Analysis of a Developmentally Regulated Enhancer Pattern Expressed in Apodemes and the Peripheral Nervous System in Drosophila melanogaster

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ABSTRACT

slinky, a novel enhancer trap pattern, located on the third chromosome in region 71B, has been identified and described in this study. The slinky pattern is expressed both in the ectodermal portion of the muscle attachments to the epidermis and in the peripheral nervous system, which are ectodermal in origin. Based on its expression pattern, the gene associated with the slinky pattern may be involved in axon guidance and muscle precursor cell guidance to their attachment site on the epidermis. In this study as well as numerous others, P-elements have been very useful in identifying new genes.

INTRODUCTION

Model organisms, such as Drosophila melanogaster, are particularly useful in studying developmentally regulated genes for many reasons. One of these reasons is that the Drosophila genome is very well characterized; there have been classical genetic screens carried out in the past 20 years, that have identified potentially all genes that are developmentally important (Gans et al., 1975; Nüsslein-Volhard and Wüchs, 1980). The use of P-elements in enhancer trap screens has also been very useful in identifying many genes based on their expression pattern (Bellen et al., 1989; Wilson et al., 1989). There are also techniques that allow for controlled misexpression of genes that may help to define gene function (Brand and Perrimon, 1993). These techniques have allowed researchers to find genes that help to determine the developmental processes that allow the Drosophila egg to go from a single cell to a fully functioning adult. Another reason that using Drosophila as a model system has proven so useful is that many genes found in Drosophila have vertebrate homologues (Gehring and Hiromi, 1986; Adams et al., 2000) and may aid in the understanding of similar processes in more complex eukaryotes. The rapidly growing amount of information that we have about developmentally regulated genes of model organisms may bring us to an era that allows one to determine the
biological function of genes simply by looking at sequence data (Miklos and Rubin, 1996).

This study describes an enhancer trap pattern that has been found in multiple lines of flies and we believe is associated with a novel gene. This novel enhancer trap pattern, that we have called *slinky*, drives *LacZ* expression in the apodemes, which are cells at the sites of muscle attachments, and peripheral nervous system (PNS). Both of these cells types are ectodermal in origin. It has been suggested that the interaction between early muscle precursors and the epidermis is important in defining the muscle attachment sites and play a key role in the patterning of somatic muscles (Callahan et al., 1996).

A few other genes have been identified which are expressed in the apodemes. These include genes that code for cell adhesion proteins like the PS integrins (Bogaert et al., 1987) and the basement membrane glycoprotein *glutactin* (Olson et al., 1990). Other apodeme expressed proteins, like the *Drosophila* homologue of the epidermal growth factor receptor, *DER/fib*, have a role in cell-cell communication (Zak et al., 1990). One of the redundant enhancer elements associated with *β1 tubulin*, also drives expression in the apodemes (Buttgereit et al., 1991; Buttgereit, 1993). The receptor tyrosine kinase, *derailed*, is expressed in the PNS and in the apodemes which is very similar to the expression pattern of *slinky* (Callahan et al., 1996).

Many of the previously mentioned genes as well as numerous other genes have been found through the use of P-element based enhancer detectors (enhancer traps). Enhancer traps identify segments of the genome that can serve as gene regulatory elements. Essentially, enhancers are transcription factor binding sites that can activate a basal promoter. In the case of an enhancer trap, the basal promoter is driving a marker gene like *LacZ*. The enhancer trap thus reflects the regulation of gene expression conferred by nearby enhancers. P-element mediated enhancer detector screens have served as an efficient way of identifying genes based primarily on their expression patterns. These screens have also been helpful in the rapid analysis of developmentally regulated gene expression patterns at the genetic and molecular level (Wilson et al; 1989; Bellen et al., 1989). Enhancer trap screens can often provide information about the spatial and temporal expression of genes and may give some indication to particular gene functions. The use of P-elements may also facilitate cloning of any selected gene that is of particular interest (Wilson et al., 1989). P-element screens have also been used to generate a mutation of a specific gene. If a P-element is transposed into the open reading frame of a gene or interrupts the effectiveness of one of the regulatory elements of the gene, the gene may be effectively disrupted. Mutations may also be generated if a P-element is located near a gene or a regulatory region of a gene and the P-element is imprecisely excised. If some of the flanking DNA that has been excised is part of the gene or regulatory region of a gene, then a mutation will result.

The line of flies analyzed in this study was generated during a mutagenesis screen that was looking for a female sterile mutation in the *dropout* gene (Galewsky and Schulz, 1992). The original mutagenesis scheme made use of the P-*LacW* transposon insertion line B204. This transposon has been mapped to chromosome region 71 D1-D2 (Flybase, 1994). The location of the P-element in the B204 strain allowed us to take advantage of preferential transposition of P-elements to nearby chromosomal sites (Tower et al., 1993).
This phenomenon, known as “local hop” transposition, is associated with P-element duplication events, and allowed us to look for mutations and pattern elements strictly in the 71 region.

The P-element was mobilized by the stable transposase source Δ2-3 (Robertson et al., 1988). The P-LacW transposon contains the LacZ gene enhancer detector system, bacterial plasmid sequences for rapid genomic cloning, and the eye color gene White for convenient scoring of insertions. The dosage sensitive nature of the White gene makes it possible to look at eye color as an indication of transposon duplication as well as a loss of a P-element.

An advantage of using the P-LacW transposon in the B204 fly line is its ability to function as an enhancer pattern detector. Expression of the LacZ gene in the transposon is driven by a minimal promoter. In the presence of a genomic enhancer near the site of transposon insertion, this marker gene is expressed at high levels in an enhancer specific manner. The B204 insertion shows LacZ expression primarily in the salivary glands and proventriculus relatively late during embryonic development. Since the local insertion phenomenon is associated with a duplication of the P-element, new patterns are easy to detect along with the simple B204 expression pattern in the background.

Because the line of flies that is being analyzed in this study was the product of a P-element duplication event, the flies express the B204 staining pattern as well as the expression pattern that we have called slinky. To facilitate analysis of the slinky pattern, a P-element excision screen was performed to transpose out the B204 P-element. This generated a line of flies with the single P-element associated with the slinky expression pattern. Generating a single P-element line makes cloning the gene associated with the slinky enhancer pattern easier. The LacZ expression pattern of slinky suggests that it is associated with a gene expressed in developing ectoderm, muscle attachment sites, the chordotonal sense organs and other PNS components. In this study we have performed double antibody stains in order to determine whether slinky is being expressed in the PNS. We have also begun mutant analysis on a line of flies that show recessive lethality that is associated with the slinky P-element.

MATERIALS AND METHODS

Flies
Flies were raised on standard cornmeal agar food at 20°C. All fly stocks used were transferred weekly. Unless noted, all stocks used are described in Lindsley and Zimm (1992). slinky645 was generated from a P-element transposition screen in which a series of local hops were made from an initial P-element enhancer trap line called B204 P-lacW. The original B204 transposon has been mapped previously to chromosome region 71 D1-D2 and is inserted in the control region for CrebA (Smolik-Utlaut et al., 1992). The B204 P-element was mobilized by the stable transposase source, Δ2-3,Ki. Insertions were maintained as heterozygotes balanced over either TM3,Sb or TM6B,Tb (Figure 1).

Staining for LacZ Expression
Embryos were dechorionated in undiluted household strength bleach, rinsed with water, and placed into a 1:1 mixture of heptane and 4% paraformaldehyde in 1x PBS (phosphate
buffered saline: 130 mM NaCl, 7 mM Na₂HPO₄·2H₂O, 3 mM NaH₂PO₄·2H₂O, pH 7.2) for 15-45 min. Embryos were then washed with 1x PBT (phosphate buffered saline with 0.1% Tween-20) and incubated overnight in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) in FeNaP buffer (10 mM Na phosphate pH 7.2, 3.1 mM K₃[Fe(II)[CN]₆], 3.1 mM K₃[Fe(III)[CN]₆], 150 mM NaCl, 1.0 mM MgCl₂ₙ) according to standard protocols (Ashburner, 1989).

**Generating the slinky⁶⁴⁵ Single P-element Line**

Slinky⁶⁴⁵ arose from a duplication of the B204 P-element. To facilitate further analysis of this line, we removed the original B204 P-element generating a line of flies that carried only the slinky P-element. This was accomplished by crossing slinky⁶⁴⁵ with the transposase stock, Δ2-3. After removing the Δ2-3 transposase chromosome in the next generation, single fly lines were established. The flies selected had a reduced eye color, indicating the potential loss of one of the P-elements. Embryos were collected from the new lines, stained for LacZ expression and scored for expression of the slinky LacZ expression pattern, and the absence of the B204 expression pattern (Figure 2).

**Antibody Staining**

Embryos were dechorionated in 100% bleach, rinsed with water, and placed into a 1:1 mixture of heptane and 4% paraformaldehyde in 1x PBS for 30-60 minutes. The paraformaldehyde was removed and replaced with methanol to devitellinize the embryos. The embryos were washed twice with methanol and twice with ethanol. The embryos were then rehydrated in a 1:1 mixture of PBT and ethanol followed by three washes with PBT. The embryos were then blocked with 5% fetal calf serum in PBT for 60 minutes. The embryos were subsequently incubated overnight at 4°C in primary antibody (anti-ßgal 1:500; 22C10 1:50). After three PBT washes the embryos were incubated in enzyme conjugated secondary antibody (1:300) for 60 minutes at 4°C. The embryos were washed five times in PBT and then the appropriate enzymatic treatment was performed.

Embryos were photographed with a Zeiss Photomicroscope III (10x objective na 0.25) using Kodak Gold 400 film. Negatives were scanned into a Power Macintosh 7100 and manipulated using Adobe Photoshop.

**Mutant Analysis**

One of our single P-element lines of flies, slinky⁶⁴⁵k, was found to be homozygous lethal. It is possible that the P-element localized to the open reading frame or regulatory region of the gene associated with the enhancer trap pattern. To insure that the mutation is associated with the slinky P-element we crossed slinky⁶⁴⁵ flies to Df(3L)fzₐₙ₂¹ that have a deficiency in the region 70D-71E.

In order to determine the stage of lethality for slinky⁶⁴⁵ flies, 500 embryos from slinky⁶⁴⁵k back crosses, slinky⁶⁴⁵ flies crossed to Df(3L)fzₐₙ₂¹ flies or control crosses were placed on a grape agar plate and the number of eggs that hatched into larvae were counted. For some crosses, the larvae were left on the plate and the number of larvae that pupated were also counted.
RESULTS

Original P-element Screen
The line of flies that we have been working on, slinky\(^{645}\), was generated during a P-element mutagenesis screen looking for a mutation in the gene dropout (Figure 1). The screen was conducted by making a series of local hops with the intention of localizing a second P-element into the dropout gene. It has been demonstrated that local transposition events often arise through a P-element duplication event (Tower et al., 1993). This is in contrast to an excision and re-integration event. Fortunately, the White gene is dosage sensitive, thus two copies of a P-element in the genome give rise to stronger red eye color making duplication events identifiable. Additionally, the original P-element line, B204, shows relatively weak White expression. Thus, P-element duplication was scored as those males that showed an increase in White expression (stronger red eye color). The slinky pattern was found 15 times out of the 802 lines that were generated during this screen.

High Resolution Analysis of the slinky Expression Pattern
The slinky expression pattern is shown in Figure 3. Earliest LacZ expression is seen in segmental patches during late germ band extension (Figure 3A). Expression is restricted to clusters of cells in the abdominal segments. This pattern suggests that expression is initially localized to the developing ectoderm because staining never crosses the ventral midline (Figure 3B). Expression in the segmental patches remains strong until after germ band retraction begins. During germ band retraction, the expression becomes localized to the intersegmental regions of the ectoderm (Figure 3C). At this point in development, expression appears to be localized to the attachment sites of muscles to the body wall. These attachment points, called apodemes, are infoldings of the ectoderm (Bate, 1993). Expression remains strong in these intersegmental regions through the dorsal closure stage. A small cluster of cells in each segment, ventral to intersegmental staining, begins to show LacZ expression during the late stages of germ band extension. This specific cell staining pattern is consistent with cells of the chordotonal organs. Some expression is also seen in the developing head segments (Figure 3D).

Generation of a Single P-element Insertion Line
For higher resolution of the expression pattern, embryos were also stained with anti-βGalactosidase (anti-βGal). Because slinky\(^{645}\) was generated by a P-element duplication event, the original line contained both the B204 P-element and the P-element associated with the slinky pattern (Figure 4A). Staining is seen in the salivary glands and in the amnioserosa, which is driven by the B204 P-element, as well as in the apodemes and PNS which is driven by the slinky P-element. To better analyze the slinky pattern, the original B204 P-element was transposed out of one of our double P-element lines, slinky\(^{645}\), leaving a single P-element line that we have named slinky\(^{645k}\) (Figure 4B). Staining is no longer seen in the salivary glands or in the amnioserosa, but there is staining in the apodemes and in the PNS.

To determine the location of some of the P-elements we have been working on, polytene chromosomal in situ hybridizations were performed. Chromosomal localization of the P-elements in the slinky\(^{645}\) line show two distinct sites of hybridization in polytene region 71. In situ hybridization using a LacZ probe on slinky\(^{645k}\) polytene chromosomes show a
single hybridization in the region 71B (data not shown). The B204 P-element has been mapped to chromosome region 71 D1-D2 (Flybase, 1994).

Colocalization of the slinky Expression Pattern
In order to localize the staining pattern of slinky, a series of antibody stains were carried out. Some embryos were stained with 22C10 which is an antibody that is specific for an antigen that is located on the membranes of all cells in the PNS. This staining pattern was compared to the slinky staining pattern (Figure 5). The cluster of cells that are staining in chordotonal organs of the PNS in the 22C10-stained embryos (Figure 5A) seem to be the same cells as those in the slinky645k embryos stained with anti-ßGal (Figure 5B). To confirm this observation, slinky645 embryos were double-stained with anti-ßGal and 22C10. A subset of cells in the embryo, that presumably are the chordotonal organs of the peripheral nervous system, stained with both antibodies (data not shown).

Analysis of a Recessive Lethal Mutation Associated with the slinky P-element
One of the fly lines containing a slinky associated P-element, slinky645k, was found to be homozygous lethal. Lethality was associated with the P-element insertion by crossing slinky645k flies to flies with a deficiency in the same region, Df(3L)fz2m21. The slinky645k P-element is located in region 71B and the deficiency in Df(3L)fz2m21 spans the region 70D-71E. There were not any viable progeny that contained both the slinky645k chromosome 3 and the Df(3L)fz2m21 chromosome 3.

To determine the stage of lethality of slinky645k, egg and pupal case counts were performed. Ninety-eight percent of the slinky645k embryos hatched into first instar larvae but only 73% survived to the pupal stage. The larvae that survived to the pupal stage carried at least one wild type chromosome. As a control, observations were made of the hatching frequencies and number of larvae that reached the pupal stage in wild type embryos. Ninety-six percent of the wild type embryos hatched into larvae and 94% survived to the pupal stage. This indicates that the slinky645k homozygous line is larval lethal. When placed in trans with the deficiency, the slinky645k hemizygote appears to be embryonic lethal with only 67% of the eggs hatching. To date, we have been unable to restore viability in slinky645k by P-element excision.

DISCUSSION

This study has potentially identified a novel gene that is being expressed in the apodemes in the PNS. Because the pattern resembles a spring, we have named the pattern slinky in honor of the 50th anniversary of the slinky© toy. The P-element associated with the slinky expression pattern is located on the third chromosome in region 71B. This leads us to believe that the gene associated with the slinky staining pattern is also in the region 71B. We have not identified an existing P-element line in the growing number of insertion stocks listed in Flybase that corresponds to the slinky P-element. We also have a line of flies that contains a homozygous larval lethal mutation that is associated with the slinky P-element. We believe that this mutation possibly results in a reduction of the gene product and not a complete removal. Two copies of the mutation result in embryo viability but end with larval lethality, while a single copy of the mutation (when crossed to the deficiency Df(3L)fz2m21) results in embryo lethality. Further analysis of this mutation is required.
To understand the development of apodemes, it is first important to discuss the formation of mesoderm and ectoderm as well as the development of early muscle precursors and the epidermis. During gastrulation, cells move into the interior of the embryo along the invaginating furrow located along the ventral midline of the embryo (Sonnenblick, 1941; Poulson, 1950; Fulllilove et al., 1978). These invaginated cells will form the mesoderm.

Some of the mesodermal cells that have been formed differentiate into muscle cells which will form attachments to the epidermis with the help of some neighboring ectodermal cells. The first visible sign of muscle development is the fusion of mesodermal cells midway through embryogenesis at specific locations within the embryo (Bate, 1990). These cells are thought to fuse with surrounding mesodermal cells (Rushton et al., 1995) to give rise to multinucleate muscle precursors (Bate, 1990). After gastrulation, myoblasts migrate laterally from the midline to the appropriate position next to the epidermis. The muscle precursors extend growth-cone-like processes that come into contact with the overlying epidermis while navigating along specific routes toward attachment sites. Establishment of appropriate epidermal attachment gives muscle its proper orientation and length (Bate 1990; Bier et al., 1990). It has also been shown that mutations in Drosophila that disrupt normal epidermal formation cause defects in muscle patterns (Bier et al., 1990; Volk and Vijayaraghavan, 1994).

Although little is known about how individual muscles acquire their specific morphology, genes such as stripe, Toll, and myospheroid have been shown to be involved in muscle attachment (Callahan et al., 1996). The gene derailed is expressed in tissues similar to the expression pattern of slinky. derailed is expressed in the PNS and at the sites of muscle attachments (Callahan et al., 1996). In the PNS, derailed has been found to be responsible for axon guidance. The neural precursors of derailed mutants fail to extend along their appropriate pathways and derailed seems to play an important role in the recognition between growing neurons and their pathways (Callahan et al., 1996). derailed is also expressed in embryonic muscle fibers and neighboring epidermal cells during muscle growth and attachment. This muscle guidance function has been shown not to be due to gross alterations in the epidermis or loss of epidermal attachment cell precursors. The role that derailed plays in the guidance of muscle precursor cells has been shown to be analogous to axon pathway selection in the nervous system (Callahan et al., 1996).

Further study of the function of the gene associated with the slinky enhancer trap pattern will provide basic information on a spatially restricted protein expressed in the apodemes and PNS. Mechanisms of determination and development are at the leading edge of developmental biology research. With the molecular and genetic tools that are currently being developed, information about genes involved in embryonic development will be gathered more easily and efficiently.
REFERENCES


Figure 1. Localized P-element transposition screen.

Cross 1: \( \text{B}204\![P\text{-LacW}^+] \)  
\( \text{B}204\![P\text{-LacW}^+] \)  
\( \text{X}^{\text{A 2-3, Kl}} \)  
\( \text{X}^{\text{2-3, Kl}} \)  
Select mosaic-eyed, Kl virgin females

Cross 2: \( \text{TM}6\text{B, Th} \)  
\( \text{TM}3\text{, Sb} \)  
\( \text{X} \)  
\( \text{B}204\![P\text{-LacW}^+] \)  
\( \text{X}^{\text{A 2-3, Kl}} \)  
Select Non-Kl, Non-Sb males with darker red eyes (X)

Cross 3: \( \text{R}204\![P\text{-LacW}^+] \)  
\( \text{TM}6\text{B, Tb} \)  
\( \text{X} \)  
\( \text{TM}3\text{, Sb} \)  
\( \text{TM}6\text{B, Tb} \)  
Stain embryos for \( \beta \)-galactosidase expression
Figure 2. Generating a single P-element line. In one of the lines of flies that expressed the *slinky* pattern, the B204 P-element was specifically transposed out for better resolution of the new pattern. This was done by re-introducing the transposase, Δ 2-3, Kl. Male flies with light orange eyes were used to establish new balanced lines. The individual lines of flies were X-Gal stained to determine which P-element was transposed out.

Cross 1:
(mass-mating)

\[
\begin{array}{ccc}
\odot & \odot & \begin{array}{c}
\text{[P-LacW]^[645]}^{\star} \\
\text{TM6B, Tb}
\end{array} \\
& \times & \\
\Delta 2-3, Kl & \Delta 2-3, Kl
\end{array}
\]

\*contains both 645 and B204 p-element

Select mosaic eyed, Kl males

Cross 2:
(mass-mating)

\[
\begin{array}{ccc}
\odot & \odot & \begin{array}{c}
\text{[P-LacW]^[645]}^{\star} \\
\Delta 2-3, Kl
\end{array} \\
& \times & \\
\Delta 2-3, Kl
\end{array}
\]

Select non-Kl males with light orange eyes

Cross 3
(single-pair matings)

\[
\begin{array}{ccc}
\odot & \odot & \begin{array}{c}
\text{[P-LacW]^[slinky]} \\
\text{TM6B, Tb}
\end{array} \\
& \times & \\
\text{TM6B, Tb}
\end{array}
\]

Embryos were stained with lacZ to determine which p-element hopped out.
Figure 3. Developmental expression of the *slinky* pattern. (A) Lateral view of a stage 11 embryo. Expression of the *LacZ* reporter gene is seen in a segmental pattern. (B) Dorsal view of a stage 11 embryo; staining does not cross the ventral midline. (C) Lateral view of an early stage 12 embryo. Expression is becoming restricted to the posterior margin of each segment. (D) Lateral view of an early stage 14 embryo. Expression of the *LacZ* reporter gene is still seen in the intersegmental apodemes (iapo) and is now seen in the chordotonal organs of the peripheral nervous system (ch).
Figure 4. Anti-βGal stains of apodeme and chordotonal expression pattern. (A) Lateral view of a stage 14 slinky$^{645}$ embryo. This line of flies contains two P-elements. Expression of the LacZ reporter gene is driven in the salivary glands (sg) and the amnioserosa (as) by the B204 P-element and also in the intersegmental apodemes (iapo) and in the chordotonal organs of the peripheral nervous system (ch) by the slinky P-element. (B) Lateral view of a stage 14 slinky$^{645k}$ embryo. This line of flies contains only the slinky P-element. LacZ expression is seen in the intersegmental apodemes (iapo) and the chordotonal organs of the peripheral nervous system (ch). There is no longer staining in the salivary glands or in the amnioserosa.
Figure 5. Comparison of 22C10 stained embryos and anti-ßGal stains of the *slinky* expression pattern. (A) Lateral view of a stage 14 embryo stained with 22C10 antibody stain. Staining is seen in all of the cells of the peripheral nervous system. (B) Lateral view of a stage 14 embryo stained with anti-ßGal. Arrows are pointing to cells that appear to be staining with anti-ßGal as well as with 22C10.