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ANALYSIS OF A TRANSCRIPTIONAL REPRESSOR IN DROSOPHILA MELANOGASTER

presented by

YIFAN MAO

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of the requirements for

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Major professor

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ANALYSIS OF A TRANSCRIPTIONAL REPRESSOR IN *DROSOPHILA MELANOGASTER*

By

Yifan Mao

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ABSTRACT

ANALYSIS OF A TRANSCRIPTIONAL REPRESSOR IN *DROSOPHILA* MELANOGASTER

By

YIFAN MAO

The repressor protein Knirps regulates gene expression in *Drosophila* embryonic development. I have been using an *in vivo* repression assay to define the repression domains of the Knirps protein. A panel of mutant knirps gene constructs were generated and transformed into *Drosophila* embryos by microinjection.

The repression activities monitored in embryos suggested that two portions of the Knirps protein can mediate repression. One region contains the binding site for dCtBP, a cofactor previously shown to be important for Knirps function. In addition, a more N-terminal portion of Knirps is able to repress transcription without the dCtBP binding sites. Thus, Knirps may function via two different pathways. Residues 202-358 and residues 139-330 have been mapped to the minimal C-terminal repressor and N-terminal repressor respectively. By the comparing the repression activities of repression domain with different DNA binding domains, the Knirps DNA binding domain is proposed to exert negative interference on the repression domain.

To Mum and Dad

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Finally, I want to give my special thanks to my family, for their support and faith.

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CHAPTER 1

LITERATURE REVIEW

Overview of the transcriptional regulation in eukaryotes

Eukaryotic transcription is carried out by three DNA-dependent RNA polymerases (reviewed by Young 1991): RNA polymerase I, II and III. RNA polymerase I synthesizes ribosomal RNA; RNA polymerase II synthesizes messenger RNA and some small nuclear RNA; RNA polymerase II synthesizes the 5S rRNA, tRNA and some small nuclear RNA. Each of these three RNA polymerases is composed of 8-14 polypeptides. Most eukaryotic Pol II promoters that recognized by RNA polymerase II contain common elements such as a TATA box, GC rich sequences or CAAT boxes. To initiate transcription, multiple transcriptional factors (TFs) are required for accurate initiation from a promoter DNA sequence. These transcriptional factors include TBP (TATA-binding protein) or TFIID, TFIIA, TFIIB, TFIIF, TFIIE and TFIIH (Zawel and Reinberg, 1993, Roeder, 1996, Orphanides et al., 1996). Similar TFs have been identified in yeast, *Drosophila*, rat and human systems. These TFs along with RNA polymerase II are often referred to as the general transcriptional machinery.

In eukaryotes, DNA was packaged into chromatin and DNA sequences within the chromatin structure are generally inaccessible to transcription factors and Pol II. Transcriptional activators can relieve this repression caused by chromatin structure by direct or indirect covalent modification of the histones to loosen the packaging of the nucleosomal DNA. Activators also possess the ability to facilitate transcription by acting on the general machinery to increase the efficiency of the process.

Like transcriptional activation, transcriptional repression is important for appropriate gene expression *in vivo* by turning off transcription (Hanna Rose and Hansen, 1996). Repressors play a major role in many biological processes. Mutations in repressor genes may cause tumor growth, developmental disorders or disregulation of the cell cycle (Wang et al., 1999).

There are four different models proposed regarding the mechanisms of repressors in eukaryotes. The first one is called the competition model; in which the DNA binding sites of the repressor overlap with those of the activator. The repressor binds to the enhancer and prevents the activator from binding to DNA (Foulkes et al., 1991). The second model is called the quenching model. Both the activator and the repressor are capable of binding to DNA simultaneously, but the repressor keeps the activator from functioning by masking the activation surface and preventing it from making proper contact with the basal machinery. Some repressors can repress multiple enhancers from long distances (over 500 bp).

The third model of repression is that the repressor somehow prevents the formation of the transcription complex, thus blocking transcription. The *Drosophila* protein Even-skipped is an example of a repressor that uses this mechanism. This protein represses transcription by binding to TBP and blocking the TFIID-TATA box interaction (Li et al., 1998).

The fourth model is the squelching model. In this model, the repressor interacts neither with DNA nor the activator protein, but represses transcription by sequestering factors required for the function of the activator. The estrogen receptor (ER) represses the transcriptional activation by progesterone receptor (PR) in this manner (Etienne et al., 1989).

Transcriptional regulation in *Drosophila* embryonic development

Transcription is regulated by factors that result in highly selective gene expression in organisms. In *Drosophila*, enhancers integrate both positive and negative regulatory information to direct sharp patterns of gene expression in the embryo. These enhancers contain tightly clustered binding sites for both transcriptional activators and repressors (Jäckle 1992) and lead to the cross and hierachical gene regulation cascade. Activators, such as Bicoid (Driever et al., 1989), turn on gene expression, while repressors, such as Knirps (Arnosti et al., 1996), either reduce or turn off gene expression completely. Thus, transcriptional regulation of one gene in the *Drosophila* embryo usually involves multiple transacting factors. For example, in *Drosophila melanogaster*, *even-skipped* stripe 7 is activated by Tailless and repressed by Knirps. The interplay of these multiple transcriptional regulators causes the specific developmental pattern of *Drosophila* embryos. *Drosophila* segmentation also depends on the successive activities of several classes of genes; The maternal gene products Bicoid, localized in the anterior pole of the embryos, and Nanos, localized in the posterior pole of the embryo, set up the anterior-posterior axis of body pattern formation. Those maternal gene products provide the position information for the zygotic genes, including the gap genes, pair-rule genes and segment-polarity genes (for reviews, see Nüsslein-Volhard et al., 1987). In the hierarchical gene regulation process involved in embryonic development, the gap genes respond to the regulation of maternal gene products. In turn, the gap genes regulate expression of the pair-rule genes and the pair-rule genes regulate their downstream genes

including the segment polarity genes (for reviews, see Pankratz and Jäckle, 1993). This sequential regulation of gene expression determines the fate of the embryonic development of *Drosophila*.

Transcriptional repression is a complicated issue which people are addressing from different respects. *Drosophila* is an ideal system to study transcriptional repression because of several advantages: *Drosophila* is an eukaryotic system; it has a relatively short generation time; it is relatively easy to maintain and manipulate; *Drosophila* genetics has been well studied. Most importantly, the same types of proteins are found in other higher eukaryotes. Since Knirps is a transcriptional repressor that plays a major role in the embryonic development of *Drosophila melanogaster*, the study of Knirps will have significance in both developmental biology and the study of transcription mechanisms.

Introduction to the *knirps* gene and the Knirps protein

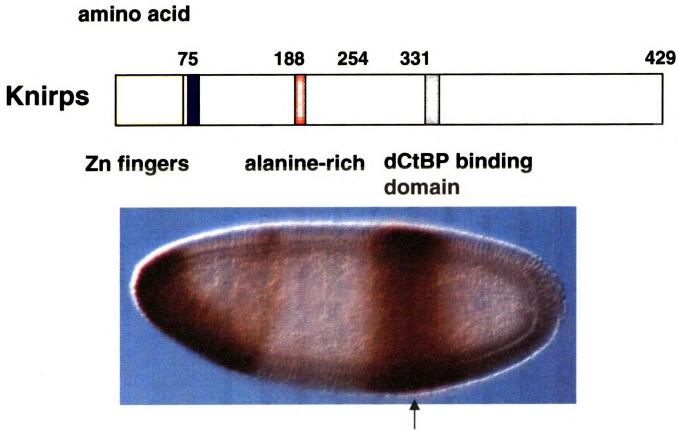
The *knirps* gene is one of the gap genes involved in early embryonic development of *Drosophila melanogaster*. It encodes a transcriptional repressor that regulates correct spatial and temporal gene expression in the early *Drosophila* embryo. The *knirps* gene was identified in a screen for early developmental defects, when a type of *knirps* mutant embryo was found to form only two abdominal segments instead of the normal eight segments in the embryo (Nüsslein-Volhard and Wieschaus, 1980). Knirps protein is expressed in the anterior-ventral region and the posterior region of the blastoderm embryo, which will give rise to the abdomen between nuclear division cycle 11 and 14 (Figure 1) (Rothe et al., 1992). It is known that the products of other genes including

activators such as Krüppel, Caudal, Bicoid (Rivera-Pomar, et al., 1995) and repressors such as Giant and Tailless, set the boundaries of expression of Knirps (Pankratz, et al., 1993).

Knirps has been well studied genetically and a series of mutant alleles have been identified: 1. a number of null alleles that causes the embryo to lack six out of eight abdominal segments; 2. an intermediate hypomorphic allele that result in the loss of three or four abdominal segments; and 3. a weak hypomorphic allele that leads to the loss of one or two abdominal segments (Nauber et al., 1988). The *knirps* gene has been cloned and located on the left arm of the third chromosome, based on the evidence that certain chromosomal rearrangements are associated with a *knirps* mutation (Nauber et al., 1988).

Knirps protein is a member of the nuclear hormone receptor superfamily (Nauber et al., 1988). A Cys2Cys2 type zinc-finger DNA binding domain (residues 1-74) and a repression domain (residues 75-429) (Figure 1) functional when separated (Gerwin 1994, Arnosti et al., 1996). The "Kni box" between residue 52 and 93 is highly conserved in the Knirps-like proteins of *Drosophila melanogaster*, *Drosophila virilis* and the more distantly related *Musca domestica*. Knirps protein possesses a zinc-finger DNA binding domain and binds to DNA sequence 5'-ACTGAACTAAATCCGG-3'. Knirps is capable of binding to DNA as a monomer (Gerwin et al., 1994). Therefore, its DNA binding properties can be distinguished from those of the known Cys2Cys2 type nuclear receptors, which require homodimer or heterodimer formation. *In vivo*, Knirps acts as a repressor to define the posterior boundary of *even-skipped* stripe 3 (Small et al., 1992) and to define the *hairy* stripe 7 in the anterior boundary (Pankcratz, M. and Jäckle, H., 1993). Mutating *knirps* gene in embryos results in the expanding of the expression

Figure 1. The schematic representation of the Knirps protein (top) and the expression pattern of the endogenous Knirps protein (bottom). Images in this thesis are presented in color.


amino acid
75
188
254
331
429
Knirps
Zn fingers
alanine-rich
dCtBP binding domain
Endogenous Knirps protein

patterns of those stripes due to the loss of repression (Small et al., 1996), eventually causing embryonic lethality (Nüsslein-Volhard, C. and Wieschaus. 1980).

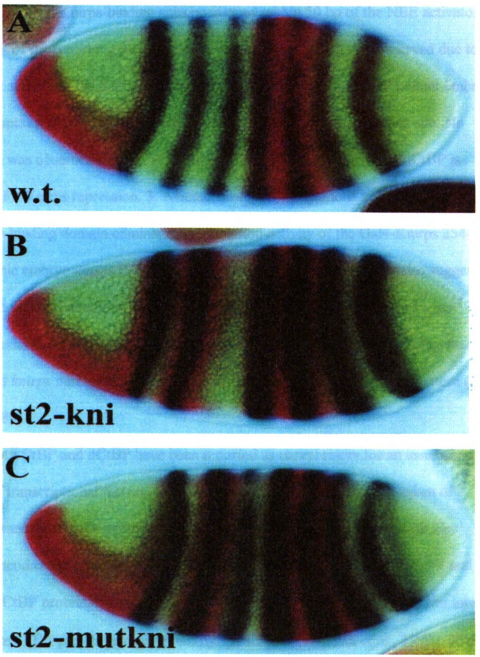
There are two classes of repressors: long-range repressor versus short-range repressor. The *Drosophila* long-range repressor Hairy is capable of working over 1 kb away from the upstream activator to repress the rhomboid NEE enhancer (Barolo et al., 1997). Knirps is a member of the short-range class of repressors, which includes Snail (Gray et al., 1994), Krüppel (Gray et al., 1996) and Giant (Hewitt et al., 1999). These factors act on nearby DNA sequences to repress gene expression. For example, Knirps represses an *even-skipped* stripe 2 enhancer element, when it is bound less than 100 bp away from this enhancer (Arnosti et al., 1996). Knirps can also function as a dominant repressor, blocking multiple enhancers, such as the *twist* enhancers and the *rhomboid* enhancer, when it is bound 75 bp or less away from the transcription start site (Arnosti et al., 1996). It has been shown that Knirps protein contains several functional domains which are arranged in a modular fashion. The alanine-rich part of the Knirps protein (encoded by codons 189-254) acting as a functional repressor in *Drosophila* Schneider cells (Gerwin et al., 1994).

The transcriptional corepressor of Knirps, dCtBP

Co-factor mediated repression has been commonly discovered in a variety of systems. In mammalian cells, the transcriptional repression by the methyl-CpG-binding protein MeCP2 requires a corepressor complex containing the transcriptional repressor mSin3A and histone deacetylase (Nan et al., 1998). During an investigation of the

mechanisms of Knirps repression function, dCtBP, the *Drosophila* homologue of the mammalian C-terminal binding protein, was shown to be involved in the repression activities of Knirps protein (Nibu et al., 1998b). The mammalian protein, CtBP (C-terminal binding protein) interacts with the adenovirus E1a protein and decreases the transformation activity of the E1a protein (Schaeper et al., 1995). This interaction was suggested to be critical for adenovirus replication and oncogenic tranformation. A specific sequence motif P-DLS-K, highly conserved among E1A proteins of various human and animal adenovirus, is required for the CtBP-E1A interaction (Schaeper et al., 1995). dCtBP protein is found to be maternally deposited into *Drosophila* embryos, involving in developmental processes (Nibu et al., 1998b). dCtBP protein recognizes the same motif P-DLS-K in Knirps protein, and alanine substitution mutation in the DLS sequence abolished interaction between dCtBP and Knirps in *in vitro* glutathione-S transferase pull-downs (Nibu et al., 1998b). The evidence as following supports the hypothesis that the P-DLS-K motif is essential for Knirps-mediated repression: 1. St2-Knirps was constructed by fusing the *even-skipped* stripe 2 enhancer to the *knirps* gene and was transformed into embryo. The transgenic embryo displays an ectopic Knirps expression pattern localized as a stripe in the anterior of the embryo (Figure 2A). The products of this St2-knirps repress the *even-skipped* stripe 3 (Figure2B). The repression is not seen when a st2-knirps mutant gene with the alanine substitutions in the dCtBP-binding motif was transformed into the embryo (Figure 2C) (Nibu et al. 1998a). 2. The results from analyzing the expression of synthetic transgenes in dCtBP mutant embryos. The transgene contains a modified form of 700 bp rhomboid lateral stripe enhancer (NEE) and directs strong expression in both lateral and ventral regions. The wild type

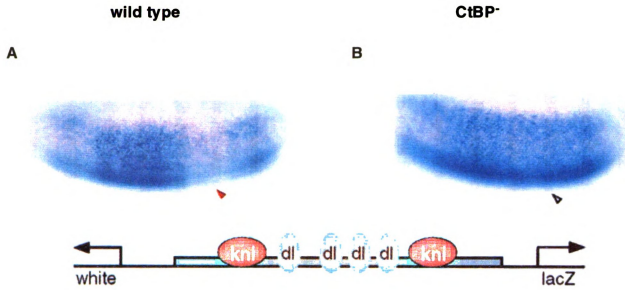
Figure 2. The P-DLS-K motif is essential for Knirps-mediated repression. Cellularizing embryos were hybridized with mixtures of a digoxigenin-labeled knirps antisense RNA (red) and a fluorescein-labeled eve antisense RNA (black). They are orientated with anterior to the left and dorsal up. (A) Double staining pattern in a wild-type embryo. *eve* is expressed in a series of seven stripes, while *knirps* is expressed at the anterior pole and antero-ventral regions, as well as in a broad posterior band which encompasses eve stripe 4 and 5. (B) Same as (A) except that the embryo contains a transgene with the full-length *knirps* coding region was mutagenized to disrupt the P-DLS-K motif (PMDLSMK to AAAASMA). The ectopic *knirps* stripe does not cause an obvious change in the *eve* pattern; in particular, stripe 3 pattern, suggesting that the mutant Knirps protein retains weak repressor activity


A
w.t.
B
st2-kni
C
st2-mutkni

embryo, where dCtBP protein is maternally deposited, contains a modified enhancer NEE with two synthetic Knirps-binding sites positioned within 50 bp of the NEE activators. Repression of the reporter gene expression in the ventral region was observed due to the repression activity of the Knirps protein (Figure 3); whereas the dCtBP mutant embryo without contains the same NEE enhancer and the synthetic Knirps binding site. No repression was observed (Figure 3) (Zhang et al., 1998), suggesting that dCtBP is necessary for Knirps repression. 3. Alanine substitution mutations in PMDL sequence in the dCtBP-binding domain eliminate the repression activity of the Gal4-Knirps 254-429 in transgenic embryo assays (Nibu et al., 1998b). 4. Gene dosage assays also suggest that Knirps and dCtBP interact *in vivo*. Embryos that are heterozygous for the *knirps* ^{9} null mutation exhibited occasional defects in the eve expression pattern. Combining the *dCtBP* and *knirps* mutations resulted in more severe disruptions in the *eve* pattern (Nibu et al., 1998b).

Both CtBP and dCtBP have been reported as corepressors for an increasing number of transcriptional factors. CtBP is reported involved in the mechanism of Rb/p130 transcriptional repression (Meloni et al., 1999). CtBP interacts with zinc finger/homeodomain protein ZEB and its *Drosophila* homologue zfh-1. CtBP 1 and CtBP 2 are two CtBP proteins with slight sequence variations that were discovered to interact with mouse δEF 1 protein in a yeast two hybrid screen (Furusawa et al., 1999). dCtBP protein mediates transcriptional repression by Knirps, Krüppel and Snail in the *Drosophila* embryo, and interacts with *Drosophila* protein Hairy in the embryo (Poortinga et al., 1998). dCtBP is also suggested to interact with polycomb group proteins

Figure 3. Loss of Knirps-mediated repression in a dCtBP mutant embryo. *LacZ-white* reporter genes were introduced into dCtBP mutant embryos and stained after in situ hybridization with a white antisense RNA probe. Cellularized embryos are oriented with anterior to the left and dorsal up (A and B). *white* staining patterns in a wild-type (A) and dCtBP mutant embryo (B). The reporter gene contains a *rhomboid* lateral stripe enhancer (NEE) and two synthetic Knirps-binding sites flanking the four Dorsal activator sites (see diagram beneathe the embryos) (From NIbu et al., 1998a).


wild type
CtBP-
A
B
knl
dl
dl
dl
dl
knl
white
lacZ

(Sewalt 1999). Recent studies suggest that ectopic expression of the native Krüppel protein causes patterning defects in early embryos, which are reversed when the P-DLS-H motif is mutagenized (Zhang et al. 1999). These results suggest that Krüppel mediated repression also depends on the recruitment of the dCtBP corepressor.

Although both CtBP and dCtBP have been shown to interact with a number of transcriptional regulator proteins, the mechanisms through which they affect developmental processes are unclear. Both CtBP and dCtBP were reported to display significant homology to the D isomer 2-hydroxy acid dehydrogenase family (Schaper et al., 1995, Nibu et al., 1998b). Members of this family have so far been identified only from bacteria, plant and lower fungi (Arthur et al., 1991, Taguchi et al., 1991, Chow et al., 1993). However, neither CtBP nor dCtBP shows any dehydrogenase enzyme activity or NAD binding activity (Schaper et al., 1995). The mammalian CtBP protein has been shown to interact with the histone deacetylase HDAC1 (Sundqvist et al., 1998). Meanwhile, it was also reported that the SV 40 promoter, shown to be repressed by CtBP, is relatively insensitive to histone deacetylase inhibitors (Meloni et al., 1999), suggesting that this is unlikely to be the primary mechanism of CtBP mediated repression.

Previous reports showed that dCtBP interacts with the repression domain of Knirps protein though the conserved motif P-DLS-K. Meanwhile, it is a common phenomenon that one repressor could contain multiple repression domains which can independently exert repression function on its target genes. One example is the *Drosophila* Engrailed protein, in which two separate repression activities have been identified. One domain provides most of the activity in embryonic assays and on

integrated genes, and binds the Groucho corepressor, a protein that also interacts with the Hairy, Runt, and Dorsal repressors. Another region of Engrailed contains an activity that appears to be primarily active on transiently transfected reporter genes and does not bind Groucho (Tolkunova et al., 1998). The Hairy protein has also been found to contain multiple activities; Hairy also interacts with the Groucho cofactor via a C terminal WRPW motif, and a separate "Orange" domain mediates antagonistic activities toward Scute protein. Recent work shows that Hairy can also interact with the dCtBP protein via a third region of the protein (Poortinga et al., 1998), although it is not clear whether this interaction contributes to repression (Zhang et al., 1999). Krüppel has also been found to contain two separable repression domains, both of which are active in cell culture assays; these two domains have been reported to have different activator specificity (Hanna-Rose et al., 1997). Knirps repressor has been suggested to possess at least two independent repression domains. These two repression domains function autonomously to repress transcription through distinct mechanisms (Mao et al., manuscript in preparation). One is the C-terminal portion of the knirps protein, acting through a dCtBP dependent mechanism, and the other repression domain is located in the N-terminal region. The N-terminal repressor apparently functions through a dCtBP independent mechanism although the specific residues involved and molecular mechanisms of this repression activity are not clear.

CHAPTER 2

THE REPRESSION ACITIVITIES OF KNIRPS

Short-range repression activity of the Knirps protein

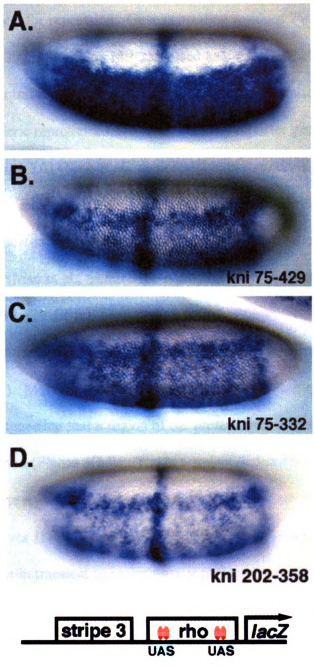
Short-range repression activities have been observed in the behavior of a number of repressors, in which repression occurs only when the targets are near the repressor. Krüppel and Snail are examples of short-range repressors. Knirps represses transcription within a certain distance from either the promoter or the enhancer. Previous studies indicated that endogenous Knirps was capable of blocking transcription when the binding sites were situated at –55 bp, but not at –130 bp from a heterologous promoter (Arnosti et al., 1996). Knirps can also repress a heterologous enhancer over a short distance (Arnosti et al., 1996). To determine the exact distance dependence of Knirps repression, and test whether the loss of activity with increasing distance represents a "step function" or a gradual tapering off of activity, transgenes were designed with tandem binding sites situated at -55, -70, -75, -100, -130 or –180 bp. These genes were introduced into *Drosophila* by P-element mediated germ line transformation, and the embryonic expression patterns of the transgenes were analyzed by in situ hybridization. Strong repression was observed in genes with Knirps binding sites whose 3' edges were situated at -55bp, -70, or -75 bp. The Knirps binding sites used in this gene have been shown to confer repression in a Knirps-dependent manner (Arnosti et al., 1996), and the repression was observed only in the presumptive abdomen and ventral anterior regions, where the *knirps* gene is expressed (Jäckle 1992). Repression was less effective in the –100 bp and –130 bp constructs, and almost undetectable in the construct with the sites at -180 bp. No differences were observed in the pattern of genes with repressor sites situated at -70 or -

75bp, indicating phasing effects are not important on this reporter (Mao et al., manuscript in preparation).

Gal4-Knirps, the model for the study of Knirps

Mutagenesis of the endogenous *knirps* gene leads to embryonic lethality. To study the mechanisms of the repression behavior of Knirps protein in embryos without interfering with the normal embryonic development, various recombinant knirps genes were constructed by replacing the DNA binding domain codons 1-74 of Knirps with the DNA binding domain codons 1-93 of the yeast Gal4 protein. Two reporter systems were constructed and subjected to the *in vivo* repression assay, in which a *lac Z* reporter gene was under the control of two enhancer elements. One was the *even-skipped* stripe 2 and stripe 3 enhancers and the Gal 4 cognate sites (UAS) are located adjacent to stripe 2 enhancer. The other was the *even skipped* stripe 3 and *rhomboid* enhancers with the Gal 4 recognition sites located within the rhomboid enhancer. In both cases, the proximal enhancers, stripe 2 in the first reporter and rhomboid in the second reporter, were repressed in the region where knirps was present. These results indicate that the Gal4-Knirps chimeric proteins are causing the same effect as the endogenous protein and Gal4-Knirps is a good model for study the repression mechanisms of Knirps protein in *Drosophila* embryos (Figure 4) (Mao et al., manuscript in preparation).

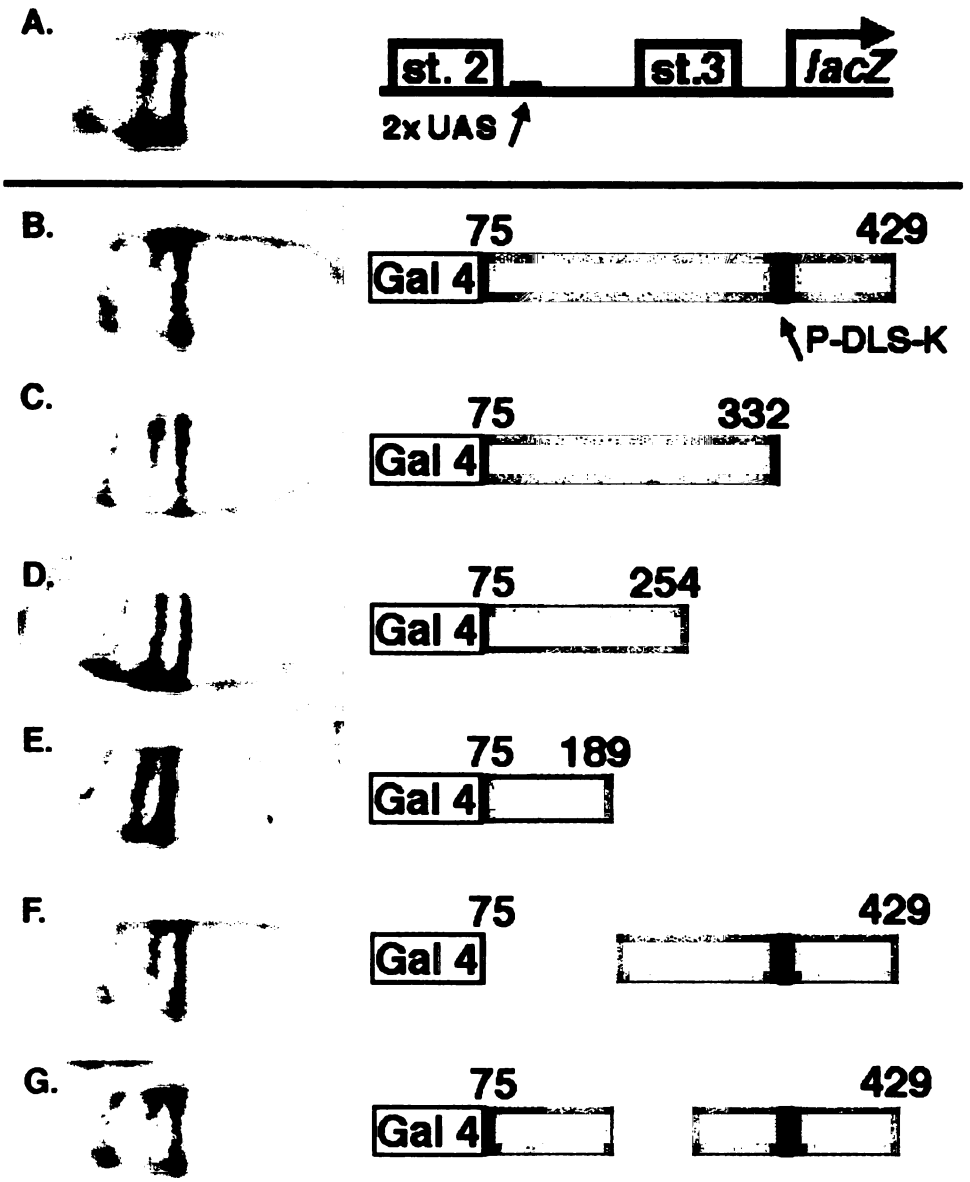
Figure 4. Gal4-Knirps chimeric repressors mimic the activity of endogenous Knirps portein on a *rhomboid* enhancer element. (A) Expression pattern of the *eve* stripe 3/*rho lacZ* reporter gene showing robust ventral expression directed from a *rho* enhancer element lacking endogenous Snail binding sites (Gray et al., 1996), and a central stripe from the *eve* stripe 3 enhancer. (B) Repression in ventral regions mediated by Gal4-Knirps 75-429 repressor protein expressed in ventral regions of the embryo under control of a *twist* promoter construct. (C) Repression mediated by the Gal4-Knirps 75-332 chimera, lacking the dCtBP interaction motif. (D) Repression mediated by the Gal4-Knirps 202-358 chimera, which contains the dCtBP interaction motif. Gal4 binding sites (marked UAS in the figure were introduced in a 600 bp *rho* enhancer at the positions previously used for targeting Knirps protein to this gene complex (Arnosti et al., 1996). Ventrolateral views are shown, with anterior to the left (From Mao et al., manuscript in preparation).


A.
B.
kni 75-429
C.
kni 75-332
D.
kni 202-358
stripe 3
rho
lacZ
UAS
UAS

Mutational analysis of the Gal4-Knirps chimera.

A series of truncation mutations were introduced in the Gal4-Knirps gene to identify residues critical for repression. Genes encoding residues 75-332, 75-254, 75-189, 75-189 + 254-429, 188-429, or 189-254 fused to the Gal4 DNA binding domain were expressed in ventral regions of transgenic embryos under control of a *twist* promoter element. These chimeric repressors were tested on *lacZ* reporter genes activated by *eve* stripe enhancers (Figure 5A) (Mao et al., manuscript in preparation). A full-length 75-429 chimera showed effective repression of the proximal stripe 2 enhancer, while, consistent with the short-range of Knirps activity, the distal stripe 3 enhancer was not affected (Figure 5B) (Mao et al., manuscript in preparation). A C-terminal truncation removing residues 333-429 did not compromise activity (Figure 5C) (Mao et al., manuscript in preparation), although it does remove most of the dCtBP interaction region of Knirps (see below). More extensive C-terminal truncations starting at residues 254 or 189 were inactive, suggesting that a region of the protein from 254-332 might be necessary for repression (Figure 5D-E). The N terminal region 75-187 was dispensable for activity, as was the region of the protein from 189-254 (Figure 5F,G). The chimera containing only residues 189-254 was not active (Figure 5H), unlike the result obtained for a similar construct in transient transfection assays (Gerwin et al., 1994). Constructs were also tested on the *eve* stripe 3/ *rho* reporter gene as in Figure 3 and a stripe 2 lacZ gene (Arnosti et al., 1996), with identical results (data not shown). All constructs scored as active showed at least 20% of the embryos were repressed in blinded scoring assays,

Figure 5. dCtBP interaction region of Knirps is dispensible for repression activity in C-terminal truncated proteins. (A) Expression pattern of the *eve* stripe 2/stripe 3 reporter gene, with UAS binding sites shown adjacent to the stripe 2 element. (B) Expression of the Gal4-Knirps 75-429 gene represses stripe 2, but not stripe 3, in ventral regions. (C) Gal4-Knirps 75-332 chimera, lacking an intact dCtBP binding motif (gray box), is a functional repressor. (D, E) Truncation at codon 254 or 189 leads to loss of activity. (F) Deletion of residues 75-187 does not compromise repression activity. (G) Deletion of residues 189-254 does not compromise repression activity. Genes shown here were produced by introducing stop codons into the gene at appropriate sites, thus the overall transcript size and 3'UTR is conserved . Embryos are shown with anterior to left, dorsal side up (Mao et al., manuscript in preparation).


A.
st. 2
st.3
lacZ
2x UAS
B.
75
429
Gal 4
P-DLS-K
C.
75
332
Gal 4
D.
75
254
Gal 4
E.
75
189
Gal 4
F.
75
429
Gal 4
G.
75
429
Gal 4

while inactive repressor genes and the reporter construct alone had less than 2% of the embryos scored as repressed (Mao et al., manuscript in preparation).

One of the Gal4-Knirps chimeric proteins, Gal4-Knirps 75-330, which contains the residues 75-330 of Knirps, and Gal4-Knirps 75-332, which contains the residues 75-332, do not have the dCtBP-binding motif but do show *in vivo* repression activity (Dr. Scott Keller unpublished results). In addition, Gal4-Knirps 75-332 protein does not interact with dCtBP in GST pull down assays (Dr. Carla Margulies unpublished results). Thus, binding to dCtBP *in vitro* depends on an intact interaction motif. However, this interaction is not a requirement for the activity of the 75-332 protein. These results suggest that dCtBP-binding is dispensable for the repression function of the N-terminal portion of Knirps protein, between residues 75 and 332.

CHAPTER 3

THE MINIMAL REPRESSION DOMAIN OF KNIRPS

Introduction

Like transcriptional activators (Mitchell and Tjian, 1989), many transcriptional repressors are modular factors since the effector domain can be separated from the DNA binding domain. Repression domains in many repressors can be classified according to the amino acid sequence, such as charged, alanine-rich and proline-rich domains (Gray et al., 1996). Previous work has demonstrated that a region between residue 75 and 429 is sufficient to confer transcriptional repression when separated from the DNA binding domain (Arnosti et al., 1996). To further identify the repression domains within this region, various recombinant *knirps* genes were constructed by replacing the DNA binding domain codons 1-74 of Knirps with a DNA binding domain to identify residues and regions critical for repression.

Materials and methods

Plasmids

Minimal repression domains: Fragments of the *knirps* open reading frame were generated by PCR from the pCarnegie 20 vector pN741 containing the *knirps* cDNA (G. Struhl, unpublished) with *Pfu* DNA polymerase (Stratagene), using different pairs of primers. The products were subcloned into the *Kpn*I and *Xba*I sites of the pTwiggy vector (Arnosti et al., 1996) containing *twi* enhancer element 2xPEe-Et and *twist* basal promoter. The constructs and the corresponding primers used were:

202-358-flag: oligo 1,

5'-CGGGGTACCGCTGCCGCTGCAGCGGCTTCTGCTGCCGATGCCGCT-3';

202-358-flag: oligo 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

CACCTCCACTTCTTGATCCTCGGA-3';

211-358-flag: oligo 1,

5'- CGGGGTACCGATGCCGCTTACCGGCAGGAGATGTACAAGCACCGC-3';

211-358-flag: oligo 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

CACCTCCACTTCTTGATCCTCGGA-3';

The amplified fragments were digested with *Cla*I and *Xba*I and used to replace the *Cla*I-*Xba*I fragment of the *knirps* cDNA clone in pBluescript(SK+). The *Kpn*I-*Xba*I fragments of these clones were then inserted in pTwiggy (Arnosti et al., 1996). The final constructs encode Knirps amino acids followed by an eight amino-acid sequence including the FLAG epitope, DYKDDDDK.

248-358-flag: oligo 1,

5'-GGG TCGGTACCGCAGCCTCGGCCCGCCAGTCGCCCATCGAT-3'

248-358-flag: oligo 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

CACCTCCACTTCTTGATCCTCGGA-3'

202-429-flag: oligo 1,

5'-CGGGGTACCGCTGCCGCTGCAGCGGCTTCTGCTGCCGATGCCGCT-3'

202-429-flag: oligo 2,

GTAATCGACACACACGAATATTCCCCTCAT-3'

211-429-flag: oligo 1,

5'- CGGGGTACCGATGCCGCTTACCGGCAGGAGATGTACAAGCACCGC-3'

211-429-flag: oligo 2,

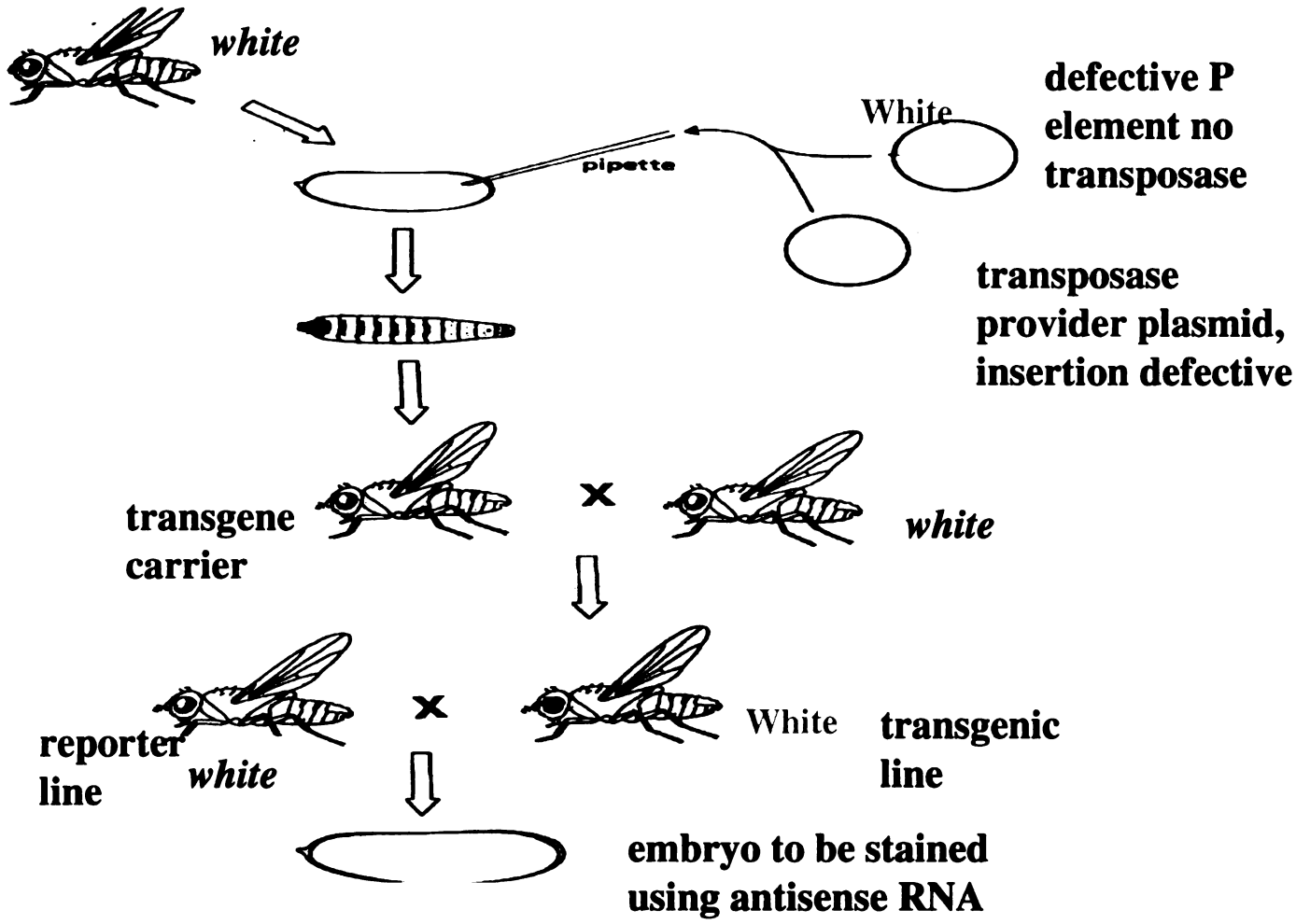
5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCGTCATCCTT

GTAATCGACACACACGAATATTCCCCTCAT-3'

P-element transformation, whole-mount in situ hybridization of embryos, and crosses to reporter lines. P-element transformation vectors were introduced into the *Drosophila* germline by injection of $y\ w^{67}$ embryos as described (Small et al., 1992) (Figure 6). For each gene construct, at least three separate lines were tested, and similar results were obtained in each case. In situ hybridizations were performed as described (Small et al., 1992) using digoxigenin-UTP labeled antisense RNA probes to *lacZ*. The Gal4-dCtBP chimeric lines used in Figure 11 were kindly supplied by Dr. Yutaka Nibu and Dr. Michael Levine.

Assays of in vivo repression activity. Transformant offsprings were crossed with reporter lines containing one of three reporters: 1) *even-skipped* stripe 2 linked to *eve-lacZ* (Arnosti et al., 1996), 2) *even-skipped* stripe 2 and stripe 3 enhancers linked to *eve-lacZ* (Nibu et al., 1998b). The stripe 2 and stripe 3 enhancers of even-skipped were placed 1.6 kb apart from each other, and two copies of the UAS sequence were introduced adjacent to the proximal enhancer, the stripe 2 enhancer. or 3) *eve* stripe 3 and *rhomboid* enhancers linked to the transposase-*lacZ* fusion gene (Hewitt et al., 1999).

Figure 6. The making of a transgenic fly.


white
White
defective P element no transposase
pipette
transposase provider plasmid, insertion defective
transgene carrier
x
white
reporter line
white
x
White
transgenic line
embryo to be stained using antisense RNA

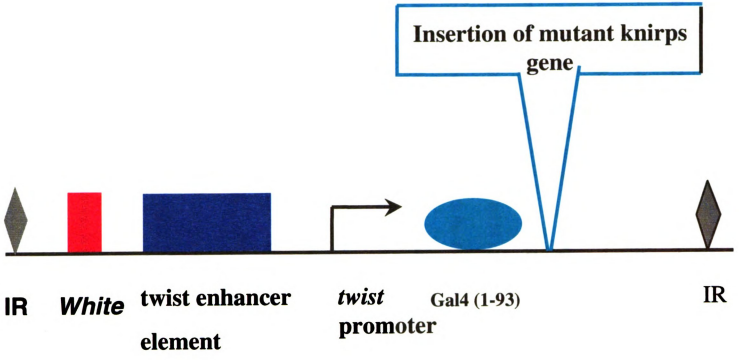
Embryos generated by the crosses were fixed and stained by in situ hybridizations. To quantitate repression activity of Gal4-Knirps chimeras, heterozygous effector lines were crossed to homozygous reporter lines, and several hundred embryos aged to 2-4 hours from each cross were collected at room temperature, fixed and stained as described in Small et al., 1992. After mounting on microscope slides, embryos were visually scored in a blinded experiment for evidence of repression. Most functional repressors completely abolished ventral staining in the *eve* stripe 2 region; embryos exhibiting weakened but not complete repression were scored in a separate category. Typically, a larger proportion of older embryos exhibited repression, presumably because of the lag between the activation of the *eve* or *rho* enhancer and the production of adequate amounts of the Gal4-Knirps protein after its gene's transcription under the control of the *twist* enhancer. In this assay, the maximum percentage of embryos exhibiting repression in the assay would be 50%, because only half of the fertilized embryos receive the Gal4-Knirps effector gene from the heterozygous male parent. The actual percentages are lower than this because all embryos showing reporter gene expression were counted, including younger embryos in which repressors had not yet reached appreciable levels.

Results:

202-358 is a minimal repression domain of Knirps protein

Genes encoding portions of the *knirps* open reading frame and the Gal4DNA binding domain were expressed in ventral regions of transgenic embryos under control of a *twist* promoter element (Figure 7). These chimeric repressors were tested on a *lacZ*

Figure 7. The injection vector pTwiggy contains a *twist* enhancer element, which drives the expression of Gal4-Knirps proteins in the ventral region of the embryo, and a *white* gene, which serves as a marker of transgenics and the inverted repeats of the P element, which can mediate the transposition of the injection construct into the germline of *Drosophila* embryos.


Insertion of mutant knirps gene
IR
White
twist enhancer element
twist promoter
Gal4 (1-93)
IR

reporter gene activated by eve stripe 2 and stripe 3 enhancers, with Gal4 binding UAS sequences adjacent to the stripe 2 element. An alanine-rich sequence is located within the region from residue 202 to 211. Constructs Gal4-Knirps 202-429, Gal4-Knirps 211-429, Gal4-Knirps 202-358, Gal4-Knirps 211-358 were designed to test the whether the alanine rich region is related to repression function of Knirps protein. A *PvuII* restriction site, located at codon 189 within the cDNA sequence of knirps, was used to generate the construct Gal4-knirps 189-358. Gal4-Knirps 189-358, Gal4-Knirps 202-429, Gal4-Knirps 211-429, Gal4-Knirps 202-358, Gal4-Knirps 211-358 showed effective repression of the proximal stripe 2 enhancer, while, consistent with the short-range of Knirps activity, the distal stripe 3 enhancer was not affected. (Representative examples of repressed and non-repressed embryos from *in vivo* repression assays are shown in Figure 8). In construct Gal4-knirps 248-358, the similar repression was observed in a smaller proportion of embryos. That is to say, the ratio of the numbers of the repressed embryos to that of the non-repressed embryo is six times lower than knirps 189-358, knirps 202-358 etc., indicating that amino acids 248-358 may encode a weak repressor (Figure 9).

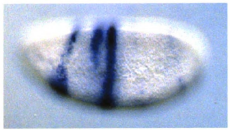
To further identify residues important for repression, Drs Keller and Foley tested Gal4 fusion proteins containing residues 202-358 and derivatives in transgenic assays. Their results have shown that deletions of residues 202-210 (202-358 ΔA), (alanines previously identified in a minimal repression domain in cell culture assays), 220-227 (202-358 ΔB), 228-251 (202-358 ΔC), or 292-313 (202-358 ΔD) did not impair repression activity, while deletions of residues 330-343 (202-358 ΔE) abolished repression completely (Figure 10). The region affected in 202-358 ΔE includes the residues P-DLS-K, recently shown to mediate Knirps interaction with the dCtBP protein

Figure 8. Representative embryos show the repression of *eve* stripe 2 by Gal4-Knirps. Transgenic embryos show the absence of stripe 2 expression in the ventral sides (A and B), while embryos lacking the Gal4-knirps transgene show equally well-expressed stripe 2 an stripe 3 (C and D).

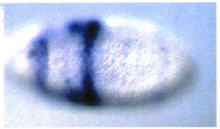
Lateral view

Ventral view

A

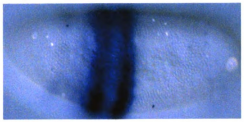


B

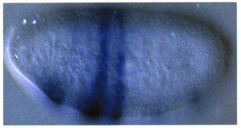


Transgenic embryos (St2 is repressed)

C



D



Control embryos (St2 and St3 are expressed)

Figure 9. The schematic structures and the in vivo repression activities of the Gal4-Knirps mutant proteins. Three transgenic lines of each construct were assayed except that only one line of 189-358 and one line of 248-358 were generated and assayed.

Activities of the C-terminal Knirps mutants

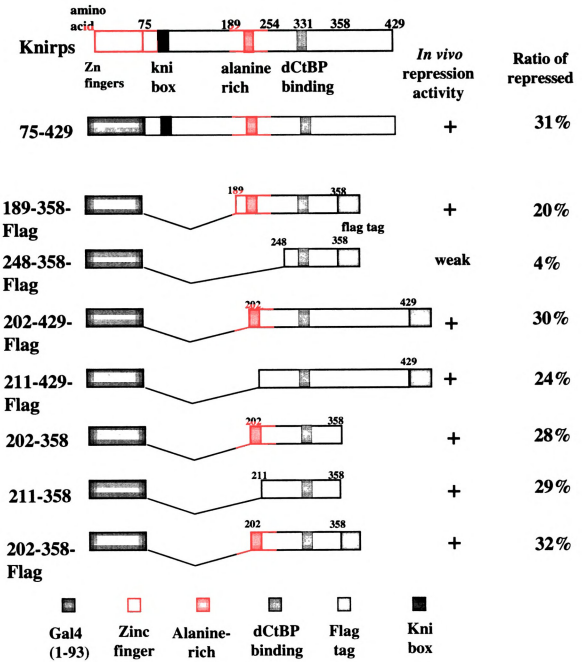
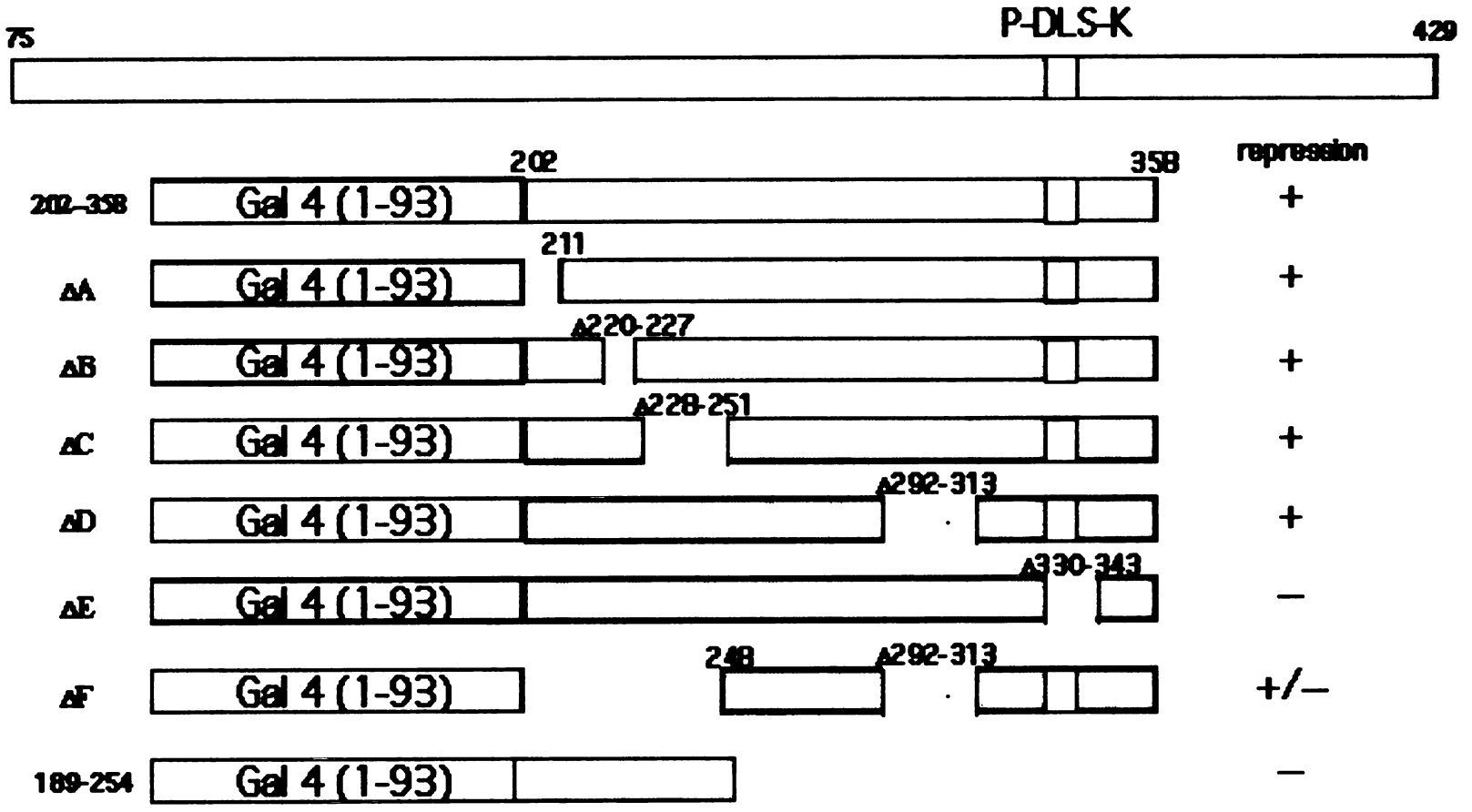


Figure 10. A central region of Knirps requires the dCtBP binding motif for activity. Genes were generated by PCR amplification of regions of the *knirps* gene, followed by site-directed mutagenesis (ΔA-ΔE) to remove internal conserved residues (Gerwin et al., 1994) and non-conserved residues , ΔC. The dCtBP binding motif is removed in ΔE. The activities of the genes are indicated to the right of the construct.


75
P-DLS-K
429
202
358
repression
202-358
Gal 4 (1-93)
+
211
ΔA
Gal 4 (1-93)
+
Δ220-227
ΔB
Gal 4 (1-93)
+
Δ228-251
ΔC
Gal 4 (1-93)
+
Δ292-313
ΔD
Gal 4 (1-93)
+
Δ330-343
ΔE
Gal 4 (1-93)
−
248
Δ292-313
ΔF
Gal 4 (1-93)
+/−
189-254
Gal 4 (1-93)
−

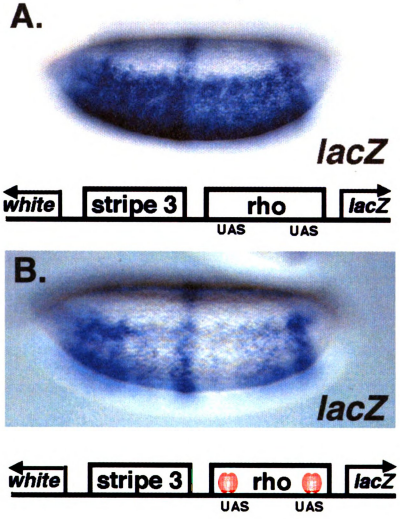
(Nibu et al., 1998a, 1998b). The lack of repression activity of protein 202-358 ΔE could be caused by the lack of important repression residues, but also could be due to the lack of protein expression in embryos. To address this question, gel mobility shifts experiments have been carried out and the mobility shift complexes formed by the mutant protein 202-358 ΔE and the Gal4 binding site-containing probe were detected in the polyacrylamide gel (see Chapter 6), indicating that the non-functional protein is expressed. Alanine scanning mutations affecting these residues have been shown to compromise the repression activity of a chimeric Gal4-Knirps protein containing residues 255-429 (Nibu et al., 1998a) and by ectopically expressed Knirps (Nibu et al., 1998b). Our results indicate that the minimal 202-358 repression construct is also dependent on dCtBP for activity. A minimal construct containing only residues 248-291 and 314-358 was also active, consistent with earlier reports that residues N terminal to 255 were dispensable for activity (Nibu et al., 1998a). Our result was consistent with Nibu et al., who found that the dCtBP-binding is important for the repression function of knirps. On the other hand, two other constructs, Gal4-knirps 75-332, Gal4-knirps 75-330 lack the dCtBP-binding motif and display robust repression activity *in vivo*. Given the fact that Knirps 75-332 do not bind to dCtBP in vitro in the glutathione S-transferase pull down assay (Dr. Carla Margulies unpublished results), our results indicate that the dCtBP-binding motif is dispensible in the N-terminal portion between residues 75 and 330.

Activity of the dCtBP repressor protein from a distal enhancer position.

Our results suggest that for the N-terminal repressor, residues 75-330, binding of the dCtBP repressor protein to Knirps is not required for repression. We considered the

possibility that dCtBP may merely affect an allosteric alteration of the Knirps protein, allowing the Knirps protein's own repression domain to contact a target in the transcription machinery. In this case, dCtBP would not directly mediate transcriptional repression. Previous experiments have established that tethering dCtBP or a murine homologue, CtBP2, to a promoter inhibited gene expression, suggesting that the cofactor is itself capable of repression (Turner and Crossley, 1998; Nibu et al., 1998). However, these assays used promoter-proximal Gal4 binding sites that may be subject to steric hindrance effects. Therefore, we tested whether the Gal4-dCtBP protein was active from a distal enhancer site within the *rho* enhancer (Figure 11). Expression of a Gal4-dCtBP chimera under the control of a Krüppel promoter inhibited activity of the *rho* enhancer, but not the *eve* stripe 3 enhancer that is far away from the Gal 4 binding sites, demonstrating that this cofactor is capable of independent action as a repressor when targeted to a specific enhancer. Thus, the central 202-358 portion of Knirps is likely to act through dCtBP, and the N terminal portion of Knirps that does not bind to dCtBP may repress via an alternative pathway.

Figure 11. The Gal4-dCtBP chimeric protein acts as an independent repressor within an enhancer. (A) Expression pattern of the *eve* stripe /*rho lacZ* reporter gene, showing robust ventral expression directed from a *rho* enhancer element lacking Snail binding sites (1), and a central stripe from the *eve* stripe 3 enhancer. (B) Repression of *rho* enhancer activity in the central portion of the embryo mediated by the Gal4-dCtBP chimera. The repressor is driven by a Krüppel promoter (25). Gal4 binding sites were introduced in a 600 bp *rho* enhancer at the positions previously used for targeting Knirps protein to this gene complex (1). Ventrolateral views are shown, with anterior to the left.


A.
lacZ
white
stripe 3
rho
lacZ
UAS
UAS
B.
lacZ
white
stripe 3
rho
lacZ
UAS
UAS

CHAPTER 4

THE ANALYSIS OF THE N-TERMINAL REPRESSOR OF KNIRPS

Introduction

Many repressor proteins have multiple repression domains. Engrailed, Hairy and Krüppel are examples (Tolkunova et al., 1998, Poortinga et al., 1998, Light et al., 1994). Our data suggest that Knirps protein also possesses more than one repression domain. As previous work has pointed out, the residues 331-337 PMDLSMK is the dCtBP-binding motif and disruption of this motif will abolish dCtBP-Knirps interaction. The mutant proteins, which have this particular motif PMDLSMK, are potent repressors in embryos, for example, the Gal4-Knirps 202-358. Meanwhile the mutant protein Gal4-Knirps 75-330 is a functional repressor although it does not contain the dCtBP-binding motif, which indicates that there is another repression domain located in the N-terminal region between residue 75 to 330 and this N-terminal region confers transcriptional repression independent of recruiting dCtBP protein. To reveal the possible mechanisms involved and to define the important amino acids or motifs involved in repression for the N-terminal repressor, the polymerase chain reactions with corresponding primers were used to generate a series of N-terminal truncation Gal4-Knirps mutants (See Materials and Methods). These mutants contain the cDNA sequence of *knirps*, starting at various positions and ending at codon 330. They are constructs 94-330, 124-330, 139-330, 169-330, 189-330, 200-330 and 75-304.

Materials and Methods:

N-terminal deletions:

Fragment of the knirps open reading were generated from the pCarnegie 20 vector pN741 containing the knirps cDNA (G. Struhl, unpublished) with *PfuI* DNA polymerase (Stratagene) with polymerase chain reactions, using different pairs of primers. The products were subcloned into the KpnI and XbaI sites of the pTwiggy vector containing *twist* enhancer element 2xpEe-Et and twist basal promoter. The constructs and the corresponding primers were:

94-330: primer 1,

5'-GGGTCGGTACCCACGAACAGGCCGCCGCAGCGGCGGGCAAG-3'

94-330: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

TCCTTCTTGAGCGGAAACGGTGGG-3'

124-330: primer 1,

5'-GGGTCGGTACCGCCGCAGCGGGCTCGCCACACACTCCCGGATTTGGG-3'

124-330: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

TCCTTCTTGAGCGGAAACGGTGGG-3'

136-330: primer 1,

5'-GGGTCGGTACCCACCACCATCATCAGCAGCAGCAGCAC-3'

136-330: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

TCCTTCTTGAGCGGAAACGGTGGG-3'

169-330: primer 1,

5'-GGGTCGGTACCGCCGCAGCGTCCGCCGCCCTGCCCTTCTTCAGC-3'

169-330: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

TCCTTCTTGAGCGGAAACGGTGGG-3'

189-330: primer 1,

5'-GGGTCGGTACCCTGCCCCCACACCTCCTCTTCCCAGGCTAC-3'

189-330: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

TCCTTCTTGAGCGGAAACGGTGGG-3'

200-300: primer 1,

5'-GGGTCGGTACCGCAAGTGCTGCCGCTGCAGCGGCTTCTGCT-3'

200-300: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

TCCTTCTTGAGCGGAAACGGTGGG-3'

75-304: primer 1,

5'- GGGTCGGTACCGCAGCCTCGGCCCGCCAGTCGCCCATCGAT-3'

75-304: primer 2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

GGGCGACAAGCTCTGCATCTTGGC-3'

75-304 plasmid was constructed by polymerase chain reaction and subcloned into the ClaI and XbaI site of 75-429 in Twiggy plasmid.

DNA sequencing reactions confirmed all the constructs except 75-304 and 94-330. Both construct 75-304 and construct 94-330 contain a point mutation from base T to base C within residue 107.

P-element transformation, whole-mount in situ hybridization of embryos, and crosses to reporter lines.

The methods are the same as described in Chapter 3

Assays of in vivo repression activity.

The methods are the same as described in Chapter 3.

Results:

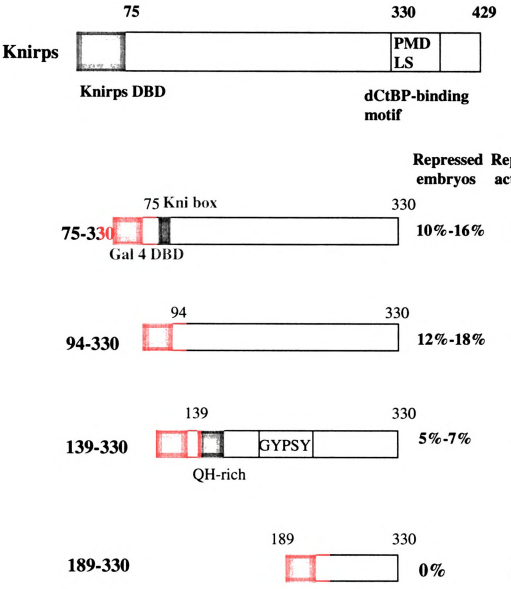
The mutant *knirps* genes 94-330, 124-330, 139-330, 169-330 189-330, 200-330 and 75-304 were constructed as described in the materials and methods. 124-330, 169-330 and 200-330 were constructed but to be transformed into embryos. Construct 75-304 was constructed and transformed into embryos but to be assayed for repression activity. 94-330, 139-330 and 189-330 were transformed into yw^{67} fly embryos. 19, 4 and 10 transgenic lines were generated from construct 94-330, 139-330 and 189-330 respectively. The 94-330 transgenic lines were divided into three pools by mixing the transgenic males from individual lines with each approximately 50 flies per pool. 4 lines of 139-330 were mixed into one pool, and 10 lines of 189-330 were mixed into one pool to obtain sufficient number of male flies. The reporter lines contain the stripe 2 and stripe 3 enhancer and two copies of UAS sequences (the Gal4-Knirps binding sites) adjacent to the stripe 2 enhancer. Crossing the males from the transgenic lines of 94-330, 139-330

and 189-330 respectively with the virgin females from the reporter lines was performed. Embryos from these crosses were stained and looked for the expression pattern of the *lac Z* gene under the control of stripe 2 and stripe 3 enhancers (See Materials and Methods). Gal4 Knirps 94-330 and Gal4-Knirps 139-330 repress the proximal enhancer stripe 2 but not the distal stripe 3, while Gal4-Knirps 189-330 repress neither stripe 2 nor the stripe 3 (Fig 12). These results suggest that amino acids 139 to 189 contain a motif necessary for repression.

Discussion and conclusion

The repressed embryo ratio in 94-330 (12%-18%) is higher than that in 139-330 (5%-7%). This phenomenon can be explained by either 1) The repression activity of the N-terminal repressor is additive, i. e. An auxiliary element located between residue 94 and 139 is facilitating the repression in addition to residues 139-189. Or 2) Position effect altered observed repression activities in P element mediated transformations. When a transposon is translocated into the locus of a heterochromatin region, the transgene will be silenced. Therefore, even a transgene capable of encoding a potent repressor may not express repression activities in some transgenic lines. The repression assays presented in this chapter are done by mixing several transgenic lines together due to the insufficient number of male flies present in each individual line. The embryos scored for repression in this study are derived from the pool of lines with different sites of integration. Thus, the observed repression activities monitored by the average repressed embryo ratios are expected to be small if a particular transgene is particularly sensitive to position effects.

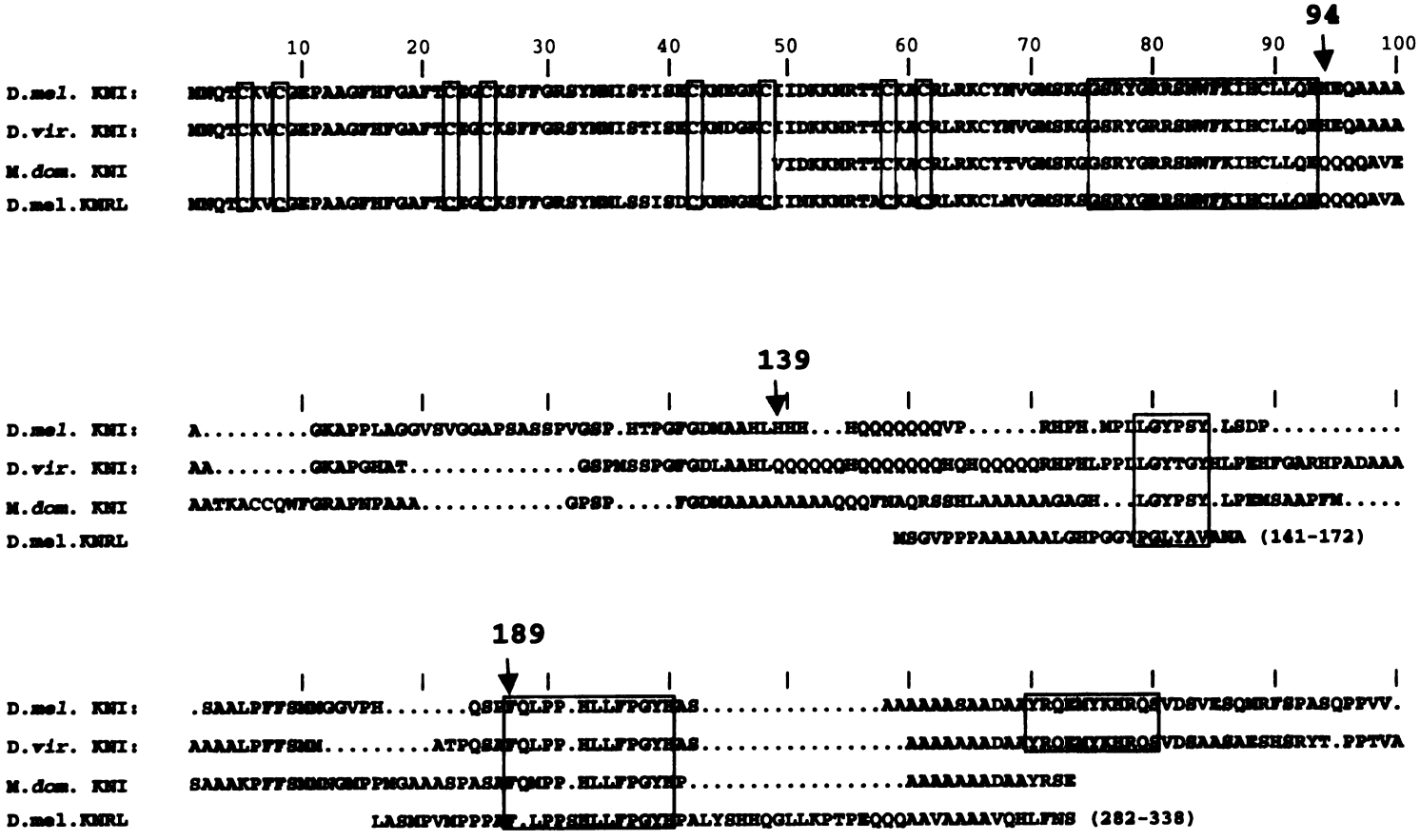
Figure 12. The schematic structures of the amino-terminal Knirps mutants. The Knirps DNA binding domain is indicated in green, while the Gal4 DNA binding domain is indicated in red. Gal4 fusion proteins containing residues 75-330, 94-330, 139-330 showed effective in vivo repression activities, whereas the Gal4-fusion protein 189-330 does not possesses repression activity.


75
330
429
Knirps
PMD
LS
Knirps DBD
dCtBP-binding
motif
Repressed
embryos
Repression
activity
75 Kni box
330
75-330
Gal 4 DBD
10%-16%
+
94
330
94-330
12%-18%
+
139
330
139-330
GYPSY
5%-7%
+
QH-rich
189
330
189-330
0%
-

The lack of region from amino acid 139-189 inactivates the protein 189-330. This region contains a LGYPSY box that is conserved in Knirps derived from several different insect species: *Drosophila melanogaster, Drosophila virilis* and *Drosphila domestica* (Figure 13). In addition, the region between amino acid 139 and 149 contains multiple charged residues of histidine and glutamine and is also well conserved (Figure 13). Many transcriptional regulator proteins function through protein-protein interactions, for example activator VP16 interacts with TAF (the TBP associated factors) and *Drosophila* eve protein interact with TBP. Regulations of transcription appear to be the result of multiple interactions between the regulator proteins and the targets. It was previously reported a glutamine-rich region constitute the activation domain of VP16 and Sp1. Therefore, it is likely that the glutamine-rich region present in Knirps is also important for repression activity. These predictions are consistent with the observations that the multiple glutamine-rich regions spanning protein 94-330 and 139-330 contribute to repression while the region 189-330 does not (Figure 12). The LGYPSY box contained in the functional proteins 94-330 and 139-330 but not in the non-functional 189-330 constitutes the repression domain. Proline has the tendency of introducing turns into the protein secondary structure. It is likely that proline residue in the LGYPSY box contributes to the repression function of Knirps by helping the protein maintain proper conformation.

Although the Kni box is conserved in all the three species: *Drosophila melanogaster, Drosophila virilis* and *Drosphila domestica*, the lack of the Kni box in the

Figure 13. Sequence comparison of Knirps-homologous proteins from different species: *Drosophila melanogaster*, *Drosophila virilis* and *Drosophila domestica*. Conservation of amino acids between the different proteins are indicated by a grey background. Positions 75-93 are located as the absolutely conserved Kni box. Other conserved protein motifs are marked by boxes as well, such as LGYPSY box. The first N-terminal Knirps residues of recombinant proteins 94-330, 139-330 and 189-339 are indicated by the arrows. (From Gerwin et al., 1994)


94
139
189
D.mel. KNI:
D.vir. KNI:
M.dom. KNI
D.mel.KNRL
(141-172)
(282-338)

protein 139-330 does not impair the repression activity, it suggests that this motif is not necessary for repression.

The conclusions and discussions above were based on the presumption that all the three recombinant proteins, 94-330, 139-330 and 189-330 are expressed in embryos. 189-330 is the only one that does not display activity in vivo, thus, further experiments need to be done to detect the protein expression level of the construct 189-330 in embryos as describes in Chapter 6.

The results indicate that the region between 139-189 containing the LGYPSY and poly histidines and poly glutamines is important for the function of the amino terminal repressor and Knirps 139-330 contains the minimal N-terminal repression domain.

CHAPTER 5

DNA BINDING DOMAIN, THE INHIBITORY ELEMENT IN KNIRPS REPRESSION

Introduction

A full-length wild type Knirps protein was misexpressed in the same location as the *even-skipped* stripe 2 by experimental manipulations in previous experiments. Knirps diffused into the stripe 3 region and binds to its cognate sites within the stripe 3 enhancer, eliminating the expression of even-skipped gene in the area of stripe 3. Meanwhile it has been observed that alanine substitution mutations in the dCtBP-binding motif, (PMDL were mutated into AAAA) caused the derepression of *eve* stripe 3 (Nibu et al., EMBO, 1999) (Figure 2). The conclusions from this experiment were that dCtBP-binding motif is required for full-length knirps to repress *even-skipped* stripe 3.

However, it was observed that the Gal4-Knirps 75-330, which contains a heterologous DNA binding domain and lacks the C-terminal tail residues 331-429, including the dCtBP binding motif, repressed transcription when assayed on the *lacZ* reporter controlled by *eve* stripe 2 and stripe 3 enhancers. The differences between the repression caused by the full-length Knirps and that caused by the Gal4-Knirps 75-330 are as follows: 1) the presence or absence of the C-terminal stretch of amino acids, 2) the DNA binding domain, 3) the enhancers which drive the ectopic Knirps expression (*eve stripe* 2 versus *twist*), 4) the target genes (stripe 3 of endogenous *eve* enhancer versus a stripe 2 and stripe 3 LacZ chimera enhancers). Based on the difference 1) and 2), we can infer that either the zinc finger DNA binding domain of Knirps or the C-terminal tail abolishes the protein fucntion. Thus, a reasonable hypothesis is that there maybe an inhibitory element present in either the zinc finger DNA binding domain of Knirps or in the C-terminal tail. I chose to test the C-terminal inhibition hypothesis first. Therefore,

the following constructs were designed. Knirps cDNAs starting from 75 and ending with different residues in the C-terminal region between residue 330 and 429 with a mutated dCtBP-binding motif (Nibu et al., 1998a) fused to Gal4 DNA binding domain were constructed and tested for *in vivo* repression activities. These constructs are: Gal4 knirps 75-429*, Gal4-knirps 75-394* and Gal4-knirps 75-364* (* indicate the mutations in the dCtBP-binding motif) (See materials and methods).

Materials and Methods:

C-terminal deletions:

Fragments of the knirps open reading were generated from the plasmid Casper 22FΔKE (Nibu et al., 1998a) containing the alanine substitution of the dCtBP-binding motif in the knirps cDNA with *pfuI* DNA polymerase (Stratagene) with polymerase chain reactions, using different pairs of primers. The products were subcloned into the ClaI and XbaI sites of the 75-429 vector containing *twist* enhancer element 2xPEe-Et and twist basal promoter. The constructs and the corresponding primers used were:

75-429*: oligo1,

5'-GGGTCGGTACCGCAGCCTCGGCCCGCCAGTCGCCCATCGAT-3'

75-429*: oligo2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

GAACTTCCGGCGCGGAGCCACCTC-3'

75-394*: oligo1,

5'-GGG TCGGTACCGCAGCCTCGGCCCGCCAGTCGCCCATCGAT-3'

75-394*: oligo2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

GAACTTCCGGCGCGGAGCCACCTC- 3'

75-364*: oligo1,

5'-GGG TCGGTACCGCAGCCTCGGCCCGCCAGTCGCCCATCGAT-3'

75-364*: oligo2,

5'-GGGGAATCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATC

GAACTTCCGGCGCGGAGCCACCTC-3'

P element transformation, whole-mount in situ hybridization of embryos, and crosses to reporter lines.

The methods are the same as described in Chapter 3.

Assays of in vivo repression activity.

The methods are the same as described in chapter 3.

Results:

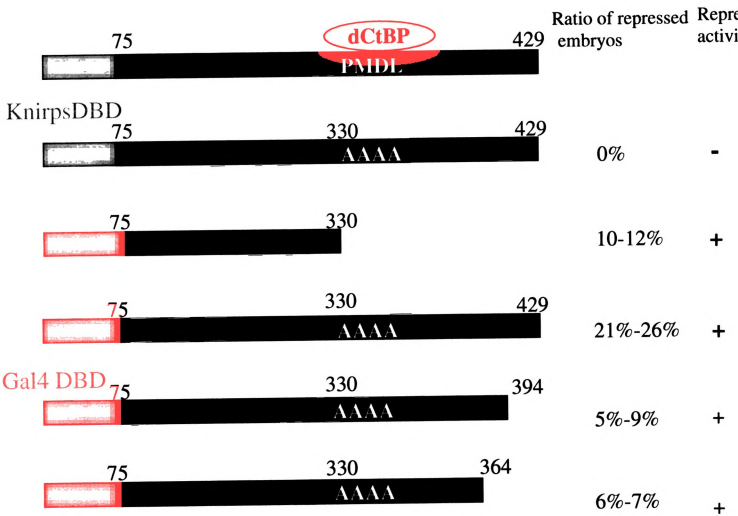
Three constructs encode the Gal4-Knirps proteins with the mutated dCtBP-binding motif, Gal4-knirps 75-429*, Gal4-knirps 75-394*and Gal4-knirps 75-364* were transformed into fly embryos and assayed on the reporter *lacZ* gene under the control of the stripe 2, 3 enhancers for repression activities. Three transgenic lines of Gal4 knirps 75-429* were tested and an average of 21-26% embryos showed robust repression of the *eve* stripe 2 enhancer in the region where Gal4-Knirps 75-429* is expressed, indicating that Gal4-Knirps75-429* is a functional repressor (Figure 14). The difference in activity

between the functional repressor, Gal4 Knirps 75-429*, with the non-functional protein, full length mutant Knirps protein (Nibu et al., EMBO 1998, also see the beginning of Chapter 5) could rely on the different DNA binding domains. Our result indicates that replacement of the Knirps DNA binding domain with the Gal4 DNA binding domain rescued the function of the mutated 75-429 portion (dCtBP-binding motif is mutated). Similarly, one of two Gal4-Knirps 75-394* lines assayed and two of three Gal4-Knirps 75-364* lines assayed also showed repression activity and elimination of the staining of the *even-skipped* stripe 2 in the ventral portion of the embryos were observed (Figure 14). However, one line of each construct failed to show repression. These variations in activity between lines of the same constructs may reflect position effects, i.e. the chromosomal position of the integrated P element causes variation in the level of expression of the transgenes.

Discussion and Conclusion:

Mutagenizing of the dCtBP-binding motif in the Gal4 Knirps 75-429*, Gal4-Knirps 75-394* and Gal4-Knirps 75-364* did not affect the repression activity, suggesting that the dCtBP-binding is not a requirement for repression by these proteins. This result not only confirms the result that protein encoded by construct Knirps 75-330 in which the dCtBP-binding motif is completely deleted showed effective repression (Mao et al., manuscript in preparation), but also provides a hint as to the role of Knirps DNA binding domain on Knirps repression function. Mutation in the dCtBP-binding motif abolished the repression activity of Knirps protein with the Knirps DNA binding domain, but did

Figure 14. The schematic representation of the carboxy terminal mutants of Knirps protein and their repression activities. Blue boxes represent the Knirps DNA binding domainand the red boxes represent the Gal4 DNA binding domain. With the same alanine mutations, Gal4 DNA fusion proteins containing residues 75-429, 75-394 and 75-364 show effective repression whereas when linked to the Knirps DNA binding domain, residues 75-429 showed no repression activity. Three transgenic lines of 75-429, two transgenic lines of 75-394 and two transgenic lines of 75-364 showed repression. The embryos from lines that showed repression were scored and average ratios of the repressed embryos for each constructs were indicated.


Ratio of repressed embryos
Repression activity
75
dCtBP
429
PMDL
KnirpsDBD
75
330
429
AAAA
0%
-
75
330
10-12%
+
75
330
429
AAAA
21%-26%
+
Gal4 DBD
75
330
394
AAAA
5%-9%
+
75
330
364
AAAA
6%-7%
+

not affect activity of the knirps protein with a Gal4 binding domain. In addition Gal4-dCtBP chimera shows good repression. These observations help to clarify the repression mechanisms of Knirps protein. Regarding what are the roles of the knirps DNA binding domain and binding to dCtBP in repression activity of knirps, I propose three models to explain the observations above:

1) dCtBP promotes dimerization of the Knirps protein with the Knirps binding domain to exert effective repression. As a member of the nuclear hormone receptor superfamily, Knirps is very likely to repress transcription as a dimer. As a matter of fact, in our transgenic assays, the Knirps binding sites consists of 16 nucleotides with inverted repeats (Arnosti et al., 1996). However, there is also evidence that Knirps can form a monomer as well as a dimer in gel retardation assays (Gerwin et al., 1994). In the embryo, whether Knirps exert repression as a monomer or as a dimer is not clear. dCtBP is known to share significant homology with the D-isomer 2-hydroxy acid dehydrogenases (Schaeper et al., 1995). Members of this particular family of dehydrogenase have been shown to form homodimers. It is possible that the homology between dCtBP and the dehydrogenase family constitutes the preservation of structural rather than enzymatic features and dCtBP-Knirps interaction facilitates Knirps dimerization and leads to effective repression (Figure 15).

2) The Knirps DNA binding domain prevents the Knirps repression domain from functioning. Mutations in the dCtBP-binding motif disrupt the repression function of the full-length Knirps protein, but not the Gal4-Knirps protein, It is likely that the

Knirps DNA binding domain acts as an inhibitory element in addition to its role of DNA binding. The recruitment of dCtBP relieves this inhibition possibly due to allosteric changes and restores activity of the repression domain (Figure 16).

3) The repression domain with the mutated dCtBP-binding motif interferes with DNA binding by the Knirps DNA binding domain. Binding to dCtBP causes conformational changes that disrupt this interference (Figure 17).

Figure 15. The schematic representation of the model I. dCtBP helps Knirps dimerization to perform effective repression. The green circles represent the Knirps DNA binding domain. The black boxes represent the repression domain(s) of Knirps protein. "AAA" represents the alanine substitution mutation within the repression domain.

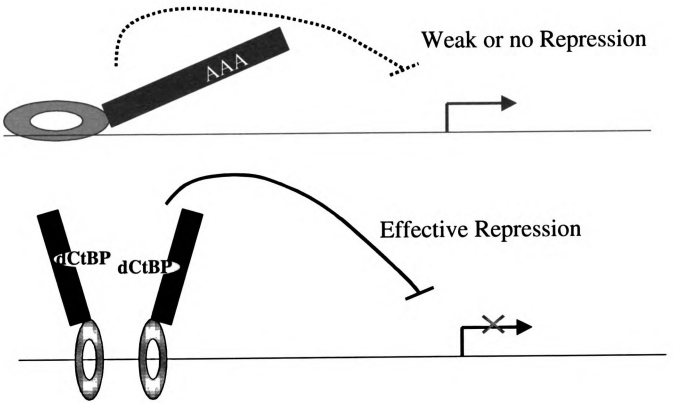

Weak or no Repression
AAA
Effective Repression
dCtBP
dCtBP

Figure 16. Schematic representation of model II. The DNA binding domain interferes with the function of the repression domain. "AAA" represents the alanine substitution mutation in the dCtBP binding motif. (A) The mutation in the dCtBP binding motif inactivates Knirps through the inhibitory element within the Knirps DNA binding domain (in blue). (B) Wild-type Knirps represses transcription through recruiting dCtBP (in yellow). (C) Mutations in the dCtBP ginding motif do not affect the repression activity of the Gal-Knirps function.

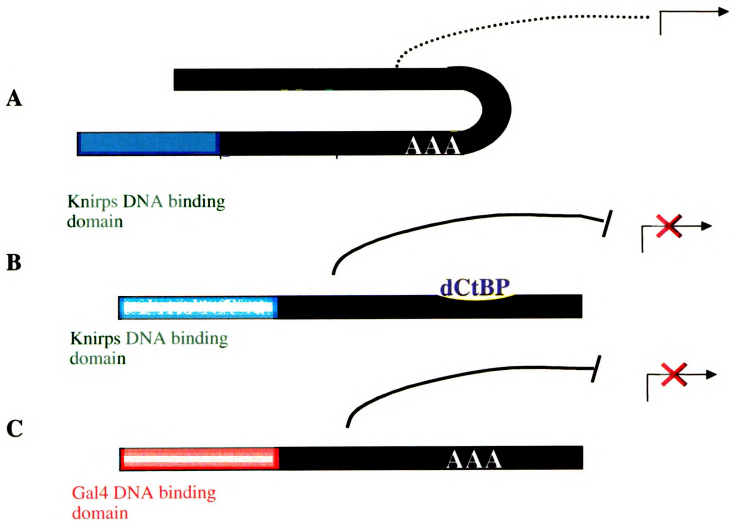
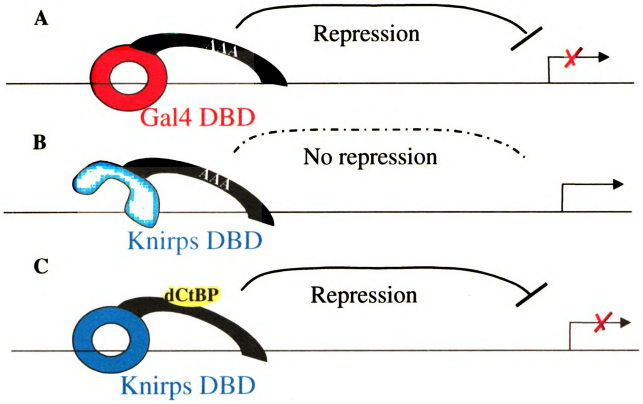

A
AAA
Knirps DNA binding domain
B
dCtBP
Knirps DNA binding domain
C
AAA
Gal4 DNA binding domain

Figure 17. The schematic representation of model III. The repression domain inhibits DNA binding of Knirps. (A) The Gal4-Knirp with the alanine substitutions in the dCtBP motif is able to repress transcription. (B) The same mutations in the Knirps protein with the Knirps binding domain (in blue) affect DNA binding of Knirps so that no repression occurs. (C) Wild –type Knirps recruits dCtBP (in yellow) so that it binds to DNA and represses transcription.


A
Repression
Gal4 DBD
B
No repression
Knirps DBD
C
dCtBP
Repression
Knirps DBD

CHAPTER 6

DETECTION OF CHIMERIC THE GAL4-KNIRPS REPRESSOR PROTEINS

Introduction

The difference in repression activities of the mutant proteins described in previous chapters could reflect some variables not directly related to repression, such as protein expression or protein stability. To measure the relative amount of protein in each transgenic line, Knirps polyclonal antibody was used to detect Gal4-Knirps proteins by in situ antibody staining. Only the expression of the wild type Knirps protein was detected in the non-transgenic embryos, while in the transgenic embryos from Knirps 189-358 line, specific additional Gal4-Knirps mutant proteins were detected (Figure 18). However, the mutant protein signal has not been found in other transgenic embryos (embryos from Knirps 202-358, Knirps 211-358 and Knirps 248-358). Anti-Flag monoclonal antibody was used in a similar assay, but no specific recombinant protein was detected. A possible explanation is that the protein expression level is not high enough.

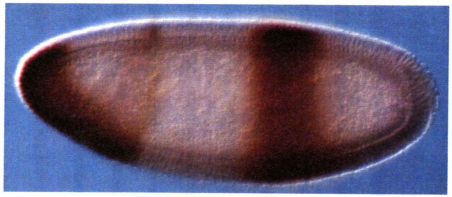
The mutant proteins from the full-length Gal4-Knirps 75-429 over-expressed in *E. coli* was detected by Western blotting using a flag antibody and Knirps polyclonal antibody respectively (data not shown), but no recombinant proteins have been detected yet. It is possible that the transgenes are expressed at levels below detection but still high enough to repress transcription.

Materials and methods:

Whole mount in situ antibody staining:

Figure 18. The protein expression patterns of a non-transgenic embryo (top) and a transgenic embryo from Gal4-Knirps 189-358 line were detected by anti-Knirps antibody staining (in dark-gray). The lateral views of both embryos show that the recombinant protein expressed specifically in the ventral region of the transgenic embryos as expected.

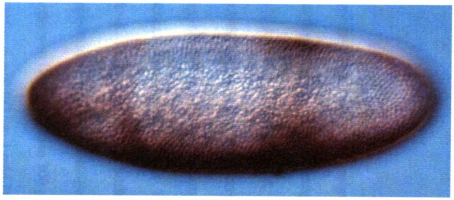
A non-transgenic embryo



Endogenous Knirps protein

A transgenic embryo

from 189-358 transgenic line



Gal4-Knirps recombinant protein

Polyclonal anti-Knirps antibody raised in guinea pig (kindly provided by D. Kosman) was used for the in situ antibody staining using the conditions reported previously (Macdonald et al., 1986).

Embryo nuclear proteins extraction:

Large scale preparation: 500 ml bleach-dechorionated embryos were washed with TBS (Tris buffered saline), the TBS was removed and the embryo pellet was resuspended in 600 μL ice-cold buffer A (10 mM HEPES pH7.4; 10 mM KCl; 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF. PMSF and DTT were added fresh). Embryos were transferred to a 35 mm petri dish and smashed and homogenized using the bottom of a scintillation vial until no intact embryos could be seen under a microscope. The homogenate was transferred back to an Eppendorf tube. The petri dish was rinsed with 200 μL buffer A twice and the wash fraction was collected into the Eppendorf as well. The homogenate was centrifuged for 30s at 250g force. The nuclear pellet was resuspended in 300 μL ice-cold buffer C (10 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF. DTT and PMSF were added fresh). The tube was gently rocked on a rotator for 30 min at 4°C. The extract was centrifuged for 5 min at 4°C and supernatant was frozen in aliquots at -70°C. The protein yield by this preparation is between 1.5 μg/μl-3.0 μg/μl.

Small scale preparation: 50 ml bleach-dechorionated embryos were washed with TBS (Tris buffered saline), the TBS was removed and the embryo pellet was resuspended in 50 μL ice-cold buffer A (10 mM HEPES pH7.4; 10 mM KCl; 0.1 mM EDTA, 1 mM

DTT, 0.5 mM PMSF. PMSF and DTT were added fresh). Embryos were transferred to an Eppendorf tube and smashed and homogenized using a small pestle (from Fisher) until no intact embryos could be seen under a microscope. The pestle was rinsed with 25 μL buffer A twice and the wash fraction was collected in the same tube. The homogenate was centrifuged for 30s at 250g force. The nuclear pellet was resuspended in 50 μL ice-cold buffer C (10 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF. DTT and PMSF were added fresh). The tube was gently rocked on a rotator for 30min, with mixing the suspension with the micropippet tip every 6 min. The extract was centrifuged for 10 min at 4°C and supernatant was frozen in aliquots in liquid nitrogen and stored at -70°C. The protein yield by this preparation is between 1.5 μg/μl - 5.0 μg/μl.

Gel mobility shifts

The DNA used in the gel mobility shift experiments has the sequence 5'-GATCTCGGAGGACTGTCCTCCGATGCG-3' on the top strand and 5'-CGCATCGGAGGACAGTCCTCCGAGATC-3' on the bottom strand. The double stranded DNA was labeled with [γ^{32}P]-ATP by T4 polynucleotide kinase. 6 μg protein from each embryo nuclear extract was incubated with 4.5 fmol radioactively labeled oligo in a binding buffer containing 7.5 μL GSB (50 mM HEPES, 10 mM $MgCl_2$, 20% glycerol, 2 mM DTT), 0.25 μL BSA (10 mg/ml) and 0.1 μL sonicated salmon sperm DNA (10 mg/ml from Gibco). Samples were loaded on a two-hour-prerun 4% polyacrylamide gel (30:1, acrylamide/bis) containing 1/2 x TBE buffer (Tris, Borate,

EDTA). Electrophoresis was at 150v at room temperature for 1.5 h and bands were visualized in a phosphorimager using ImageQuant software.

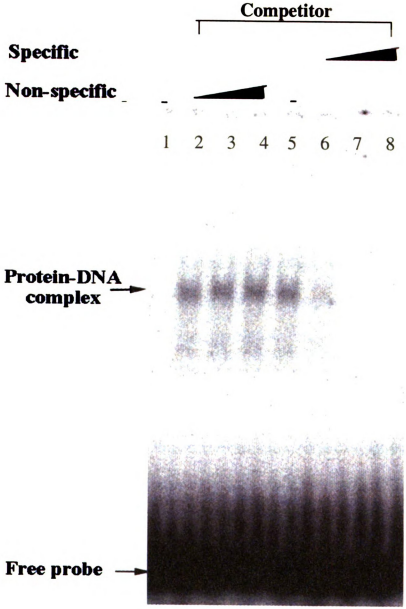
Results

Detection of fusion proteins by gel mobility shift assay

The mutant proteins retain DNA-binding ability because of the intact Gal4 DNA binding domain. Consequently, gel mobility shift assay allows us to detect the protein-DNA complex with nuclear extracts prepared from transgenic embryos by the large preparation method. Oligo UAS (double-stranded oligo consists of 5'-GATCTCGGACTGTCCTCCGATGCG-3') was phosphorylated with γ^{32}P-ATP to act as the DNA response element probe. The binding of radioactively labeled UAS oligo to the Gal4-Knirps 202-358 protein remains unchanged upon addition of a non-specific oligo (double stranded oligo: 5'-GACTACAAGGATGACGATGACAAGCAG-3') was added in increasing concentration. However, adding specific, unlabeled oligo of the same nucleotide sequence (Figure 19) competed off the binding of radioactively labeled oligo to the protein. These results indicate that the Gal4-Knirps proteins are stably expressed in the transgenic lines in detectable levels and possess the ability to interact with DNA.

Antibodies can be used in a particular kind of gel shift assay, named antibody super-shift experiments, to assist the detection of epitope-tagged protein. In these assays, two incubation mixtures were assayed by electrophoresis in an acrylamide gel in parallel.

Figure 19. Gal4-Knirps 202-358 protein binds to its cognate sites specifically. 5 μg Gal4-Knirps 202-358 protein from nuclear extract were prepared as described in Materials and Methods. 4.5 fmol ^{32}P-labeled UAS probe were incubated with non-specific oligo of different amounts 22.5 fmol, 90 fmol and 450 fmol (lanes 2, 3 and 4) and incubated with specific oligo of different amounts: 22.5 fmol, 90 fmol and 450 fmol (lanes 6,7 and 8). DNA-bound protein complexes and the free oligo are indicated by arrows.


Competitor
Specific
Non-specific
1 2 3 4 5 6 7 8
Protein-DNA complex
Free probe

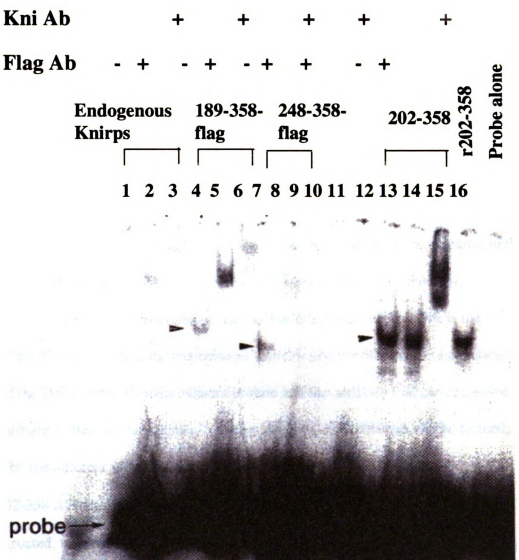
One is the mixture of the DNA probe and the recombinant proteins and the other is the incubation mixture of the DNA probe, the recombinant protein and the antibody that can recognize the recombinant protein. Consequently, two complexes of different sizes will be formed, indicated by the different mobilities in the acrylamide gel. Nuclear proteins prepared by the large preparation from transgenic embryos of Knirps 189-358-flag, Knirps 202-358-flag, Knirps 248-358-flag and non-transgenic embryos from yw^{67} were assayed in gel shift conditions. Recombinant proteins Knirps 189-358-flag, Knirps 202-358 and Knirps 248-358-flag were all able to bind to the labeled UAS oligo, form specific complexes, and migrate differently in the acrylamide gel (Figure 20 lanes 4, 7 and 12). The anti-Knirps polyclonal antibody was able to supershift both DNA bound Gal4-Knirps 189-358-flag and Gal4 Knirps 202-358-flag (lanes 6, 14 respectively), but only disrupt the Gal4-Knirps 248-358-flag and DNA complex (lane 9). Meanwhile, the anti-flag antibody was able to form supershifted complexes with Gal4-Knirps 189-358-flag and Gal4-Knirps 248-358-flag (lanes 5 and 8), but not the non-flag epitope tagged protein. In addition, the DNA/protein complex and supershift complexes are not observed in the assays with yw^{67} extracts from non-transgenic embryos except that a weak non-specific complex formed when the anti-flag antibody is added (lane2).

202-358 ΔE, a non-functional protein, is stably expressed in transgenic embryos.

The deletion mutation in the dCtBP-binding motif eliminated the repression activity of 202-358 ΔE proteins, spanning from Knirps amino acid 202 to 358, and also abolished binding to dCtBP *in vitro* (Drs Scott Keller and Carla Margulies unpublished

Figure 20. Gal4-Knirps 189-358-Flag, 248-358-Flag and 202-358-Flag are stably expressed in transgenic embryos. Nuclear protiens from wild type *yw* ^{67} embryos and trangenic lines Gal4 189-358, 248-358 and 202-358 were extracted from the embryos by the large-scale preparation as described in Materials and Methods. Approximately 6 μg nuclear protein of each line and 4.5 fmol ^{32}P-labeld UAS probe were used in each gel mobility shift reacion. Gal4-Knirps 189-358-Flag was incubated with the UAS probe alone, or the probe and the anti-Flag antibody, or the probe and the anti-Knirps antibody and assayed in the acrylamide gel under the conditions described as the Materials and Methods (lanes 4,5 and 6). The gel mobility shifts reactions were performed with Gal4-248-358-Flag (lanes 8, 9 and 10), 202-358 (lanes 13,14 and 15) and nuclear proteins from wild type *yw* ^{67} embryos (lanes 1, 2 and 3). Anti-Flag and anti-Knirps antibody were incubated with the UAS probe, and reaction mixtures were also assayed in gel shift conditions (lanes 11 and 12).

Detection of Gal4-Knirps proteins by gel-retardation



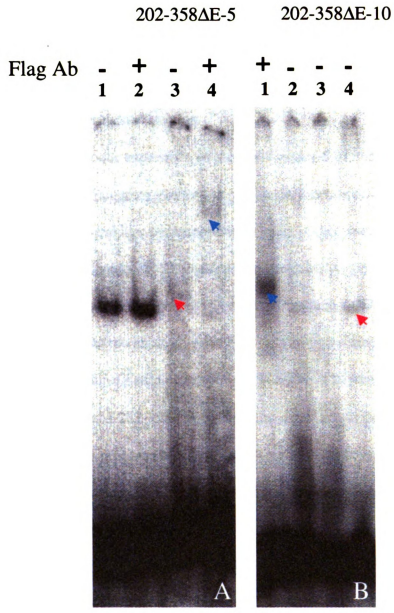
observations). Two factors could be responsible for the lack of function of the mutant proteins: the recombinant protein not being expressed and the lack of intact repression domain such as the dCtBP-binding motif. Therefore, the inactivity of the 202-358ΔE construct can be explained by three hypotheses: 1. dCtBP protein is required for stabilizing the protein and the inability of 202-358 ΔE proteins binding to dCtBP will result in the protein becoming unstable, or 2. dCtBP is acting as a corepressor for the repression activity of Knirps 202-358 protein and the inability of 202-358 ΔE proteins to bind dCtBP will result in the loss of repression activity, or 3. dCtBP plays dual roles as described as above. To test the first hypothesis, antibody supershift experiment was performed to test if there is any level of 202-358 ΔE protein expression in transgenic embryos. 202-358 ΔE protein possesses a flag peptide, N-Asp Tyr Lys Asp Asp Asp Asp Lys-C, at the carboxyl end. The flag-tagged proteins are recognizable to monoclonal anti-Flag antibodies (Pharmacia). In antibody super-shift experiments, two incubation mixtures were assayed by electrophoresis in an acrylamide gel in parallel. One is the mixture of the DNA probe and the recombinant proteins and the other is the incubation mixture of the DNA probe, the recombinant protein and the antibody that can recognize the recombinant protein. Consequently, two complexes of different sizes will be formed, indicated by the different mobilities in the acrylamide gel.

202-358 ΔE with the deletion in the coding region for the dCtBP-binding motif, was constructed, transformed into embryos and assayed by Dr. Scott Keller. No *in vivo* repression activity was observed in all the transgenic lines of this construct. Nuclear proteins of three lines of 202-358 ΔE: lines 202-358 ΔE-5, 202-358 ΔE –10 and 202-358 ΔE-13, and one line of the construct 202-358: line 202-358-4B, were extracted using the

small preparation protocol (See Materials and Methods), then incubated with the radio-labeled DNA probe containing the Gal4 recognition sites. Recombinant protein from transgenic embryos of line 202-358-4B and 202-358 ΔE-5 forms a complex with the DNA probe and migrate differently from the probe in the acrylamide gel (Figure 21A, lane 1 and 3). Adding the M2 anti-Flag antibody into the 202-358 ΔE-5 reaction mixture causes a larger complex formation, indicated by the slower mobility in the acrylamide gel (lane 4). This suggests that the protein component of the complex is the recombinant protein 202-358 ΔE-5 with the flag epitope. Adding the anti-flag antibody did not super-shift the complex of 202-358-4B and the DNA probe (Figure 21A, lanes 1 and 2). However, 202-358-4B was confirmed to contain the flag epitope in the c-terminal by the sequencing reactions and it is a transcriptional repressor in embryo assays (data not shown). This contradictory result can be explained by the following possibilities: 1. The folding of the protein of this particular recombinant protein makes the flag tag inaccessible to the anti-flag antibody. 2. The protein-DNA complex observed in Figure 21A lane 1 is formed by protein impurities from the nuclear extract preparation and the 202-358-4B protein was lost in the nuclear extract preparation step. The recombinant protein 202-358 ΔE-10 from another transgenic line of the construct 202-358 ΔE, is also able to form a ternary complex with the DNA probe and the Flag antibody (Figure 21B lane 2 and lane 5).

Figure 21. 202-358-ΔE-5 and 202-358-ΔE-10 are expressed in transgenic embryos. Nuclear protein Gal4-Knirps 202-358-4B, Gal4-Knirps 202-358-ΔE-5, Gal4-Knirps 202-358-ΔE-10 and Gal4-Knirps 202-358-ΔE-13 generated by the small-scale preparation were used in gel mobility shift experiments to detect the recombinant protein expression in embryos. (A) Gal4-Knirps 202-358-4B protein was incubated with UAS probe alone or with UAS and anti-Flag antibody (lane 1 and 2), Gal4-Knirps 202-358-ΔE-5 was incubated with UAS probe alone or with UAS and anti-Flag antibody (lane 3 and 4). (B) Gal4-Knirps 202-358-ΔE-10 was incubated with UAS probe alone or with UAS and anti-Flag (lane 1 and 4). Gal4-Knirps 202-358-4B and 202-358-ΔE-13 were also assayed in gel mobility shift reactions with UAS probe (lane 2 and 3).

Detection of the non-functional mutant Knirps proteins in embryos



Discussion:

248-358-flag was extracted by the large-scale preparation and assayed in an antibody supershift experiment. The result indicates that 248-358-flag is expressed at a similar level to 189-358-Flag protein (Figure 20). However, in the same gel shift conditions except that the recombinant proteins were extracted by the small-scale preparation, the result of the gel shift experiments showed that 248-358-flag was expressed at a lower level than that of 189-358. The contradictory results may reflect the variables in the nuclear protein extraction.

Detection of the poor expression of the non-functional proteins 202-358 ΔE-5 by the antibody super-shifts suggests that loss of repression function could be due to either the missing of important residues or the poor expression of this protein or both. It also suggests that loss of binding to dCtBP does not lead to complete protein degradation. dCtBP is playing important roles either in repression or protein stability or both.

I have detected the recombinant proteins from transgenic embryos of lines 189-358-flag, 202-358-flag (functional repressors) and line 248-358 (weak repressor) and lines and 202-358 ΔE-5 and 202-358 ΔE-10 proteins using the gel mobility shift assay. However, there are some problems to be solved before we make the safe conclusions. First, the nuclear protein extraction methods (see Materials and Methods) is modified from the similar methods which are used in nuclear protein extraction from tissue culture cell. It may not be the best one to isolate the rare amount of recombinant proteins from transgenic embryos. Especially for the small-scale nuclear protein preparation, there were variations that can not be ignored. As a result, not every functional Knirps mutant repressor we tested showed protein expression in gel mobility shift experiments. Gal4-

Knirps 75-332 protein is an example. Second, it is still possible that the 202-358 ΔE-5 and 202-358 ΔE-10 are still partially degraded and are not expressed at the comparable level with other functional Knirps mutant proteins such as 202-358. Thus, the lack of repression ability of 202-358 ΔE-5 and 202-358 ΔE-10 proteins is possibly caused by the insufficient amount of proteins present in the embryos. Third, there are some embryo proteins that form non-specific complexes with the DNA probe containing the Gal 4 recognition sequence.

Conclusion:

The evidence above indicates that the non-functional proteins are expressed in embryos. Thus, disrupting the repression domains is most likely the reason for the lacks of repression activity of non-functional mutant Knirps proteins, although possibilities exist that relatively less amount of protein expressions contribute to the inabilities of the non-functional mutant Knirps proteins. Gel mobility shift results are consistent with a role of dCtBP as a Knirps co-repressor.

SUMMARY AND PERSPECTIVES

Two independent repression domains, the C-terminal repressor and the N-terminal repressor were discovered for the Knirps repressor. Mutagenesis and *in vivo* repression assays were performed to define the minimal repression domains, 202-358 is the C-terminal repressor and 139-330 is the N-terminal repressor. The zinc-finger DNA binding domain was found to play an autoinhibitory role in Knirps repression function.

The C-terminal repressor has previously reported to repress transcription through recruiting dCtBP corepressor. The N-terminal repressor does not contain a dCtBP binding motif and does not bind to dCtBP *in vitro*. To reveal the repression mechanisms of the N-terminal repressor, the GST-75-330 fusion protein affinity column is under construction and GST pull downs will be performed to fish out the potential cofactors required for the N-terminal repressor. The repression pattern will also be looked for in embryos lacking maternal dCtBP to test whether the repression function of the N-terminal repressor requires the presence of dCtBP.

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