscript{ovt} revertant controls (Figure 9). Specifically, the parental and revertant control heads abundantly express three different protein isoforms corresponding to the apparent molecular sizes of 98, 130, and 156 kDa, whereas greatly reduced amounts of all three isoforms are present in the bem heads (Figure 9, Parisi and Lin 1999).
Figure 9. Pum protein is expressed improperly in bem and pum₉₉ mutant heads. Western blots performed on total protein extracted from the heads of 40 flies. Flies were decapitated by freezing in liquid nitrogen and subsequent vigorous shaking through an U.S.A. Standard Sieve #25, which has an opening size of 710 μm. The protein was then run on a 6% denaturing SDS/acrylamide gel and transferred to a nylon membrane using a semi-dry transfer cell. The membrane was then blocked and probed with anti-PUM-1, a rabbit polyclonal anti-Pum antibody, at a 1:3000 dilution (Zamore et al. 1997). A sheep-anti-rabbit IgG conjugated to horseradish peroxidase was used as the secondary antibody. Results were visualized using a bioluminescent DAB substrate kit and subsequent exposure of the membrane to film. The lower panel is a gel run in parallel. The same volume of the same samples was loaded and the gel was then stained with methylene blue to serve as a loading control.
Also, \( pum^{1688} \) is missing the 156-kDa isoform whereas \( pum^{3203} \) and \( pum^{6897} \) are missing both the 156 and 130-kDa isoforms (Figure 9). Because \( pum^{1688} \) is able to complement \( bem \) behavioral defects and \( pum^{1203} \) is not, these results demonstrate that the 156-kDa isoform is not necessary for neuronal Pum function (Figure 5). Also note that the \( pum^{1196} \) allele, which expresses \( pum \) mRNA in a wild type-manner in heads, expresses all of the Pum protein isoforms at wild-type levels in heads as well. This data lends further support to the possibility that the \( pum^{1196} \) P-element does not affect the neuronal function of \( pum \).

Taken together, the results from the protein expression studies reveal that the \( bem \) P-element insertion and certain \( pum^{env} \) P-element insertions result in improper Pum protein expression in heads, which is likely to be responsible for the neuronal phenotypes exhibited by these mutants.

The genetic and molecular data presented heretofore in this thesis show that all of the \( bem \) mutant phenotypes are due to improper \( pum \) function. Therefore, we assert that the \( bem \) mutation is allelic to \( pum \) and should now be known and referred to as \( pum^{bem} \).
3.3.3 Immunocytological studies

Since I discovered a new role for *pum* in the nervous system, I next performed experiments designed to determine the neuronal expression pattern of the Pum protein. These experiments were designed to compare the Pum protein expression pattern seen in wild type and *pum*<sup>bem</sup> mutants in order to investigate the hypothesis that Pum protein is expressed aberrantly in the *pum*<sup>bem</sup> mutant nervous system.

In order to visualize the expression pattern of Pum protein in the nervous system of wild-type and *pum*<sup>bem</sup> embryos, an anti-Pum antibody (from MacDonald lab) was used to stain embryos of various stages. Results from the embryo immuno-staining are shown in Figure 10. Early stage wild type embryos exhibited staining throughout the embryo and this result is consistent with previous findings (shown in Figure 10A) (Barker, 1992). Later staged wild-type embryos exhibited staining in the ventral nerve cord and central nervous system (shown in Figure 10B). This result was initially exciting, but when the *pum* antibody was replaced with water in a negative control experiment, late stage wild type embryos exhibited the same
Figure 10. Immunocytological Studies. Embryos were fixed, washed, incubated with the *pum* antibody (from MacDonald lab), incubated with a peroxidase conjugated secondary antibody, and finally subjected to a DAB color reaction in order to visualize the staining. A. Example of an early staged wild-type embryo. B. Example of a late staged wild-type embryo. C. Negative control experiment where the *pum* antibody was replaced with water.
expression pattern seen in the experimental embryos (shown in Figure 10C).

This result suggests that there is cross-reactivity between the secondary antibody or some part of the color reaction mix and the embryonic nervous system. This result made it impossible to draw any conclusions from the embryo immuno-staining results and resulted in the discontinuation of the immunocytological studies.

3.4 *pum* over-expression studies

3.4.1 Production of transgenic flies that have *pum* over-expressing neurons

Next, we utilized the UAS/GAL4 system in order to examine the effect of over-expressing Pum protein in the nervous system. Because *pum* mutants exhibit neuronal hyper-excitability we predicted that over-expression of *pum* in the nervous system might lead to the opposite effect and cause reduced neuronal excitability. We produced transgenic flies that expressed *pum* under the control of the UAS promoter. These transgenic flies were then crossed to an *elav*-GAL4 line, which produces GAL4
specifically in post-mitotic neurons. The resulting larvae were predicted to over-express Pum protein in their neurons. Third instar larvae were then analyzed electrophysiologically in order to assess the ramifications of neuronal pum over-expression on excitability.

3.4.2 Neurons that over-express pum do not exhibit LTF onset

We found that over-expression of pum in neurons results in a decreased rate of LTF onset at the nmj when compared to parental control larvae, and larvae that are expressing either UAS-pum or elav-GAL4 alone. In fact, neurons that were over-expressing pum were never observed to exhibit LTF onset even after repetitive stimulation at 10 Hz for 90 seconds, whereas control larvae expressing either UAS-pum or elav-GAL4 alone exhibited LTF onset rates that were similar to the parental wild type control rates (data not shown).

3.4.3 Neurons that over-express pum exhibit increased ejp failure rates

During the LTF onset rate studies we noticed that larvae over-expressing neuronal pum appeared to respond to nerve stimulation with a higher frequency of ejp failure than controls in Ringers containing low
[Ca\(^{2+}\)] (0.15 mM). Sample traces showing example ejp failures and successes are shown in Figure 11A. These experiments revealed that nerve stimuli were successful in eliciting an ejp only 20% of the time in the over-expression lines, whereas nerve stimuli elicited an ejp about 80% of the time at nmjs from wild type and control lines (Figure 11B). Thus, *pum* over-expression reduces transmitter release at low [Ca\(^{2+}\)]. In contrast, nmjs from the hyper-excitabile *pum\(^{bem}\)* loss of function mutants were found to respond to stimuli with an ejp 95% of the time. This result suggests that loss of *pum* in neurons increases transmitter release at low [Ca\(^{2+}\)] (Figure 11B). The difference between *pum\(^{bem}\)* and wild-type failure rates is even more evident at lower external [Ca\(^{2+}\)] (0.10 mM) at which stimulations to wild-type neurons successfully elicit an ejp following only 35% of the stimuli, and stimulations to *pum\(^{bem}\)* neurons elicit an ejp following 85% of the stimuli (Figure 11C). Flies over-expressing *pum* in their nervous system were also tested for behavioral defects and temperature sensitive paralysis (over a temperature range of 37\(^\circ\) to 45\(^\circ\)) but no differences from wild type behavior or temperature response were evident (data not shown). The data collected
Figure 11. Over-expression of pum in the nervous system causes an increased failure rate in motor neurons. Failure rates were determined using third instar larvae of the appropriate genotypes that were dissected in the same manner described for LTF onset experiments. The nerves were then stimulated for 10 seconds at a frequency of one Hz. The number of stimuli per 10-second stimulation train that failed to evoke any muscular response was recorded. Failure rate analysis was performed in the absence of quinidine and at an external Ca$^{2+}$ concentration of either 0.15 mM (A and B) or 0.10 mM (C). A. Representative traces of failures and successes from nerves of the indicated genotypes. Black arrowheads indicate failures. B and C. Six larvae from each genotype were tested. Means and standard errors (unpaired t-test) of failure rates from nerves of the indicated genotypes are presented. B. The following pairwise combinations had statistically significant differences in failure rates: UAS-pum / elav-GAL4 vs. bem$^+$/bem$^-$, UAS-pum / elav-GAL4 vs. UAS-pum / +, and UAS-pum / elav-GAL4 vs. elav-GAL4 / + (*, $P < 0.0005$). C. The difference in failure rates between bem$^+$ / bem$^-$ and bem$^-$ / bem$^-$ is statistically significant (*, $P = 0.0001$).
from neurons over-expressing *pum* support the conclusion that the level of Pum protein expression in neurons regulates neuronal excitability. In particular, insufficient neuronal Pum causes hyper-excitability, whereas excess neuronal Pum reduces excitability.

**3.5 *hb pum*\(^{bem}\) / *hb*\(^{+}\) *pum*\(^{bem}\) mutant nerves exhibit decreased neuronal excitability**

Because *pum* functions in embryogenesis to repress *hb* mRNA translation, we wanted to see whether the neuronal phenotypes observed in *pum*\(^{bem}\) mutants were also related to altered *hb* expression levels. We hypothesized that if *pum* functions in the nervous system to repress *hb* translation, thus leading to increased Hb protein in *pum* mutant neurons, and if this increased level of Hb protein causes the increased rate of LTF onset, then reducing *hb* gene dosage from two copies to one might cause some suppression of the *pum*\(^{bem}\) phenotypes. To test this possibility, we constructed a *hb pum*\(^{bem}\) and a *hb*\(^{+}\) *pum*\(^{bem}\) control chromosome, crossed flies from each line to *pum*\(^{bem}\) mutants and compared the rates of onset of LTF. The *hb* mutant used (*hb*\(^{4}\), from the Bloomington Stock Center) is an EMS
generated amorphic allele that causes a stop codon before the first zinc finger domain thereby rendering the Hb protein unable to perform its role as a transcription factor. We found that the mutant larvae with \( hb \) gene dosage reduced from two copies to one exhibited a reduced facilitation onset rate compared to controls (Figure 12). The difference in facilitation onset rates was significant when stimulation occurred at three, five, and seven Hz (Figure 12). However, the facilitation onset rate was not reduced to wild-type levels, which raises the possibility that these neurons may still be over-expressing \( hb \). Alternatively, improper \( hb \) translational regulation may not account for the entirety of the hyper-excitability defect and other, as of yet undetermined, roles of \( pum \) may account for the remainder of the hyper-excitability defect.

Overall, these findings suggest a possible mechanism for \( pum \) function in regulation of neuronal excitability that involves the translational repression of \( hb \) mRNA, a mechanism that is possibly very similar to the known mechanism by which \( pum \) functions in embryogenesis.
Figure 12. The addition of a hb mutation into bem+/bem− larvae results in slower LTF onset rates. LTF onset rates were determined in the same manner described in the legend of Figure 4. Means and standard errors (unpaired t-test) of LTF onset rates for the indicated genotypes at the indicated stimulation frequencies are presented. (for bem+/bem−, n = 22; for hb−pum+bem/ hb−pum+bem, n = 16; and for hb−pum+bem/ hb−pum+bem, n = 16).
CHAPTER 4

CONCLUSIONS AND DISCUSSION

4.1 Summary

Here I have shown by many criteria that \textit{pum}^{bem} \textit{is a new allele of pum}. The data proving this finding is summarized in Tables 3 and 4. First, several \textit{pum} alleles failed to complement the \textit{pum}^{bem} fertility defects.

Second, female fertility in \textit{pum}^{bem} mutants was significantly restored by the expression of \textit{pum} under the transcriptional control of the \textit{nos} promoter.

Third, several \textit{pum} alleles failed to complement both the behavioral defects and defective neuronal excitability of \textit{pum}^{bem}. Finally, both \textit{pum} mRNA and protein are expressed improperly in the heads of \textit{pum}^{bem} adults. I was also able to show that previously identified \textit{pum} mutants exhibit the same increased motor neuron excitability phenotype seen in \textit{pum}^{bem}, and that over-expression of \textit{pum} in larval neurons decreases motor neuron excitability.

Therefore, I have discovered a new role of \textit{pum} in regulating neuronal excitability. I have also begun to assign specific functions to individual Pum protein isoforms. In particular, I found that \textit{pum}^{1688} eliminates only the 156-
### TABLE 3
summary of complementation data

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<td>--</td>
</tr>
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</tr>
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<td>(pum^{4897})</td>
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-- indicates experiment was not performed

### TABLE 4
summary of experiments on \(pum\) homozygotes

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<th>protein expression</th>
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-- indicates experiment was not performed
kDa isoform and retains expression of both the 130- and 95-kDa Pum isoforms, whereas \( pum^{bem} \) eliminates both the 156- and 130-kDa isoforms and expresses greatly reduced amounts of the 95-kDa isoform. This mutation \( (pum^{1688}) \) complements \( pum^{bem} \) behavioral defects. These findings suggest that either increased levels of the 95-kDa isoform or presence of the 130-kDa isoform, or both, is sufficient to rescue \( pum^{bem} \) neuronal defects, whereas the 156-kDa isoform is not required for Pum neuronal function.

4.2 Model of \( pum \) function in maintenance of proper neuronal excitability

4.2.1 Improper regulation of \( hb \) mRNA by Pum during nervous system development may result in hyper-excitability

Complex regulation of gene expression is of great importance to Drosophila nervous system development (Brody and Odenwald, 2000). In particular, temporal regulation of the expression of the transcription factor genes \( hb \) and \( cas \) is necessary to assign the proper sub-lineage to developing neuroblasts (Brody and Odenwald, 2000). This developmental scenario is described in greater detail in section 1.8 of this thesis. The mechanism by
which \( hb \) expression is regulated in the developing nervous system is unknown but it is possible that translational repression by Pum is involved. This repression could regulate \( hb \) expression in the developing nervous system and allow for the proper transcription factor to be expressed at the appropriate time. Thus, in \( pum \) mutants, improper regulation of \( hb \) in the developing nervous system could cause neuroblasts to develop into the improper sub-lineage, and this could in turn lead to adult neurons that exhibit improper anatomy and physiology. Specifically, the aberrant development may result in the production of adult neurons that exhibit an increased ratio of functional \( Na^+ \) channels to functional \( K^+ \) channels and are thereby hyper-excitible. My findings that show subtle but significant repression of \( pum \) hyper-excitability by reduction of \( hb \) gene dosage from two copies to one support this possibility (Figure 10). This possibility is similar to the manner in which neuronal identity is specified through \( ttk69 \) repression by \( musashi \) in mechanosensory bristle development and to the manner in which \( cyclin B \) mRNA translation is repressed by Pum in germ line stem cell differentiation (Asaoka-Taguchi et al., 1999; Okabe et al.,
2001). Both of these scenarios are discussed in greater detail in sections 1.5.1 and 1.7.3 of this thesis.

Also interesting is the fact that \textit{pum}^{13}, while failing to complement \textit{bem} female fertility defects, does not exhibit increased LTF onset rates (Table 1 and Figure 4B). These findings bring up the possibility that if \textit{hb} translational repression by Pum is important for maintenance of proper neuronal excitability, this repression may occur by a mechanism that is distinct from the embryological mechanism. This possibility is supported by the finding that Pum^{13} can bind \textit{hb} mRNA and recruit Nos protein but cannot recruit Brat protein to the complex (Sonoda and Wharton, 2001). This defect results in failure to form the tertiary complex needed to repress \textit{hb} translation during embryogenesis (Sonoda and Wharton, 2001). It is possible that Brat, while essential for \textit{hb} translational repression in the developing embryo, is not essential for neuronal \textit{hb} repression. That possibility would account for the normal neuronal excitability of \textit{pum}^{13} homozygous larvae. The neuronal \textit{hb} repression may involve as of yet unidentified binding partners distinct from Brat. Furthermore, recruitment
of the potential neuronal binding partners may be independent of the \textit{pum}^{13}\textsuperscript{ }

molecular lesion. Alternatively, neuronal \textit{hb} repression by Pum may occur

in the absence of other binding partners.

\textbf{4.2.2 Improper regulation of neuronal mRNAs in adult neurons may}

\textbf{result in hyper-excitability}

As discussed in detail in section 1.7 of this thesis, there is

considerable scientific precedent illustrating the importance of local protein

synthesis to the development and maintenance of proper neuronal anatomy

and physiology. This precedent extends to the regulation of local protein

synthesis, which in many cases has been shown to be achieved at the

translational level. Therefore, it is possible that \textit{pum} acts at the nerve

terminal and regulates neuronal excitability by repressing an as of yet

unidentified mRNA. In the case of the \textit{pum} mutant, improper translational

regulation of the appropriate target may result in an adult neuron that has an

increased ratio of functional Na\textsuperscript{+} to K\textsuperscript{+} channels and is thereby hyper-

excitable. Recent findings (discussed in detail in section 1.5.2 of this thesis)

showing that Pum protein is expressed in adult neurons and that \textit{pum}
mutants exhibit aberrant neuronal anatomy lend support to this possibility (Menon, 2001). Translational regulation by Pum in the adult nervous system does not necessarily rule out the possibility of a developmental role for Pum repression of *hb* in neuroblasts. Alternatively, the developmental regulation and subsequent adult neuron regulation may both be necessary and function together in order to produce and maintain proper neuronal anatomy and physiology.

4.2.3 The *pum* mutation may cause neuronal hyper-excitability through effects on K⁺ channels

Some insight into which type of channel might be the end target of *pum* neuronal function is given by the observation that *pum* over-expression mutants possess neurons that are hypo-excitable but do not exhibit temperature sensitive paralysis (Figure 9 and data not shown). Neurons that are hypo-excitable can result from either elevated number of K⁺ channels or from reduced number of Na⁺ channels. Thus, Pum could either repress Na⁺ channels or activate K⁺ channels. Pum repression of Na⁺ channels is most consistent with its role as a translational repressor. However, if so, we
would have expected over-expression of *pum* to result in reduced number of 
Na⁺ channels and temperature induced paralysis. This expectation is based 
on scientific precedent gained from studies on Drosophila excitability 
mutants. For example, Drosophila mutants such as *paralytic (para), no 
action potential (nap), kinesin heavy chain (khc) and seizure (sei)* all 
produce neurons that are hypo-excitable by decreasing the level of 
functional Na⁺ channels (Ganetzky, 1984; Ganetzky, 1986; Hurd et al., 
1996; Jackson et al., 1984). All of these mutants also exhibit temperature 
sensitive paralysis at temperatures below those required to cause paralysis in 
wild type flies (Ganetzky, 1984; Ganetzky, 1986; Hurd et al., 1996; Jackson 
et al., 1984).

Drosophila temperature-induced paralysis is hypothesized to result 
from increased Na⁺ channel inactivation and K⁺ channel activation rates, 
which begin to approach and potentially equal the Na⁺ channel activation 
rate (Hodgkin and Huxley, 1952; Nelson and Wyman, 1990). This is 
predicted to lead to sizeable decreases or abolishment of action potentials in 
affected portions of the nerve cell (Nelson and Wyman, 1990). When a
mutation that decreases the number of functional Na⁺ channels is present, the system is sensitized and temperature-induced paralysis occurs at a much lower temperature (≈37°C vs. 45°C) than in wild type flies (Nelson and Wyman, 1990). If the proposed mechanism of temperature-induced paralysis is correct, temperature-induced paralysis would only be observed at low temperatures in mutants that exhibit hypo-excitability due to decreased number of Na⁺ channels. Conversely, mutants that exhibit hypo-excitability due to increased number of K⁺ channels would not exhibit low temperature-induced paralysis. However, no examples of this situation are present in the literature.

As mentioned, *pum* over-expression lines exhibit decreased neuronal excitability (characterized by decreased LTF onset rates and increased ejp failure rates) but they do not exhibit temperature-induced paralysis. This raises the possibility that decreased excitability of these mutants is due to effects on K⁺ channels. Whereas there is no data present in the literature showing decreased LTF onset rates and increased failure rates resulting from increased K⁺ channel number, there is data showing that the opposite
phenotype (increased LTF onset rates and decreased ejp failure rates) can result from decreased K⁺ channel number (Stern and Ganetzky, 1989).

Since the *pum* over-expression line exhibited the opposite phenotype from loss of function K⁺ channel mutants, I interpret my results to suggest an increased level of functional K⁺ channels are present in the *pum* over-expression line.

This finding allows me to propose the model that Pum represses a repressor of K⁺ channel functional activity. Therefore, when *pum* is mutated, K⁺ channel repression is over-active leading to reduced K⁺ channel functional activity and subsequently hyper-excitable neurons. Conversely, when *pum* is over-expressed, K⁺ channel repression is reduced leading to the production of an increased number of K⁺ channels and nerves that are less excitable than normal.

Since the molecular data present in the literature shows that Pum represses translation of target mRNAs (*hb* and *cyclinB*), I favor a model where Pum represses a repressor of K⁺ channel activity, but it should also be considered possible that Pum activates K⁺ channel translation in the nervous
system. This opposite function may be achieved by recruitment of different neuronal binding partners and the formation of a mRNA protein complex that promotes translation, potentially by inhibiting de-adenylation. If this model is correct, pum loss of function mutants would not sufficiently activate K⁺ channel translation and the resulting neurons would be hyper-excitabile. Conversely, pum over-expressing mutants would over-activate K⁺ channel translation and the resulting neurons would exhibit decreased excitability.

4.2.4 pum may function by a novel mechanism in maintenance of proper neuronal excitability

The wealth of knowledge surrounding the mechanism by which Pum regulates translation as well as the emerging view that translational regulation is important to the development and function of the nervous system make it attractive to hypothesize that pum functions by translational regulation in the nervous system. However, the possibility that pum functions to maintain neuronal excitability by a novel mechanism cannot be eliminated.
4.3 Future Work

4.3.1 Attempts to identify additional/neuronal *pum* target mRNAs

While it is possible that improper translational repression of *hb* mRNA accounts for the entirety of the neuronal defects present in *pum* mutants, it is also possible that regulation of other neuronal targets by Pum is necessary for proper neuronal function. The elucidation of the mechanism by which *pum* functions in germ line stem cell maintenance (repression of *cyclinB* mRNA) has shown that Pum is able to repress translation of more than one mRNA target (Asaoka-Taguchi et al., 1999; Sonoda and Wharton, 2001). This finding also brings up the possibility that the development, anatomy, and physiology of various Drosophila systems may require Pum-based translational regulation of various mRNA targets. This possibility would account for the pleiotropic defects seen in *pum* mutants.

Presently, no neuronal mRNA targets for Pum have been identified; however discovery of these potential targets remains a compelling future goal. One potential approach to identifying neuronal targets of Pum would utilize a *pum* antibody in immuno-precipitation experiments. Briefly,
mRNA could be extracted from Drosophila heads. The *pum* antibody could then be incubated with the head mRNA extracts and subsequent centrifugation would precipitate the *pum* antibody along with Pum and any bound mRNA. RT-PCR and DNA sequencing of the bound mRNA could be used to identify the mRNA that was precipitated by the *pum* antibody. Identification of *hb* mRNA from embryo extracts would serve as a good positive control to show that the procedure was functioning as predicted. This approach could lead to the identification of either known or novel genes involved in the maintenance for proper neuronal excitability, which are regulated translationally by Pum.

Some potential difficulties associated with this experiment do exist. Since most but not all of the Drosophila head is neuronal tissue, the mRNA extract should be enriched for neuronal mRNA but some non-neuronal mRNA will be present as well. This could lead to difficulty in discriminating between precipitated mRNA that was from neuronal versus non-neuronal sources. Also, since Pum mRNA binding is known to be stabilized by other protein binding partners (Nos and Brat) it is possible that
Pum may not be able to precipitate neuronal targets in the absence of neuronal binding partners. Addition of Nos or Brat to the extract could help with this problem but the possibility that Nos and or Brat are not involved in neuronal Pum translational regulation must be considered. Nonetheless, this approach may still be worth pursuing due to the potential reward of identifying the neuronal target of Pum.

4.3.2 Behavioral and electrophysiological analysis of nos and brat mutants

Another potentially interesting experiment involves performing negative gravitaxis and electrophysiological experiments on brat and nos mutants. Initially, non-lethal homozygous mutant lines would be identified and obtained. Subsequent behavior and electrophysiological experiments would be performed in the same manner described in sections 2.3 and 2.4. The results from these experiments would give further insight into the mechanism by which Pum represses translation in the nervous system. If the brat and/or nos mutants exhibit behavioral defects and increased LTF onset rates, that would suggest that Brat and Nos are required for neuronal
translational repression by Pum. If these mutants are behaviorally and
electrophysiologically normal, that suggests that Pum functions to repress
translation in the nervous system with as of yet unidentified binding partners
or in the absence of other binding partners all together.

4.3.3 Electrophysiological analysis of pum mutants that are not
expressing neuronal hb

Since I noticed a subtle reduction in LTF onset rates in pum mutants
that were only expressing one functional copy of the hb gene (Figure 10), it
would be interesting to reduce the hb gene dosage by one more copy and see
if this causes the increased LTF onset rate phenotype to be suppressed even
more. Unfortunately, no existing hb mutants are viable to third instar larval
stages as homozygotes.

A potential approach that may allow this problem to be bypassed
utilizes the yeast FLP recombinase system (Dang and Perrimon, 1992;
Konsolaki et al., 1992). This technique takes advantage of site specific
recombination between FRT (FLP Recombination Target) sequences which
is mediated by the FLP recombinase (Dang and Perrimon, 1992; Konsolaki
et al., 1992). In this system, a neuronal promoter (such as elav) is used to drive expression of FLP in the nervous system. This in turn causes recombination events to occur between FRT sites that are flanking hb, the target gene. This would result in the abolishment of hb expression in all neuronal tissues expressing the promoter.

Use of this system may allow production of pum mutants that lack hb expression in their nervous system but retain all other hb expression. This may allow the resulting mutants to develop into third instar larvae on which electrophysiological experiments can be performed. If electrophysiological experiments reveal that these mutants exhibit LTF onset rates that are close to wild type rates, that would suggest that Pum repression of hb is responsible for the entirety of the hyper-excitability seen in pum mutants. Alternatively, if these mutants exhibit LTF onset rates similar to those seen in the pum mutants that have their hb gene dosage reduced by only one copy, that would suggest that pum’s role in maintaining proper nervous system excitability extends beyond hb repression. A potential problem with this experiment is that since hb is already known to function in neuronal
development, removal of both copies of *hb* from the nervous system may
still result in lethality prior to third instar larval stages.
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