UNRAVELING THE FUNCTIONAL MOTIFS OF THE \textit{yan} ENHANCER IN \textit{Drosophila melanogaster}

A Thesis in
Genetics
by
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ABSTRACT

The organization of eukaryotic genomes can be divided primarily into two regions, those areas that are transcribed and those that regulate transcription. This thesis focuses on improving our understanding of cis-regulatory modules and how they direct transcription. To address this issue we used the Drosophila melanogaster yan gene as our model. The yan gene encodes a general inhibitor of differentiation and proliferation and is expressed dynamically throughout Drosophila development including oogenesis, embryogenesis and the larval central nervous system (CNS) and eye imaginal disc. We were interested in identifying module elements required for these various stages of expression. In this work we describe analysis of a 20 Kb genomic region surrounding the first exon of the yan gene. We show that discrete parts of this genomic region are responsible for specific subportions of the Yan protein expression pattern. In particular, we isolated a 122 bp fragment that is sufficient to specify yan expression within the developing eye. In this eye-specific enhancer we identified a number of elements critical to directing yan expression including a boundary element associated with insulator activity. One such regulatory element requires Su(H), a component of the Notch pathway, for gene transcription. Experimental data also demonstrated that this activation by the Notch pathway can be negatively modulated by receptor tyrosine kinase (RTK) signaling. Pointed, a nuclear target of the RTK pathway, is able to directly bind to the yan enhancer and compete with Su(H) for DNA binding. This is one of the first instances where these two pathways have been shown to interact directly in an antagonistic
manner. In summary, this work not only describes the various genomic components necessary for Yan expression throughout development but also provides a mechanism for regulation of yan transcription during eye development.
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Dedication

This thesis is dedicated to my family.

Carmen and Florentino
Louis, Timothy, Veronica and Tina
Sharon and Azaray
and
My Wife Margaret

Much love to you all.
Chapter 1

Introduction

1.1 Transcriptional Regulation

Throughout development the transcriptional regulatory regions within the genomic DNA carry out their function by activating or inactivating transcription in a time, space and sometimes lineage dependent manner. In eukaryotes, gene regulation is a multifaceted phenomenon that requires intercellular cooperation at many levels. At a broad level gene regulation is dependent on chromatin activity or inactivity. This activation of chromatin requires sequential and precise coordination of large enzymatic ATP-dependent complexes such as the SWI/SNF complex. These enzymes are recruited to active transcription sites by sequence-specific promoter/enhancer binding proteins (for review, see Workman and Kingston 1998; Kadam and Emerson 2002; Horn and Peterson 2002). Once at these active sites, SWI/SNF remodels nucleosomes and increases DNA accessibility to transcription factors. During this process, regulation of histones is also occurring by reversible chemical modifications that include methylation, ubiquitination and/or acetylation. These modifications allow for greater access to the DNA template. The best studied process is the acetylation of histones by the histone acetyltransferases (HATs) such as CBP/p300, PCAF/GNC5, SRC/p160 (for review, see Workman and Kingston 1998; Kadam and Emerson 2002; Horn and Peterson 2002). These modifications by SWI/SNF and HATs act collectively to establish a local chromatin
structure that is permissive to the general transcriptional machinery as well as other transcription regulators.

Another aspect of transcription is control in cis by promoters and enhancers. These cis-regulatory elements tend to be either basal or regulatory transcription factors. The basal transcription factors [e.g. TATA binding protein (TBP) and TBP associated factors (TAFs)] act upon most genes by binding to initiator sequences such as TATA boxes and/or Downstream Promoter Elements (DPEs). These elements are the very minimal factors needed to turn on gene expression. The regulatory transcription factors, on the other hand, act on specific genes by binding to upstream regulatory elements leading to specified or increased expression of those genes. During development separate cis-regulatory elements carry out different parts of the overall regulatory function of a gene in a time and space dependent manner. Each element or module is formally defined by Arnone and Davidson (1997) as “a fragment of cis-regulatory DNA that, when linked to a reporter gene and transferred into an appropriate cell, executes a regulatory function that is a subfraction of the overall combined regulatory function executed by the complete system.” Understanding the organization of the genome requires that we understand how cis-regulatory modules are structured. To begin, module elements are complex and regulated by many factors that include activators, coactivators, repressors, and corepressors often in a combinatorial mechanism (Flores et al. 2000; Halfon et al. 2000; Xu et al. 2000). An example of this complexity is seen in the regulation of the gene even-skipped (eve). The eve gene encodes a homeodomain protein that is distributed in a series of seven pair-ruled stripes during embryogenesis (Frasch and Levine 1987). Each stripe expression is modular and is controlled by separate enhancers.
(Small et al. 1992; Small 1993). In the eve stripe 2 enhancer, there are twelve known factor binding sites. Six sites are for activators of which five sites are for *bicoid* (*bcd*) and one is for *hunchback* (*hb*). These sites function synergistically to activate the stripe 2 enhancer in the anterior part of the embryo. Of the remaining six factor binding sites, three are recognized by the *giant* (*gt*) repressor protein and three are recognized by the repressor *Kruppel* (*Kr*). The function of these repressor proteins is to establish anterior and posterior borders for *bcd* and *hb* activation (Arnosti et al. 1996; Small et al. 1996). As this example demonstrates, understanding and identifying modular elements is of critical importance because it is these modules that define and allow a gene to have varying roles and expression patterns at different stages of development. This type of *cis*-regulatory analysis has been the focus in a number of papers and is the major interest of this thesis (Nellesen et al. 1999; Cooper et al. 2000; Miller et al. 2002; for review, see Arnone and Davidson 1997; Arnosti 2002). Also, studies in the area of *cis*-regulation are allowing for the development of computational models that could be used to understand expression patterns in a genomic context by allowing one to identify potential enhancer elements and then specifically testing for regulatory expression (Berman et al. 2002; Markstein et al. 2002; Markstein and Levine 2002).

A third mechanism for regulating transcription is with boundary elements. As described above, enhancers and modules influence gene expression by communicating with promoter elements and can influence transcription over long distances. One issue to consider is the mechanism employed to prevent control regions from influencing transcription at adjacent loci. Boundary elements or insulators have been identified which block the ability of enhancers to activate transcription when present between the
enhancer and target promoter. Insulator elements have been found throughout species from yeast, *Xenopus*, chicken and mammals as well as *Drosophila* (reviewed by Bell *et al.* 2001; Gerasimova and Corces 2001; West *et al.* 2002). In *Drosophila*, the two best studied insulator elements are the gypsy insulator found within the gypsy retroposon and the scs (specialized chromatin structure) and scs’ insultators found flanking the heat shock protein 70 (hsp70) locus. The gypsy insulator is a 340 bp retroposon sequence that contains 12 direct repeats as binding sites for the Suppressor of Hairywing [Su(Hw)] protein. These repeats contain a 12 bp consensus (YRTTGCATACCY) with a 6 bp invariant core sequence TGCATA (Y = C or T, R = A or G). Between each repeat is an AT rich spacer ranging from 20 to 29 base pairs. The Su(Hw) protein is essential for gypsy insulator function. Loss-of-function mutations in Su(Hw) abolish the enhancer blocking activity of the gypsy insulator. Modifier of mdg4 [Mod(mdg4)] is another key component of the gypsy insulator. Mod(mdg4) interacts directly with Su(Hw) to form a protein complex required for insulator function (Gerasimova *et al.* 1995; Geyer 1997; Gerasimova and Corces 2001). For the scs’ a protein called boundary element-associated factor (BEAF) 32 binds cooperatively to DNA with three copies of a CGATA sequence. This site appears to be required for its function but as yet, no mutations in BEAF 32 have been identified to test for a genetic interaction (for review, see Hart and Laemmlli 1998). Nevertheless, both Su(Hw) and BEAF proteins are present at several hundred sites on polytene chromosomes indicating a role or potential for establishing regulatory domains and/or chromatin organization within the genome. Together, all these elements from chromatin organizers to module elements along with a myriad of other mechanisms regulate gene expression within the genome.
To better understand the organization of the genome, studies of cis-regulatory regions show that separate cis-regulatory modules carry out different parts of the overall regulatory function needed for the complete expression of a gene. Studies from these modules have given some insight to understanding the structural organization presented in the noncoding regions of eukaryotic genomes. Still, the regulatory elements from relatively few genes have been studied despite their vital role and informative nature in the developmental process. To address this issue, we characterized cis-acting elements that are responsible for driving *Drosophila melanogaster* yan gene expression in the developing embryo, central nervous system (CNS) and imaginal eye disc. In particular, we sought to identify and characterize the smallest enhancer element necessary to drive proper yan expression within the developing eye.

### 1.2 Development of the *Drosophila* Eye.

The *Drosophila* adult eye is composed of an ordered array of approximately 800 facets or ommatidia. Each ommatidia is comprised of approximately 20 cells; eight photoreceptor cells (R1-R8), four cone cells, pigment and bristle cells. Early in embryogenesis retinal development begins when about 20 cells are fated to form the eye-antennal disc. These cells proliferate forming a monolayer epithelium until differentiation begins in the mid-third instar larval stage. At this step eye development occurs in two phases: the initiation and propagation of the morphogenetic furrow (MF) succeeded by an ordered process of ommatidium assembly in the third instar larval eye disc (Wolf and Ready 1993).
Progression of the MF within the first phase of eye development moves in a wavelike manner from posterior to anterior across the eye disc. The MF is a constriction of the actin fibers creating a physical indentation in the eye disc that demarcates the start point for eye differentiation. Prior to MF progression all cells are rapidly dividing in an unorganized fashion. In the furrow, all cells become arrested in the G1 phase of the cell cycle awaiting differentiation. The progression of neuronal differentiation, leading to the formation of ommatidial clusters, is initially coordinated by the signaling molecules encoded by *hedgehog (hh)*, *decapentaplegic (dpp)*, and *wingless (wg)* (figure 1) (Treisman and Heberlein 1998). Together Hh and Dpp are essential for maintaining differentiation at the posterior margin of the eye disc while Wg prohibits differentiation.

**Figure 1. Initiation and Progression of the Morphogenetic Furrow**

Progression of the morphogenetic furrow (MF) (blue) is from posterior to anterior across the eye disc and is regulated by the secreted protein Hedgehog (*hh*). Hh is expressed in the developing photoreceptors (red) and directly regulates transcription of *Decapentaplegic (Dpp)* and the the proneural gene *atonal (ato)* within the MF. *ato* expression is refined to the future R8 photoreceptors and is required for the development of that cell type. Wingless (*wg*) expression at the dorsal/ventral margins inhibits differentiation. This inhibition is blocked by *Dpp* thus allowing MF progression and differentiation. Anterior is to the left. This figure adapted from Jessica Treisman, Skirball Institute 1999.
at the dorsal and ventral margins (Ma and Moses 1995; Treisman and Rubin 1995; Heslip et al. 1997). Hh is also involved in initiating the progression of the MF by its forward diffusion and subsequent activation of the proneural gene *atonal* (Heberlein and Moses 1995; Borod and Heberlein 1998). As a result, *atonal* expression helps establish the initial cell clusters that will form the ommatidium as well as the specification and differentiation of the photoreceptor R8 “founder cell”. The founder (R8) cell then initiates the recruitment of the remaining photoreceptors sequentially in pairs beginning with R2/R5 and R3/R4 (figure 2). At this point cells, not in this five cell precluster,

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**Figure 2. Cell Recruitment and Differentiation of the Ommatidium in the Third Instar Eye Disc**

A schematic segmental diagram depicts the order of photoreceptor recruitment in ommatidium. Photoreceptor differentiation begins with the establishment of R8 (red). R8 then initiates the recruitment of the remaining photoreceptors sequentially in pairs beginning with R2/R5 (blue), R3/R4 (green), R1/R6 (green) and finally ending with R7 (yellow). Cone cells and other accessory cells are added later. Yan protein is seen in basally located nuclei (pink) of undifferentiated cells within and posterior to the morphogenetic furrow. Adapted from T. Wolff and D.F. Ready 1993.
undergo an additional round of synchronized cell division to form the remaining precursor cells. These new precursors are recruited to form photoreceptor cells R1/R6 and the last photoreceptor cell R7. This is followed by accessory cell recruitment of 4 lens-secreting cone cells and later during pupation by the recruitment of pigment and bristle cells. This process of progressive recruitment of differentiating cells is known to require at least 15 nuclear factors during ommatidium assembly. These include nuclear factors Atonal (ato), Rough (ro), Bar (B), Cut (ct), Lozenge (lz), Phyllopod (phyl), Prospero (pros), Seven in absentia (sina), Seven-up (svp), Splat (sal), Tramtrack (ttk), Pointed (pnt), Suppressor of hairless [Su(H)], Yan and D-Pax2 most of which are transcription factors (TF). It is through an orderly temporal combination of these TF along with proper receptor tyrosine kinase (RTK) and Notch signaling cascades that ommatidium development transpires. For more details see Wolff and Ready (1993) and Kumar and Moses (1997).

1.3 Receptor Tyrosine Kinase Signaling Elicits Diverse Outputs

Numerous studies have demonstrated that the sequential specification of photoreceptor cells is a consequence of the reiterative activation of the Ras/MAPK pathway. In particular, two receptor tyrosine kinases (RTK’s), the *Drosophila* homolog of epidermal growth factor receptor (DER) and Sevenless (Sev) function during photoreceptor development. DER is functional in all photoreceptor cells and is vital in other processes of *Drosophila* development including proliferation, recruitment and cell survival in the eye disc (reviewed by Freeman 1997; Perrimon and Perkins 1997; Rebay 2002). Sev is primarily restricted to R7 specification but is also expressed in R3/R4 and
R1/R6 (reviewed by Raabe 2000). Because RTK signaling is so profuse in eye development it is believed that its classical “instructive” function alone does not convey any fate-specifying information but RTK may serve as a switch turning on or allowing cells to commence differentiation.

To initiate differentiation, DER and Sevenless activate the downstream signaling molecule MAPK that subsequently is translocated into the nucleus to regulate activity of factors involved in photoreceptor cell fate specification (figure 3). Two RTK nuclear targets in eye development are Pointed and Yan, members of the E twenty six (ETS) DNA binding family of proteins (Lai and Rubin 1992; Brunner et al. 1994; O’Neill et al. 1994; reviewed by Dickson 1995; Dickson 1998). ETS proteins are a family of proteins

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Figure 3. RTK Signaling is Essential for Cell Differentiation
The RTK signaling pathway is essential for cell fate and differentiation. Ligand activation of the RTK pathway triggers a signaling cascade that leads to activation of the MAPK encoded by the rolled gene. Activation of MAPK leads to the phosphorylation of Yan and Pointed (Pnt). Upon phosphorylation, Yan is degraded and no longer represses differentiation while conversely Pnt phosphorylation allows differentiation to occur.
composed of a broad array of molecules involved in many diverse processes including proliferation, differentiation, transformation and apoptosis (Yordy and Muise-Helmericks 2000). Pointed is a transcriptional regulator of genes essential for photoreceptor cell fate, while Yan is an antagonist of the MAPK proneural signal. In response to RTK induction, phosphorylation activates Pointed allowing differentiation while conversely, Yan is phosphorylated and targeted for degradation (Lai and Rubin 1992; O'Neill et al. 1994; Rebay and Rubin 1995). Recently, Pnt and Yan in conjunction with Lozenge and the inclusion or absence of Su(H) have been shown to activate target genes in R7 and cone cells at the transcriptional level in a combinatorial mechanism (Flores et al. 2000; Xu et al. 2000). In our studies of the yan gene we have examples of complex interplay between some of these same factors regulating yan transcription.

1.4 Notch Signaling During Eye Development

In eye development Notch signaling is required at several consecutive steps, first for stable expression of the proneural gene atonal during MF progression and subsequently in the process of lateral inhibition to restrict atonal expression to the presumptive R8 cell (Baker and Yu 1997; Ligoxygakis et al. 1998). This lateral inhibition function of the Notch pathway in the posterior precursor cells is mediated by Suppressor of Hairless [Su(H)] through activation of the Enhancer of split complex [E(spl)-C] genes (Bailey and Posakony 1995; de Celis et al. 1995; Nellesen et al. 1999). Su(H)-dependent Notch signaling involves the interaction between the cleaved Notch intracellular domain (NICD) and Su(H) in combination to activate gene transcription (figure 4). In the nucleus, interaction between Su(H) and the intracellular portion of Notch is required but may not be sufficient for transcriptional transactivation (Lecourtois
and Schweisguth 1995). Recently it has been suggested that the intracellular portion of Notch in conjunction with Su(H) activates transcription by disrupting the formation of a repressor complex between Su(H) and a histone deacetylase complex (Kidd et al. 1998). In the posterior precursor cells, Su(H) acts as a transcription factor to upregulate $E(spl)$-C expression which helps these cells to maintain an undifferentiated state. Yan protein is also expressed in these precursor cells and like $E(spl)$-C proteins, functions to inhibit

**Figure 4. Notch Signaling Pathway**
A schematic drawing showing Notch intercellular domain in conjunction with Su(H) activating the $E(spl)$-complex genes. Upon ligand binding to the Notch receptor cleavage occurs releasing the Notch intercellular domain (NICD). The NICD is then translocated into the nucleus where its acts as a transcriptional regulator with its cofactor Suppressor of Hairless Su(H).

The imaginal disc expression patterns for each of the 12 E(spl)-C genes have been observed and appear to be distinct, suggesting a diversity of function. Seven genes in the E(spl)-C encode basic-helix-loop-helix (bHLH) transcriptional repressors (m3, m5, m7, m8, m , m and m ), while 4 others encode members of the Bearded family of small proteins (m2, m4, m6, m ). Finally the last gene, m1, encodes a putative Kazel-type protease inhibitor (Leviten and Posakony 1996; Leviten et al. 1997; Nellesen et al. 1999). Still, the mechanistic basis for the expression pattern variation has not been identified.

![Diagram of E(spl) complex genes and groucho]

**Figure 5. E(spl) – Complex Organization.** Above is a diagram showing the E(spl)-complex organization and its neighboring gene groucho. This complex encodes basic-helix-loop-helix (bHLH) repressors, members of the Bearded family proteins and a Kazel-type protease inhibitor. E(spl) genes m7, m8, m , m and m are expressed in the posterior undifferentiated cells in the eye disc. The E(spl)-C shown with distal to the right.

Nevertheless, strong evidence from enhancer-reporter transgene experiments suggests that regulation is at the transcriptional level. Certain E(spl)-C genes are direct transcriptional targets of Su(H) and the proneural bHLH proteins Achaete and Scute (Nellesen et al. 1999). This is likely true for 10 of the 12 transcription units in the E(spl)-C due to extensive sequence similarity from analysis of the promoter-proximal upstream regions of the 12 transcription units. In a specific example, a 234 bp E(spl)m enhancer is sufficient to specify the normal pattern of gene expression in the eye and in the wing.
imaginal disc (Nellesen et al. 1999). This enhancer contains three Su(H) binding sites, two bHLH E-box activator-binding sites, and a direct repeat of the hexamer motif GAAAGT. Intriguingly, a 155 bp truncated $E(spl)m$ enhancer derived from the 234-bp $E(spl)m$ enhancer continues to direct reporter gene expression in the wing disc, but lacks expression in the eye, although it contains all of the Su(H) binding sites, E-boxes and the GAAAGT repeat. This suggests that additional elements present within the 234 bp $E(spl)m$ enhancer aid in proper eye expression. It is this type of regulation that interests us in finding an eye-specific enhancer for the $yan$ gene to understand the regulatory elements necessary for eye-specific expression.

1.5 $yan$ Function and Regulation

The *Drosophila yan* gene encodes a nuclear protein with an ETS DNA-Binding motif, a Pointed domain and several putative PEST sequences (Lai and Rubin 1992; Price and Lai 1999). Yan also contains 8 MAPK phosphorylation sites that function in Yan degradation through phosphorylation by the Rolled MAPK (Rebay et al. 1995). Mutating these sites creates a protein that is constitutively active (Rebay et al. 1995). This mutant is able to inhibit the differentiation of both neuronal and non-neuronal cells. These results indicate that MAPK phosphorylates Yan to inactivate it, thus removing the barrier of differentiation (O'Neill et al. 1994; Brunner et al. 1994). In the third instar eye disc, Yan functions as a repressor in and posterior to the MF to maintain retinal precursor cells in an undifferentiated state. Yan normally accumulates in basally located nuclei of undifferentiated cells in the larval eye imaginal disc (figure 6). Hypomorphic mutations
in *yan* result in extra photoreceptor cells indicating that *yan* acts in an autonomous manner (Lai and Rubin 1992). Ectopic expression of Yan in the cells anterior to the MF severely disrupts eye development resulting in total loss of eye structure (Z.C. Lai, personal communication). Meanwhile, over-expression of *yan* in all cells posterior to the MF completely blocks cellular differentiation (Rebay and Rubin 1995). After photoreceptor precursor cells start to differentiate and their nuclei migrate to an apical position, Yan protein levels are dramatically reduced (Lai and Rubin 1992) therefore allowing neuronal and non-neuronal differentiation to proceed.

In addition to *yan* function in the developing eye, *yan* is dynamically expressed in the embryonic ectoderm and mesoderm (Gabay *et al.* 1996). During embryonic dorsal closure Yan is a repressor of cell shape changes. This repression is down regulated following JUN phosphorylation (Hou *et al.* 1997; Kockel *et al.* 1997; Riesgo-Escovar and Hafen 1997; Noselli 1998). Additionally, *yan* functions in repressing the specification of
denticle-secreting *engrailed (en)* expressing cells (O'Keefe *et al.* 1997). In early embryogenesis, Yan functions as a repressor of ventral cell fate in the ectoderm. Loss-of-function *yan* embryos show expanded expression of ventral cell markers *orthodenticle (otd)*, *argos (aos)*, and *tartan (trn)*. In contrast, activated Yan reduces the expression of these early ectodermal markers (Gabay *et al.*, 1996). *yan* is also responsible for the differentiation of the dorsal head ectoderm. This tissue gives rise to the visual system, medial brain, and head epidermis. Loss-of-function *yan* mutants exhibit massive head defects caused by over proliferation and subsequent cell death in these precursor tissues (Rogge *et al.*, 1995). Finally, Yan expression is also seen in the larval central nervous system (CNS) and has also been detected in ovaries identified by immunostaining and by the enhancer trap line *yan*\(^P\) (Price and Lai 1999).

The *yan* gene is highly conserved between the *Drosophila* species *D. melanogaster* and *D. virilis*. These sibling species are thought to have diverged 60 million years ago. Despite the high conservation during evolution of the RTK pathway members, no *yan* orthologues have yet been identified in vertebrates to date. Phylogenetic analysis reveals that *yan's* closest relative is the human *tel* gene, a negative regulator of differentiation in hematopoetic precursors (Price and Lai 1999).

### 1.6 Characterizing *yan* Regulation

Even though there has been extensive genetic and biochemical analysis showing Yan protein regulation, nothing is known about how *yan* transcription is established and maintained. The Yan protein is known to be a general inhibitor of differentiation and proliferation in embryo and larval tissues. Studies have shown that the subcellular
nuclear localization and stability of the Yan protein are regulated through phosphorylation by Ras mediated MAPK signaling (reviewed in Schweitzer and Shilo 1997; Treisman and Heberlein 1998; Hsu and Schulz 2000; Rebay 2002). These studies elucidate the major regulatory components of the Yan protein but fail to address the transcriptional regulatory elements of the yan gene. The goal of this project is to characterize cis-acting elements and their interacting proteins. Specifically we want to determine regulation of the temporal and spatial restricted expression pattern of yan in the developing eye. To investigate how yan expression is established in precursor cells prior to differentiation we examined a 20 kb region surrounding the first exon of the D. melanogaster yan gene locus. Using the bacterial lacZ gene as a reporter, yan genomic fragments were used to generate transgenic flies through P-element mediated transformation. We show that specific sections of this 20 kb region are sufficient to drive expression of the lacZ reporter in a spatial and temporal pattern similar to wild type yan expression and use this information to elucidate regulatory components necessary to regulate yan transcription.
Chapter 2

Material and Methods

2.1 Drosophila Stocks

All stocks were maintained on standard yeast-agar medium at 18°C or 25°C. In all cases, Canton-S flies served as wild type controls. The yan\textsuperscript{P} allele, an enhancer trap which has been previously described (Lai and Rubin 1992), was used to show endogenous yan expression. \textit{w}\textsuperscript{1118} embryos were used for all germ line transformations. \textit{w}; \textit{TM3/TM6B}, and \textit{w}; \textit{Adv/CyO} flies were subsequently used to determine chromosomal location of the transgenes. When needed, jumping of the P-element was done using \textit{w}; \textit{Dr\textsuperscript{1}/TM3, Sb, Δ2-3} flies. Two other stocks used in genetic crosses were \textit{w}; \textit{S/SM1}; \textit{Sb/TM6} and a line containing \textit{SM6-TM6B} the double balancer (\textit{w}; \textit{gp1503/SM6-TM6B}). Other fly strains used in this study include \textit{Su(H)}\textsuperscript{AR9} and \textit{Su(H)}\textsuperscript{SF8} (Ashburner), \textit{Su(H)}\textsuperscript{del47} (Morel and Schweisguth 2000), \textit{UAS-Su(H)}\textsuperscript{VP16} (Bray), \textit{N\textsuperscript{6}}, \textit{hs-Notch, hs-Su(H)} (The Blomington Drosophila Stock Center), \textit{UAS-Notch\textsuperscript{Intra}} (Treisman), \textit{UAS-Notch\textsuperscript{ECN}} (Muskavitch), \textit{pnt}\textsuperscript{A78}, \textit{pnt}\textsuperscript{A88} (O’Neill et al. 1994), \textit{pnt}\textsuperscript{T6} (Brunner et al. 1994), \textit{UAS-pntP1} and \textit{UAS-pntP2} (Klaes et al. 1994), \textit{UAS-E(spl)m7} and \textit{UAS-E(spl)m8} (Ligoxygakis et al. 1998), \textit{UAS-yan\textsuperscript{ACT}} (Rebay and Rubin 1995), \textit{UAS-tor\textsuperscript{D}-DER} (Dominguez et al. 1998), \textit{UAS-Ras1\textsuperscript{V12}} (Karim and Rubin 1998), \textit{spen\textsuperscript{EP}} (The Szeged Drosophila melanogaster P Insertion Mutant Stock Centre) and \textit{GMR-Gal4} (Hay et al. 1994), \textit{Su(Hw)}\textsuperscript{v}/\textit{Su(Hw)}\textsuperscript{f},.
Su(Hw)\(^2\)/Su(Hw)\(^f\), Su(Hw)\(^{wp[w+RPI215]}\), Mod(mdg4)\(^u1\) (Corses, Geyer, Dorsett, Georgiev) and *D. simulans*, *D. mauritiana*, and *D. sechellia* were a gift from A. Clark.

### 2.2 Target Overexpression Analysis Using the Gal4/UAS System

The Gal4/UAS system is a bipartite system in *Drosophila*. The two components of the system are the UAS responder element and the Gal4 driver. The Gal4 driver is regulated in *cis* in a particular pattern depending on its regulator. The UAS responder is only active when both the responder and driver are present in the same lineage (Brand and Perrimon 1993). The driver used for all overexpression in this thesis is the Glass Multiple Repeat (GMR) driver that activates expression in all cells within and posterior to the eye disc.

### 2.3 Mosaic Analysis by FRT/FLP Generation of Clones

To make mosaic animals the yeast FRT-FLP site-specific recombination system was used. The FLP is the recombinase source and the FRT is the recombination target site. Clonal analysis was done as described in Xu and Rubin 1993. The FRT lines used are *w; p{ry=neoFRT}42D, p{Ubi-GFP}2R/Cyo* and the FLP source is *w, ey-FLP*. The clones are negatively marked by absence of Green Fluorescence Protein (GFP).

### 2.4 Transgenic Fly lines

Transgenic fly lines used in this study are listed below. The chart lists constructs name and genotype. Chromosome location when known is given as follows 1 = on X, 2 = on second, 3 = on third and na = not known. The expression patterns are as follows:
= no expression, 1 = embryo, 2 = CNS and 3 = Eye. For genotype the first number indicates chromosome location of transgene and the second number indicates an independent line for that construct.

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2.5 Generation of Reporter Constructs

Genomic DNA encompassing the 5' yan locus was subcloned from FIX genomic clones (Lai and Rubin 1992). Constructs were cloned into the pCaSpR-AUG- gal, pwnβE (vector containing a nuclear localization signal) or pBluescript KSII+ vectors (Stratagene). Constructs A through J were generated by M. Price. Constructs K through P were generated by PCR and standard molecular techniques. Constructs K-N were made by PCR with fragment J as the template. For making construct K, primers pCaP (GGTACTTCAAATACCCTTGGGA TCG) and p227m (TTGGATCCACTTCTGTCA TATTCC) were used. For making construct L, primers p198p (AAGAATTTCGAA TATGCAGAAGTGC) and p420m (TTGGATCCT TGAGCCACTCTTTGCC) were used. For making construct M, primers p390p (GAGATTCAAGGGAGTGGCTCAACC) and p602m (GCGGATCCAGGGGC TCAGTGG) were used. For making construct N, primers p560p (CGGAATTCGATCA CGTGCCA CC) and pCaM (GCGCCTCTATTATATACTCCGG CG) were used. For making construct O and P fragment L was used as the template. For making construct O, primers p198p and p330m (AGGGATCCTTACCACAAGAACGC) were used. For making construct P, primers p304p (CTGAATTCTTGTGGTAAGGTGCCC) and p420m were used. For making constructs Q6X and Q1X the following oligos were used, pCAAAG-F (GGAAACATGAAACCCGAAACCTG CA), and pCAAAG-R (GGTTTCGGGTTTCA TGTTCCTTGCA). Construct Q6X contains six copies of the oligo sequence of which 3 are in the sense direction, 2 in the antisense direction and one in undetermined orientation. Construct 1X contains one copy of the oligo in the sense orientation. Mutations for S1 (CCACGGACGT), S2 (ATCTCCGCACG), S3 (TCGCGGACAT) and
2Hex (GGAAACATCGTCTCCCTGCACT) were generated by the quick Change site-directed mutagenesis kit (Stratagene), mutated sites are in bold. Mutations in S1, S2, S3 and 2Hex were made in collaboration with Y. Wen. Su(Hw) and N-box mutations were made by PCR using the following primers pSu(Hw)m (AAGAATTCCAACCGATCG AAGCTGTACGAATTTTTT) and pN-boxm (AGGGATCCTTACAGCATCAACGC) respectively. These constructs were transformed into DH5 or XLBlue *E.coli* strains. Colonies were then screened for cloned inserts by PCR (see below). DNA was isolated from these cultures using a standard STET boiling preparation as printed in Molecular Cloning a Laboratory Manual 2nd Edition 1989 (Sambrook *et al*. 1989), Fast Miniprep (Zhoe *et al*. 1990) or by using the Wizard Plus SV Miniprep DNA purification system (Promega) for DNA sequencing. DNA isolated from positive clones was examined for proper orientation by either restriction enzyme digests and/or by DNA sequencing. Sequencing was performed at the Penn State Life Science Consortium Nucleic Acid Facility or by using the Beckman Coulter CEQ 2000XL Sequencer. Constructs were sequenced either in the pBluescript KSII+ vector using T3 or T7 primers or in pCaSpeR AUG gal vector using pCaP or pCaM primers. All P[lacZ, w+] transgenes were introduced into w^1118^ and/or w; Dr^1^/TM3, Sb 2-3 embryos as described in Rubin and Spradling (1982) and mapped to chromosomes following standard protocols. For each reporter construct multiple (at least 3) independent insertions were obtained.

### 2.6 PCR Colony Screening

Clones were analyzed by suspending each colony in 30 l water. 10 l of this cell suspension was then placed in PCR tubes with 10 l of a PCR reaction mix (1X PCR
buffer, 2 mM dNTP's, 20 pmol/1 primers and 1 unit of Taq DNA polymerase) using the pBluescript or pCaSpeR primers. The PCR products were then analyzed on 0.8-1.0% agarose gel and inserts were detected by product size.

2.7 Immunocytochemistry and Histology in Embryos

Embryos were fixed and stained using a mouse anti-α-galactosidase antibody (Price and Lai 1999). Eye discs from third instar larvae were immunostained with mouse -Yan antibody (1:50 dilution) fixed with 4% formaldehyde as described by Wolff 2000. In both embryo and eye tissues primary antibodies were detected using a biotinylated goat anti-mouse antibody (1:200) and vecta staining reagents for color development (Vector Laboratories). Staining to detect -galactosidase ( -gal) in third instar tissue was performed as in Wolff 2000. For double labeling antibody detection was followed by -gal staining. All images were photographed on a Zeiss Axiophot compound microscope.

2.8 -galactosidase Detection in Third Instar Larval Discs

Third instar wandering larvae were picked and washed in PBS then imaginal disc complexes were dissected from the larvae. Disc complexes were then fixed in 1% glutaraldehyde/PBS for 15 minutes at room temperature. The fix/PBS solution was removed and tissue was washed with PBS for 10 minutes 2 times. Staining solution Fe/Nap (0.2M Na2HPO4, 0.2 NaH2PO4, 5M NaCl2, 1M MgCl2, 50mM K3(Fe(CN)6), 50mM K4(Fe(CN)6), H2O) was then pre-warmed for 10 minutes at 37 C. Eye disc tissue was added to the Fe/Nap solution along with 8% X-gal in a 1/30 dilution and incubated for several hours to overnight. The staining solution was removed and discs were rinsed
Once with PBS and resuspended in 80% glycerol/PBS. Eye discs were then left for a few hours in glycerol before mounting (Rubin Lab Methods Book 2nd Edition., 1990). All images were photographed on a Zeiss Axiophot compound microscope.

2.9 Immunostaining in the Third Instar Larval Eye Discs

Eye discs were dissected from third instar larvae in PBS and transferred to Buffer B fixative (16.7 mM KH₂PO₄/K₂HPO₄ (pH=6.8), 75 mM KCl, 25 mM NaCl, 3.3 mM MgCl₂, 5% paraformaldehyde) or a paraformaldehyde-lysine-phosphate (PLP) fix, buffer for 45 minutes on ice. Eye discs where washed in PBT (1X PBS and 0.1% TritonX-100) for 30 minutes on ice before being incubated in PBT/NGS (Normal Goat Serum) for 30 minutes or washed in phosphate buffer (0.1 M phosphate, 0.15% Triton X-100, and 0.15% deoxycolate) and the parapodial membrane removed from the eye disc. Primary antibody (mouse mAb Yan-AZ, mouse mAb Yan-Bam or rat anti-Elav) was added for 3-5 hours at room temperature or overnight at 4°C. Eye discs were washed in PBT or Phosphate buffer for 30 minutes at room temperature, then washed two more times for 10 minutes. Secondary antibody, goat biotinylated, FITC-conjugated or Texas Red-conjugated anti-mouse was then added for at least one hour at 25°C. Eye discs were then washed. Staining was detected using Vectastain reagents for color development with biotinylated secondaries (Vector Laboratories). Eye discs were mounted in 80% Glycerol and photographed using a Zeiss Axiophot compound microscope or collected on a Bio-Rad MRC-1024 Confocal Laser Scanning System.
2.10 *In situ* Hybridization and Whole-Mount Immunostaining

Yan antibody and -gal RNA double staining was done as described by Cadigan *et al.* 1998. Yan antibody was used at the same concentration as above. *LacZ* DNA to be transcribed by RNA polymerases was made by using the Lig’n Scribe No-Cloning Promoter Addition Kit (Ambion). Digoxigenin-labeled single stranded RNA probe was used for hybridization. Binding of the probe was detected using an alkaline phosphatase (AP) conjugated anti-Dig-AP Fab fragment antibody (1:200) and color visualized using NBT/BCIP color detection (Roche Molecular Biochemicals). Images were photographed on a Zeiss Axiophot compound microscope.

2.11 Electrophoresis Mobility Shift Assay (EMSA)

Double-stranded oligos containing the sequence of interest were made and labeled with T4 polynucleotide Kinase (Gibco BRL) and $\gamma^{32}$P ATP in a forward reaction. Probe was purified by a non-denaturing acrylamide gel. For the binding reaction the following conditions were used: Su(H)-GST fusion protein, 10 mM HEPES pH 7.9, 1 mM EDTA, 1mM DTT, 1mM KCl$_2$, 1 mM MgCl$_2$, 10% glycerol, 1µg poly(I-C), 0.3 µg/µl BSA (20 µl total); Yan-GST fusion protein, 13 mM HEPES pH 7.9, 0.7 mM EDTA, 0.3 mM DTT, 40 mM KCl$_2$, 7% glycerol, 1 µg poly(I-C), 5 µg BSA (18 µl total); PntP2-GST fusion protein, 4.4 mM Tris pH 7.5, 0.7 mM EDTA, 0.3 mM DTT, 40 mM KCl$_2$, 9% glycerol, 1 µg poly(I-C), 5 µg BSA (18 µl total). Labeled probe and plus or minus competitor was then added and brought up to volume. This mixture was then incubated at RT for 30 min (37°C, 15min) and loaded onto 5% non-denaturing acrylamide gel. The gel was run @ 200V, 30 mA for 10 wHours (about 2 hrs) in 0.5X TBE. Subsequently the gel was then
dried on Whatman paper and exposed. Oligos used for binding are the following: S1 (ACCCGAAACCTTTTACCGTCATTGAC), S2 (TCGACATCCATCGGGAAA CATGAAACCC), S3 (GCATAGAATTTTTTCTCCATGTACGTTTG), m4S1(ATCCT TGTAGTTTCCACACTGGGTGTT), S2mETS (TCGACACTGATCTCCGCGC ATCGTCTCC) and their complement sequence. All GST-fusion proteins were purified by the method described in the GST Gene Fusion System Amersham Pharmacia Biotech 3rd Edition 1997.
Chapter 3

Results and Discussion

Module Elements

3.1 The *yan* Gene

The *yan* gene plays a critical role in regulating proliferation and differentiation throughout development (reviewed in Schweitzer and Shilo 1997; Treisman and Heberlein 1998; Hsu and Schulz 2000). It is detected in ovaries and throughout embryogenesis. Previous studies during larval development have demonstrated that Yan is only expressed in the CNS and developing eye. It is not detected in any other larval tissues (Lai and Rubin 1992). *yan’s* expression pattern shows a high degree of complexity and specificity that correlates well with Yan function. To better understand how these complex expression patterns are generated, a transgenic approach was undertaken to identify the *cis*-regulatory elements or modules necessary for *yan* expression. Through this approach, we identified key module elements capable of instructing gene expression in a manner that mimics the endogenous *yan* gene. We show that the transcriptional regulation of the *yan* gene requires a broad range of genomic information spanning over 20 kb. However, we also demonstrate that although complete *yan* expression requires a large section of genomic information smaller fragments are capable of regulating individual, temporal and spatial components of *yan’s* expression pattern. In particular we show a more comprehensive analysis of a 122 bp eye-specific
enhancer. For this module, we detail that this eye-specific *yan* enhancer is very complex combining major signaling pathways and structural organizers.

### 3.2 Identification of the *yan* Gene Regulatory Modules

In figure 7 we depict a schematic diagram of the *Drosophila melanogaster* *yan* locus. Genomic fragments used in making *lacZ* reporter constructs are also detailed. A restriction map of the genomic *yan* locus showing *Bam*HI (Bm), *Eco*RI (RI), *Sac*I (S), and *Xho*I (X) restriction enzyme sites is shown. Also, the positions of exons in the *yan* locus are represented by yellow boxes with the coding region in red. In green the location of the predicted gene CG15383 is shown. Arrows indicate direction of gene transcription. Also, shown is the position of *yanP* (blue arrow head), a p-element mediated enhancer trap line located in the first intron of the *yan* locus. (b) Genomic fragments used to make *lacZ* reporter constructs. Multiple independent transgenic lines for each construct were generated through germ line transformation. Black boxes denote fragments that drive *lacZ* expression in embryos. Orange boxes denote expression in embryos, larval eye discs and the larval CNS which include the laminar precursor cells (LPCs), the optic lobe and ventral ganglion. Green boxes denote expression within the embryos, LPCs and eye discs. The blue boxes denote expression specific for the larval eye disc. All other boxes show no expression and are non-specific. More details about each construct are provided in the text.
locus are represented by yellow boxes with the coding region in red. In green we have the predicted gene CG15383 of unknown function located upstream of the yan locus. Arrows in this schematic indicate direction of gene transcription. Additionally, in figure 7 we indicate the position of yanP an enhancer trap line used as a marker for yan gene expression. This enhancer trap line contains a P-element insertion in the first intron of the yan gene and expresses -galactosidase (-gal) in tissues and stages in which Yan protein and mRNA are detected (Lai and Rubin 1992). In figure 7b we show genomic fragments used to make lacZ reporter constructs. For each construct multiple independent transgenic lines were generated through germ line transformation. Lines A, B, C, D and E all denote module regions that mimic in part the yan gene expression pattern throughout fly development. Below the A box are smaller genomic fragments derived from A. The boxes G, J, L and O all drive reporter expression within the eye with the O fragment being the smallest region capable of specifying eye expression. All other boxes do not convey any reporter expression. As can be seen from figure 7, the transcriptional regulation of the yan gene requires a broad range of genomic information spanning over 20 kb. Each of these module elements is detailed below.

3.3 yan Cis-acting Regulatory Modules in Larval Eye Discs

In the developing third instar eye, Yan protein is expressed in all undifferentiating cells within and posterior to the morphogenetic furrow (MF). Its role in these cells is to inhibit differentiation until the proper signaling, via the RTK pathway, induces them to differentiate into photoreceptors or other cells of the ommatidium (Lai and Rubin 1992; Rebay and Rubin 1995). In our initial analysis of the large yan genomic fragments A
through E, only A and B are able to drive gene expression in the eye imaginal discs. As a control for β-gal expression we used yan\(^p\), an enhancer trap line within intron 1 of the yan gene that has been demonstrated to recapitulate yan gene endogenous expression (Lai and Rubin 1992). Fragment A drives a pattern similar to yan\(^p\) expression while B drives expression in subretinal cells likely corresponding to glial cells. This glial cell expression has not been detected in previous experiments looking for Yan protein (Lai and Rubin 1992; Price and Lai 1999). Yet, this expression seems to be genuine for the reason that it is detected in multiple transgenic lines. It is possible that previous methods used for examining Yan expression mask this detection. For example, with the yan\(^p\) enhancer trap line the β-gal expression persists even in differentiating cells making it difficult to specify glial cell expression. As for using Yan antibodies or in situ in these cells, rapid turn over of transcript and/or protein may limit detection. Another possibility is that fragment B expression is artificial and normally expression in these cells is repressed but because there is a lack of a repressor element normally present in the surrounding genomic DNA we get expression in glial cells. It should also be noted that the A

**Figure 8. yan-lacZ Reporter Expression in the Developing Third Instar Eye Disc**

The above panels show β-gal detection in the eye discs. Panel (a) shows β-gal detection for the yan\(^p\) line while (b) and (c) shows detection for different transgenic lines. The A-lacZ fragment mimics expression seen in yan\(^p\) but the B-lacZ fragment seems to be novel in detection. Anterior is to the left and the red arrow demarcates the MF.
fragment is orientation dependent. Unlike most enhancers that transcriptionally are orientation independent, the A fragment only works in the endogenous configuration. Transgenic flies were made reversing the A fragment enhancer. In these transgenic lines no reporter expression is detected in the developing eye or other tissues (data not shown). For this reason all subsequent P-element enhancer fragments derived from the A fragment are constructed in the endogenous orientation.

Figure 9. Analysis of the A Fragment
The A fragment is a 4.4 kb DNA fragment that mimics endogenous yan expression (b). Smaller DNA fragments capable of mimicking this expression are the 3.6 kb G-lacZ (c), ~800 bp J-lacZ (d), the 222 bp L-lacZ (e) and the smallest genomic fragment able to mimic yan expression is the 122 bp O-lacZ (f). Anterior is to the left and the red arrow demarcates the MF.
Since the A fragment is able to mimic an expression pattern similar to what is seen with \( yan^P \) and Yan protein we performed restricted analysis of this genomic region to identify the smallest possible region that could mimic endogenous \( yan \) expression. The analysis of the A fragment demonstrated that increasingly smaller fractions of this DNA are sufficient to drive \( lacZ \) expression within the eye (\( G, J \) and \( L-lacZ \)). A minimum regulatory sequence, \( O-lacZ \), consisting of 122 bp of the original A fragment was able to drive gene expression in the larval eye disc. Also, \( O-lacZ \) is eye-specific driving no expression in any other tissue. One question that arose from the above analysis was that -gal activity seemed to be posterior to the MF. Endogenous expression of Yan is strong in the MF so we were wondering if our enhancer lacks the ability to drive expression within the MF and a separate enhancer is needed for this expression. To determine if Yan protein and reporter expression were overlapping we double labeled eye discs with an anti-yan antibody (brown) followed by a -gal detection assay (blue). Again, \( yan^P \) was used as a control. As shown in figure 10, a low level of -gal activity for \( yan^P \) is detected within cells in the MF and a high level in cells posterior to it (figure 10a, a’). Similar results were seen in a double labeling experiment using anti-Yan and anti- -gal antibodies (Data not shown). Fragments \( A, G, J, L \) and \( O-lacZ \) (figure 10b, b’) also show limited activity within the MF. However, all drive reporter expression in all Yan positive cells posterior to the MF. This data confirmed suspicions that -gal activity is posterior to the MF.
Figure 10. Double Labeled Eye Discs
In panels (a-b’) double labeled eye discs with anti-yan antibody (brown) and β-gal (blue) show a delay of β-gal protein expression based on activity. Inserts are enlargements of boxed region. Panels (c) and (d) show antibody/in situ double staining for Yan protein (brown) and β-gal transcript (purple) within and posterior to the MF. Panel (c) shows a control with the lacZ sense probe and (d) shows the co-localization with the lacZ antisense probe. Anterior is to the left and the red arrow demarcates the MF. These experiments done with M. Rohrbaugh.

To directly address whether the yanO fragment is sufficient to drive transcription within the MF we looked at lacZ transcript by in situ analysis. Figure 10d shows a double labeling of Yan antibody and lacZ mRNA while figure 10c shows a negative
control for lacZ mRNA detection. As can be seen, lacZ transcript is present within the MF in a pattern corresponding to Yan protein. This result is in contrast to figure 10b’ which indicates the majority of -gal protein expression is posterior to the MF.

Combining the results of these two experiments demonstrates a delay between the detection of lacZ transcript and -gal protein expression. This may be due either to a delay in translation of the lacZ mRNA or a need for an accumulation of the -gal protein to sufficient detectable levels. Thus, this data demonstrates that the yanO fragment is

Figure 11. Early Detection of yanO-LacZ Reporter
-gal Detection of the yanO-lacZ Reporter From Mid-Second to Mid-Third Instrar Eye Discs. Anterior is to the left.
sufficient to regulate the complete *yan* expression pattern in the eye-imaginal disc within and posterior to the MF. Also, we further characterized the *yanO* enhancer by identifying the earliest time point for detection of expression. As can be seen is figure 11 expression begins in mid-second instar stage at the posterior most position in the eye disc.

### 3.4 *yan* Cis-acting Regulatory Modules in the Larval CNS

In the larval CNS protein data demonstrates *yan* expression in the laminar precursor cells (LPCs) of the optic lobe (figure 12; Price and Lai 1999), suggesting a role for *yan* in CNS development. However, Yan protein function at this stage has yet to be defined. In contrast, *yanP* is detected in the LPCs and in various cells throughout the brain and ventral ganglion (figure 13a). In our reporter analysis expression in the brain seems to be very compartmental in its organization. Fragments A (figure 12c, 13b) and B

![Figure 12. The Larval CNS](image)

(a) A schematic diagram depicting the eye-antennal disc and the larval CNS. The CNS consists of the optic lobe, the lamina precursor and the ventral ganglion. Also, shown is an anti-yan antibody (brown) staining of the larval CNS (b). Detection can be seen in the LPC and strongly in the eye. A general pattern is also detected in cell throughout the optic lobe and ventral ganglion. Lastly, the larval CNS and eye-antennal disc is shown for the A-lacZ reporter construct (c). -gal detection for this construct is strong in the LPC and eye. Anterior is to the top.
(figure 13c) together completely regenerate $yan^P$ expression (figure 13a). Fragment A expression mimics Yan protein in LPCs while fragment B drives expression in the ventral ganglion and in cells throughout the brain similar to $yan^P$.

**Figure 13.** -gal Detection in the Third Instar Larval CNS

In (a) $yan^P$ drives -gal expression in the laminar precursor cells (LPCs) of the optic lobes in the CNS. In addition to expression in LPCs, $yan^P$ expression is seen in a number of cells throughout the brain and ventral ganglion. $A$-lacZ (b) drives expression in the LPCs of the optic lobes while $B$-lacZ (c) drives transcription in distinct cells within the optic lobes and ventral ganglion. In G and J-lacZ (panels d and e) both show LPC expression similar to $A$-lacZ. The A fragment is a 4.4 kb DNA fragment that mimics endogenous $yan$ expression. Anterior is to the top.

Due to the similarity of fragment A and Yan expression data, further analysis of the A fragment was conducted. Smaller fragments, G-lacZ (figure 11d) and J-lacZ (figure 11e) are capable of recapitulating A fragment expression. When the J-lacZ fragment was divided even further expression in the LPCs was eliminated. The smaller fragments derived from J were not able to mimic even a portion of the J-lacZ expression within the CNS. Therefore, the 800bp J-lacZ fragment is the smallest module able to replicate Yan expression within the CNS. Together, regulatory sequences from J and B fragments appear to be sufficient to drive gene expression similar to that of the endogenous $yan$ gene.
3.5 Complex yan Cis-acting Regulatory Modules in Embryos

Previously, yan mRNA has been detected in embryos as early as stage five in the neurogenic region and dorsal ectoderm. During gastrulation (stage 6), yan mRNA and protein expression become restricted to the head region and the ectoderm layer, and are absent along the ventral midline (Gabay et al. 1996; Price and Lai 1999). Later in embryonic development (stage 11), Yan protein is detected in cells near the tracheal pits and in cells close to the ventral midline in the epidermal layer. Finally, in late embryonic development yan expression becomes confined to head and midline developing regions (Rogge et al. 1995; Scholz et al. 1997; Dumstrei et al. 1998; Price and Lai 1999).

In our analysis, -gal expression is first seen during germ band elongation. In stage 10 embryos, A-lacZ (figure 14c) drives expression in the ventral ectoderm and head in a manner resembling Yan protein pattern (figure 14a; Price and Lai 1999).
Nevertheless, some differences in expression are obvious. *A-lacZ* drives strong expression in cells close to the midline where *yan* (figure 14b) and Yan protein expression are not detected. This aberrant expression is possibly due to the absence of an inhibitory or repression element positioned outside of A that may regulate *yan* expression in midline cells. This element might prevent or minimize *yan* transcription in cells near the midline under normal developmental conditions thus precluding detectable expression.

Other fragments that drive expression during stage 10 are *D and E-lacZ* located within the first intron (figure 7). *D-lacZ* drives a fairly general pattern of expression in the ventral ectoderm (figure 14d) and head while *E-lacZ* drives expression in lateral stripes in the ectoderm and at low levels in the head (figure 14e). Interestingly, cells positive for *A-lacZ* and *E-lacZ* expression in stage 9-10 embryos together regenerate a pattern in the ventral ectoderm similar to Yan protein staining (data not shown).

Later in germ band extension (stage 11), constructs *A, D*, and *E-lacZ* still drive expression in the epidermis with *A* (figure 15c) and *D-lacZ* (figure 15e) having patterns most closely resembling Yan and *yan* (figure 15a, 15b). Expression in the head region at this stage is still evident in both *A-lacZ* and *D-lacZ*, but not in *E-lacZ* lines (figure 15f). At this stage, for the first time, *B-lacZ* (figure 15d) begins to drive reporter expression. *B-lacZ* drives a very restricted pattern of expression in cells that are consistent with the clusters of Yan positive cells located behind and beneath the tracheal pits (Gabay *et al.* 1996; Price and Lai 1999).
Figure 15. Reporter Gene Expression From Constructs A Through E in Stage 11 Embryos
In panel (a) anti-yan antibody detection for embryos is shown. In panels (a-f) a dorsolateral view of stage 11 embryos are shown. For panels (b-f) yan\(^p\), A, B, D, and E-lacZ stage 11 embryos drive -gal expression. Yan protein and yan\(^p\) expression is still seen in the ectoderm in addition to cells near the tracheal pits. A striped pattern of expression is also seen in the ectoderm of stage 11 embryos from A-lacZ, D-lacZ, and E-lacZ. For the first time B-lacZ drives expression and it can be seen in a small number of cells in each segment near the tracheal pits.

Throughout stage 13 embryos, -gal expression in yan\(^p\) (figure 16b) correlates with the Yan immunostaining (figure 16a) pattern where expression is detected at a lower level in the epidermal cells and at a higher level in the tracheal system. At this stage of

Figure 16. Reporter Gene Expression From Constructs A Through E in Stage 13 Embryos
In panel (a) anti-yan antibody detection for embryos is shown. In panels (a-f) a dorsolateral view of embryos is shown. In stage 13 embryos expression is limited to the ectoderm and tracheal system. -gal expression is detected in the ectoderm of stage 13 embryos from yan\(^p\), A-lacZ, D-lacZ and E-lacZ. Only a small number of cells in each segment express lacZ in stage 13 embryos from B-lacZ flies.
embryonic development, constructs \( A, D \) and \( E-lacZ \) (figure 16c, e, f) drive expression in a pattern consistent with \( yan^P \) and Yan protein. \( B-lacZ \), (figure 16d) continues to drive a very restricted pattern in the cells beneath the tracheal pits.

In embryos undergoing dorsal closure (stages 14-16), \( C \) and \( D-lacZ \) drive reporter expression within the head region in cells consistent with forming the optic lobes (figure 17). Specific expression is also detected in rows of cells along the dorsal midline a pattern not seen with Yan antibody staining (figure 17b, c, e, f). This midline expression can be seen as early as stage 13 in both \( C \) and \( D-lacZ \) embryos (data not shown and figure 17g). These cells along the midline are known to play a role in the circulatory system (reviewed in Cripps and Olson 2002) and may indicate a greater function than previously

**Figure 17. Reporter Gene Expression in the Dorsal Midline**
In panel (a) anti-yan antibody detection for embryos is shown. In lateral (a-c) and dorsal (d-g) views of embryos, \( C-lacZ \) and \( D-lacZ \), expression is detected in the developing head. Also, expression is seen in cells along the dorsal midline in a pattern not seen with Yan antibody. This dorsal midline expression is seen as early as stage 13 embryos for \( C-lacZ \) and \( D-lacZ \) (only \( D-lacZ \) shown).
thought for yan in cardiac development. Data from Halfon et al. 2000 supports the observation that yan is involved in cardiac development by showing that an increased number of Even skipped (Eve) positive pericardial progenitor cells can be detected in a yan mutant background. Thus, with the similarity in the expression pattern of our reporter constructs within midline cell development and the known involvement of yan in cardiac progenitor cells, there is a likely role for yan in cardiac development that has yet to be fully understood. This midline expression is unlikely due to positional effect because it can be detected with multiple independent insertions with two different transgenes.

One concern regarding the midline expression from our reporter constructs is that this pattern is not seen with yanP or Yan protein staining. In the case of yanP, it may be due to the location of its P-element insertion. Sequence analysis indicates that the enhancer trap line insertion lies within the genomic region mutual to both C and D-lacZ transgenic lines. This insertion may be disrupting an endogenous enhancer element preventing the yanP line from exhibiting midline expression. However, there may be a repression element in the endogenous situation that is missing in C and D-lacZ transgenic lines allowing for easier disclosure of –gal. As for the low level of Yan protein in midline cells, perhaps this results from a dynamic turnover of Yan protein leading to decreased immunodetection.

3.6 yan Cis-acting Regulatory Modules in Ovaries

Genetic studies demonstrate that flies homozygous mutant for yan have a fertility phenotype indicating a requirement for Yan in ovaries (Lai and Rubin 1992). Indeed,
Yan protein is expressed in follicle and nurse cells within ovaries (Price and Lai 1999). However, none of our yan genomic fragments are able to drive gene expression within these cells. Based on this data, we assume that the genomic requirement for regulating expression within ovaries may be mediated either by regulatory regions that lie outside the area covered in this analysis or by a combination of elements from different regions within the yan locus area studied.

### 3.7 Synopsis of Regulatory Modules Required For yan Gene Expression

To this point we have described how cis-acting module elements can impact yan gene expression. Also, we identified new potential areas of yan expression relating to function not detectable with previous methods. Through in vivo analysis, we demonstrated that the genomic region surrounding the yan first exon contains regulatory modules sufficient to drive gene expression in embryos, larval CNS and eye imaginal discs. Fragments A through E contain most of the information necessary for yan expression in embryos. In the larval CNS, B and J-lacZ can replicate previous expression data and in the developing eye the 122 bp O module contains the minimal information needed to specify gene expression mimicking yan\(^P\) and the Yan protein pattern. Taken in combination, this data indicates that the yan-lacZ transgenes recapitulate most of the expression patterns of the endogenous yan gene. Thus, this data highlights the significance of cis-regulatory modules in terms of regulating gene transcription.
Regulation of the *yan* Gene Eye-Specific Enhancer

### 3.8 Identification of the Eye-Specific *yan* Enhancer Regulatory Binding Sites

We identified a 122 bp enhancer that is capable of mimicking the endogenous *yan* expression pattern within the eye (figure 18). We then wanted to characterize this sequence to identify regulatory elements involved during eye development. Sequence analysis of this 122 bp *yanO* fragment revealed many potential DNA binding motifs. By homology search, we found three putative Su(H) binding sites (S1, S2 and S3) (Figure 19), two ETS DNA binding motifs, an overlapping N-box and Runt motif, a structural

![Diagram](image)

Figure 18. The 122 bp *yan* Enhancer Sequence and Structural Motifs Schematic
Highlighted sequence motifs in the 122 bp eye-specific *yan* enhancer. Two putative insulator motifs are shown in green, 3 Su(H) motifs (S1, S2 and S3) are shown in blue, a N-box/Runt motif is shown in pink and grey, two ETS DNA binding sites are shown in purple and a 22 bp sequence containing the GAAACC/A repetitive element is shown in yellow. Arrow above Su(H) sites indicate established direction or binding site.
**Su(H) Binding Site in the 122 bp *yan* Enhancer:**

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<th>5</th>
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<th>7</th>
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<th>9</th>
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<th>Su(H)-Binding Activity</th>
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<tr>
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<td>C</td>
<td>G</td>
<td>G</td>
<td>T</td>
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<tr>
<td>yanS3</td>
<td>A</td>
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**Su(H) Binding Sites from the *E(spl)*-Complex Genes:**

| E(spl)m4S1 | T | G | T | G | G | G | A | A | A | C | + |
| E(spl)m4S1 | T | G | T | G | G | G | A | A | A | C | (mutant) | − |
| E(spl)m4S2 | C | G | T | G | A | G | A | A | A | C | + |
| E(spl)m4S2 | C | G | T | G | A | C | A | A | A | C | (mutant) | − |
| E(spl)m4S3 | T | G | T | G | G | G | A | A | C | T | + |
| E(spl)m4S3 | T | G | T | G | G | C | A | A | A | C | (mutant) | − |
| E(spl)m4S4 | C | G | T | G | G | G | A | A | A | A | + |
| E(spl)m S1 | C | G | T | G | G | G | A | A | A | C | + |
| E(spl)m S2 | T | G | T | G | A | G | A | A | A | C | + |
| E(spl)m S3 | C | G | T | G | G | G | A | A | C | C | + |
| E(spl)m S4 | T | G | T | G | G | G | A | A | A | C | + |
| E(spl)m S1 | C | A | T | G | A | G | A | A | A | C | + |
| E(spl)m S2 | A | G | T | G | G | G | A | A | C | C | + |
| E(spl)m S3 | T | A | T | G | A | G | A | A | A | T | + |

**Consensus**

| N | R | T | G | R | G | A | R | S | N |

**Figure 19. Comparison of Su(H) Binding Sites**

Comparison of the *yan* enhancer Su(H) binding sites to known Su(H) consensus binding sites. The above Su(H) sites for *E(spl)*-complex genes all bind *in vitro* to Su(H) protein except for those site that are mutated. The mutated site changes an essential G-nucleotide to a C-nucleotide (Bailey and Posakony 1995; Lecourtois and Schweisguth 1995; Eastman *et al.* 1997; Nellesen *et al.* 1999).

insulator element that seems to provide a boundary function and a repetitive element with two direct copies of a GAAACC and one copy of a GAAACA sequence.

Interestingly, the Yan S1 and S2 elements together form a Su(H) paired site (SPS), a structure found in the regulatory regions of many *E(spl)* genes (Bailey and Posakony 1995; Nellesen *et al.* 1999). In the *yan* SPS element there are two direct copies of a GAAACC element and one copy of a GAAACA element. In almost all SPS elements in *E(spl)* genes there also exists an invariant hexamer motif GAAAGT (Nellesen *et al.* 1999). The functional significance of the hexamer motif in regulating *E(spl)* gene
Both the E(spl)mγ and yan enhancer have Su(H) binding sites in the same orientation and both contain a hexamer motif common to most E(spl) genes. Expression is unknown but we show these elements have a direct significance in regulating the yan enhancer. Another striking observation is the structural similarity between the 234 bp E(spl)m (Bailey and Posakony 1995) and the 122 bp yan enhancer. These Su(H) and hexamer sites in the yan enhancer have a very similar organization to the 234 bp E(spl)m enhancer. In the following sections we detail the function of all these elements for the yan gene and begin to elucidate a model for combinatorial transcriptional regulation of the yan gene during eye development.

3.9 Analysis of Notch Pathway Interaction with the yan Enhancer

The Notch signaling pathway functions in a variety of processes throughout eye development. It is involved in promoting eye development in early eye stages, it later
blocks photoreceptor specification, it is needed for ommatidial patterning and Lastly Notch is required for promoting non-neuronal cone cell differentiation. We first became interested in the Notch pathway due to the overlap of function between yan and Notch signaling during photoreceptor specification, particularly in the ability to block photoreceptor specification. In analysis of mutants for both yan and Notch a similar hyponeuronal phenotype is observed. Knowing that the yan enhancer shares similar structures to know targets of the Notch pathway and that phenotypes and function overlap significantly we investigated the role of the Notch pathway in regulating the yan expression.

The yan enhancer contains 3 Su(H) binding sites that show striking similarity in motif arrangement to a 234-bp E(spl)m enhancer which is known to be a target of Notch mediated Su(H) activity. To determine if these yan sites were targets for Su(H) interaction we conducted electromobility shift assays (EMSA). Data from this assay demonstrates that all three binding sites could be recognized by a Su(H)-GST fusion protein. To perform the EMSA we made double stranded oligonucleotides of each of the yan gene Su(H) binding sites. Also, we used a known Su(H) binding site from the E(spl)m4 S1 enhancer as a control (Bailey and Posakony 1995). In this experiment the radio labeled yan S1, S2 and S3 oligo probes demonstrate binding to Su(H) protein (figure 21). Our control also demonstrated binding to our Su(H)-GST protein and in competition assays our control oligo competed off S1, S2 and S3 for Su(H) binding. One downfall for this experiment was that secondary controls were conducted at a latter time and revealed non-specificity for Su(H)-GST binding. In secondary controls, non-specific random oligos were tested for binding to the Su(H)-GST protein. In this assay binding
Figure 21. Su(H)-GST Binding to yan S1, S2 and S3 Sites

The EMSA assay shows that Su(H) binds to yan Su(H) binding sites S1, S2 and S3. m4S1 is our competitor oligo known to bind Su(H) and is derived from the E(spl)m4 enhancer (Bailey and Posakony 1992). This experiment was done by D. Nguyen.

was still detected. In a second control assay with a mutant form of the m4S1 control oligo (TGTGGCAAC), binding was still seen. This indicated to us that Su(H)-GST protein was binding non-specifically to any oligo. With these data, we initial thought we had demonstrate in vitro interaction between the Su(H) binding sites and the Su(H) protein. It was not until the secondary controls were done that we concluded non-specific binding by the Su(H)-GST protein. Still, with genetic experiments we demonstrate an in vivo role for Su(H) in the regulation of the yan gene eye-specific enhancer.

In a subsequent analysis, to test the function of the S1, S2 and S3 Su(H) binding sites in the regulation of the yan enhancer we performed clustered mutational analysis for these binding elements. The results of these experiments reveal a drastic loss of reporter
gene expression in S1 (yan S1m-lacZ) mutant transgenic flies (figure 22b). Also, mutations in S2 (yan S2m-lacZ) show complete loss of lacZ expression (figure 22c) while mutations in S3 (yan S3m-lacZ) only show a slight down regulation of the enhancer activity (figure 22d). To this point we have shown an interaction between the yan Su(H) binding sites by Su(H) protein and a requirement for these sites in the regulation of our

Figure 22. yanO Reporter Expression with Mutated Su(H) DNA Binding Sites
Eye disc analysis is shown from transgenic flies with individually mutated Su(H) binding sites (light blue). Each eye disc shows expression with two copies of the transgene. Mutations in S1 (b) and S2 (c) completely eliminate reporter expression while mutations in S3 (d) give a reduction in reporter expression. The mutations are as follow: for S1 from (ACGGTGAAAG) to (ACG TCCGTG), for S2 from (ATCGGGAAAC) to (ATC TCCGCG) and for S3 from (ATGGAGAAAA) to (ATG TCCGCGT). Anterior is to the left.
yan-lacZ reporter. Our next step was to demonstrate whether this interaction could be indicative of in vivo regulation of yan expression.

Knowing that Su(H) is able to bind to the yan enhancer sites in vitro, we explored genetically if any interaction between yan and the Notch pathway could be established. To investigate genetic interactions between yan and Notch we used a sensitized assay that gives a slightly rough eye in adult flies. We tested interactions for a gain or reduction of this rough eye phenotype. Using the yan\(^p\) hypomorph in a homozygous background a twenty percent mutant ommatidia phenotype is observed. Upon reduction of Su(H) by fifty percent in this homozygous hypomorph background (yan\(^p\)/yan\(^p\); Su(H)\(^{del47}/+\)) we see a doubling of mutant ommatidia up to approximately forty-two percent (figure 23). This significant increase indicated a genetic interaction between yan and Notch signaling and prompted us to further investigate the role of Notch and Su(H) in the regulation of the yan gene.

![Figure 23. Genetic Interaction Between yan and Notch](image)

The eye phenotype of yan mutants is dominantly enhanced by a fifty percent reduction of Su(H). This result suggests an interaction between Su(H) activity and yan function. This experiment done by M. Rohrbaugh.
Our next goal was to determine if Su(H) and Notch are required for Yan protein expression. This was done twofold by conducting loss-of-function and gain-of-function analysis for Su(H) and Notch. In Su(H) loss-of-function assays $[Su(H)^{SF8}/Su(H)^{AR9}]$ Yan protein expression is completely lost (figure 24). To further test these results loss-of-function $Su(H)^{del47}$ clones were made in a $Su(H)^{del47/+}$ background. These results confirm the Su(H) loss-of-function assay. Clones for Su(H) demarcated by the lack of GFP also show an elimination of Yan protein expression (figure 24). To further delineate Notch pathway requirements for Yan protein expression we looked at temperature sensitive

![Figure 24. Su(H) is Required for Yan Protein Expression.](image)

Pannel (a) shows a double labeling of a wild type eye disc stained with anti-Yan antibody (Green) and the neuronal marker Elav (red). Pannel (b) shows a Su(H) mutant eye disc stained for both Yan and Elav. In this disc no Yan Protein is detected. In Pannels (c-e) a Su(H) clone is shown. The lack of GFP staining (c) defines the boundary of the clone, within which Yan expression (d) is eliminated. Pannel (e) shows the merged image of (c) and (d). Anterior is to the left and the arrow indicates the MF. This experiment was done by M. Rohrbaugh.
mutants of Notch ($N^{ts}$). In the permissive temperature (25 C) where Notch protein is active Yan protein can be detected. In the non-permissive temperature (32 C, for 7 hours) where Notch protein is inactive a loss of Yan protein detection is also observed. As can be seen in Figure 25 after recovery time when Notch activity is again restored from non-permissive to permissive Yan protein can once again be detected in the posterior margin of the eye disc.

![Figure 25. Yan Staining of $N^{ts}$ Eye Discs](image)

In the first panel (a) third instar eye disc from $N^{ts}$ flies were stained for Yan. In the second panel (b) $N^{ts}$ eye disc were stained for Yan after treatment for 7 hours at 32 C. In panel (b) no Yan detection is seen in the MF. This indicates that Notch activity is required for Yan expression. In CS flies undergoing the same heat shock treatment as above no detectable difference is seen with Yan protein detection. Anterior is to the left and the arrow demarcates the MF. This experiment was done by M. Rohrbaugh.

Knowing that both Su(H) and Notch are required for Yan protein expression we wanted to identify if this activity is mediated through our eye-specific enhancer. In a loss-of-function mutant background for Su(H) we demonstrate that the reporter expression for the eye-specific enhancer is lost in all cells posterior to the MF (figure 26).
Su(H) is required for the function of the 122 bp eye-specific yan enhancer. Panel (a) shows an eye disc stained for β-gal activity with one copy of the yanO-lacZ transgene. In panel (b) loss of Su(H) protein gives a loss of yanO-lacZ reporter activity in the posterior part of the eye disc. Anterior is to the left.

As for the staining seen in the dorsal and ventral part of the eye disc it comes from a mini white reporter in the Su(H)SF8 line and serves as an internal control. Together this data show that Su(H) is required for the function of our 122 bp eye-specific enhancer and combined with the EMSA data indicates that this function is mediated through the yan Su(H) binding sites.

In other genetic data with gain-of-function assays undertaken by overexpressing Su(H) and Notch in multiple combinations we saw a reduction in yanO-lacZ expression (data not shown). This result was contrary to our expected results based on Su(H) and Notch loss-of-function. When we overexpressed hs-Su(H) we saw a reduction. When we overexpressed the fusion protein Su(H)VP16 (GMR-Gal4/UAS-Su(H)VP16), which contains a transcriptional activator, we saw a reduction of reporter expression. Similar results were also seen with hs-N\textsuperscript{intra} or GMR-Gal4/UAS-N\textsuperscript{intra} overexpression. In these and all overexpression studies the GMR-Gal4 driver was used which drives expression in all cells within and posterior to the MF (for more information see materials and methods).
The repression effect on the *yanO* reporter construct with gain-of-function Notch/Su(H) signaling seems to contradict data from loss-of-function experiments. One possible mechanism to explain these results is an N-box basic Helix-Loop-Helix (bHLH) binding motif found within the *yanO* enhancer. The N-box motif may be a target of Notch/Su(H) activated *E(spl)* transcriptional repressor genes. As mentioned earlier the members of the *E(spl)*-complex are downstream targets of Notch/Su(H) signaling. To determine the role of *E(spl)* genes in the regulation of *yan* two experiments were done. First, *E(spl)* mutant clones were generated and monitored for *yan* gene expression. In the clone patches for *E(spl)*\textsuperscript{BX22}, a mutation that deletes the *E(spl)* genes (*m5*, *m7*, *m8*) and *groucho*, *yan*\textsuperscript{P} expression was unaffected (M. Price, personal communication). This result indicated that the *E(spl)* genes did not regulate *yan* expression. In a second experiment when concurrently overexpressing *E(spl)m7* and *E(spl)m8* *yanO* reporter expression is reduced (figure 27). This reduction with *E(spl)* genes can only be seen when simultaneously

![Figure 27](image)

**Figure 27.** *E(spl)* genes Negatively Regulate *yan* Enhancer
In panel (a) one copy of our *yanO-lacZ* transgene is shown. In panel (b) one copy of our transgene is shown with overexpression of GMR-Gal4/*UAS E(spl)m7; UAS E(spl)m8*/*yanO-lacZ*. Anterior is to the left. This experiment done with M. Rohrbaugh.
overexpressing $E(spl)m7$ and $E(spl)m8$. No reduction is seen when $m7$ or $m8$ are individually overexpressed with our reporter construct.

In cluster mutation analysis of the N-box motif we did not see an upregulation of $yan$ reporter expression. What we did see was an abolishment of reporter expression. This result may be due to a Runt domain DNA binding site. Runt domains are binding sites for Lozenge (Lz) proteins which in eye development are transcriptional activators (Flores et al. 2000). In $lz$ mutant ($lz^{15}$) eye discs the $yan$ enhancer activity is completely lost (data not shown). Thus, with the above data regulation of the $yan$ eye-enhancer seems to be regulated by the Notch pathway and mediated though Su(H) and potentially moderated through $E(spl)$ genes.

### 3.10 Regulation of the $yanO$-$lacZ$ Hexamer Motif

In the 122 bp $yan$ enhancer a repetitive GAAACC/A motif was found between Su(H) binding sites S2 and S1. These sites share a commonality with a hexamer sequence found in most $E(spl)$ genes. In the $E(spl)$ genes this hexamer sequence has not be investigated and its function is unknown. We examined these sites within the $yan$ gene by making random clustered mutations into both GAAACC sites. We did not mutate the GAAACA site because it overlaps substantially with the S2 site for Su(H) which had already been mutated and shows a functional necessity. In the remaining hexamer sequences, mutations of these sites completely abolished reporter gene expression ($yan2Hexm$-$lacZ$) (figure 28b).

To determine if the $yan$ hexamer sites provided a regulatory motif for eye-specific expression we made transgenic lines containing solely these repetitive elements. Initially
we made transgenic lines containing only the 22 bp sequence associated with the hexamer repeats \((\text{yanQ-lacZ})\). These transgenes did not show reporter gene expression (figure 28c), however, in a concatamer containing 6 copies of this 22 bp sequence \((\text{yan6Q-lacZ})\) -gal expression is detected. This -gal expression is detected in

**Figure 28. Regulation of the \text{yanO-lacZ} Hexamer Motif**

In the above photomicrographs we show -gal activity staining in each eye disc that contains two copies of its transgene except panel (d) which contains one copy of the transgene. Panel (a) shows activity staining mimicking \text{yan} endogenous expression. Panel (b) shows no activity for the \text{yanO} construct with mutated hexamer binding sites. Mutations for panel (b) are from \((\text{GGAAACATGAAACCCGAAACCT})\) to \((\text{GGAAACATCGTCTCCCTGACT})\). Panel (c) contains transgenes with one copy of the 22 bp hexamer repeat \((\text{GGAAACATGAAACCCGAAACCT})\). No activity is seen with this construct. In panel (d) activity staining is seen for a concatamer transgene containing six copies of the 22 bp hexamer repeat. For panel (d) and only for panel (d) -gal activity staining is detected as quickly as 2 to 3 minutes with the above photomicrograph taken in 10 minutes. All other eye disc images are 14-16 hours -gal activity stainings. Anterior is to the left.
eye discs (figure 28d), larval CNS and embryos in a pattern similar to yan albeit broader than normal yan expression pattern. No other expression with this construct is detected in other tissues including the ovaries. Also, all the staining assayed in the eye and CNS with the concatemer construct are with a single copy of the transgene and only stained for 10 minutes versus 14-16 hours staining for all others -gal detection in the eye or CNS for this thesis. These hexamer motifs do not seem to be specific for just eye expression but dose seem to be a vital part for the regulation of the yan gene expression within the eye and may be necessary elsewhere for yan expression.
3.11 RTK Signaling Negatively Regulates yan Gene Transcription

RTK signaling has long been known to modulate Yan protein stability but here we demonstrate a role for RTK in regulating yan gene transcription. In our 122 bp yanO enhancer there are two ETS DNA binding sites (GGAA and GGAT). These ETS DNA binding sites are located juxtaposed to the Su(H) S2 DNA binding site of the yan eye-enhancer. In EMSA binding studies using ETS DNA binding proteins Pnt-GST and Yan-GST binding specificity is detected (figure 29a). Binding is only detected with the S2 probe which contains both ETS and Su(H) binding sites the other two Su(H) probes (S1

Figure 29. Binding Competition Between ETS and Su(H) Proteins
In panel (a) the Yan-GST and Pnt-GST ETS proteins specifically bind to the S2 oligo probe. In panel (b) Pnt-GST competes off Su(H)-GST protein binding for sites on the S2 probe. In panel (c) mutations in the ETS DNA binding sites for the S2 probe prevent Yan and Pnt binding. The following mutations from (TCGACATCCATCGGAAACATGAAACCC) to (TCGACACTGATCTCGCGCATGAAACCC) were made in the ETS sites of the S2 probe. These experiments were done in conjunction with D. Nguyen.
and S3 show no binding activity. Interestingly, competition for proximal binding with Su(H) and ETS proteins can be detected. In a competition assay for the S2 probe when Su(H) is allowed to bind initially, Pnt or Yan can compete off Su(H) for binding to the S2 probe (only Pnt shown in figure 29b). The quantity of protein added in these experiments was not determined. For the competition assay a qualitative increase in protein is added. To test the fidelity of Pnt and Yan binding to the ETS DNA binding sites in the S2 probe the ETS sites were mutated within the S2 probe. With just the GGAT site mutated in the S2 probe a reduction in binding is seen with both Pnt and Yan. When both ETS sites are mutated in the S2 probe neither Yan nor Pnt is able to bind (figure 29c). Still, Su(H) continues binding to this mutated ETS S2 probe.

**Figure 30. RTK Regulation of yanO-lacZ Expression**

In (a) two copies of the yanO-lacZ is shown. Panels (b-g) contain only one copy of the yanO-lacZ transgene. GMR-Gal4 is the driver for all overexpression (b-f). All the overexpression experiments show a reduction in β-gal activity. In panel (g) an upregulation in β-gal activity is detected in a Pnt mutant background. Anterior is to the left in all discs. Above staining done by M. Rohrbaugh.
Knowing that Pnt and Yan are able to bind specifically to the yan enhancer sites \textit{in vitro}, we explored genetically if any interaction between yan and the RTK pathway could be established. To investigate the role of RTK in the regulation of yan transcription we conducted overexpression analysis of RTK components using the Gal4/UAS system. As before, the driver used in these assays is the GMR-Gal4 driver. In assays where expression of constitutively active forms of DER (\textit{UAS-tor}^{D}-\textit{DER}) or Ras (\textit{UAS-Ras}^{V12}) were overexpressed in the eye a reduction of the eye-specific reporter detection is observed (figure 30c and d). When the ETS protein Pnt was overexpressed different outcomes were detected depending on the splice form used for overexpression. With overexpression of PntP1 (\textit{UAS-PntP1}), yan \textit{O-lacZ} detection was greatly reduced (figure 30e). On the other hand, overexpression of PntP2 conveyed no change in enhancer expression. The difference in function between the two splice forms is currently not well understood. What is known is that the PntP2 is the form that gets phosphorylated and in the eye disc and embryo functions as a positive regulator of expression. This phenomenon is interesting because PntP2 is known to be an activator of gene regulation and is the target of MAPK phosphorylation but has no effect on our reporter construct while PntP1, whose function in the development of the eye is less clear, seems to function as a repressor of transcription. This is one of the few instances where Pnt functions as a repressor of transcription and maybe a mechanism where the two differentially spliced forms of Pnt may operate individually in transcriptional regulation. In mammalian studies there is documented evidence were an ETS protein may function as both an activator and repressor of transcription (Mavrothalassitis and Glysdual 2000). In a second experiment where we looked at a loss-of-function Pnt
assays (pnt$^{\text{Delta78}}$/pnt$^{T6}$; figure 27g) and (pnt$^{\text{Delta88}}$/pnt$^{T6}$; data not shown) reporter expression is up regulated for the eye-specific enhancer (figure 30g). This expression level is increased almost to the level of two copies of our transgene (figure 30a and 30g). Intriguingly, over expression of a constitutively active form of Yan also reduced the level of reporter detection (figure 30f). This active form of Yan has its phosphorylation sites mutated so that it no longer is targeted for degradation through MAPK phosphorylation. These data may indicate a mechanism where RTK and Yan itself can regulate its own transcriptional activity. Consequently, the RTK pathway functions not only in regulating Yan at the protein level but also serves as a means for regulating $yan$ at the transcriptional level.

3.12 Insulator Function in the Organization of the $yan$ Locus

Insulators are a class of regulatory elements that function as barriers between neighboring enhancers and promoter regions. Insulators work by blocking the ability of an enhancer to activate transcription when present between the enhancer and its target promoter. Insulators can block communication between enhancer and promoter, but do not activate or inactivate either the enhancer or promoter. Theses boundary elements have been shown to play an important role in the regulation of locus control regions by confining enhancer elements to specified regulatory domains (Cai and Levine 1995; Scott and Geyer 1995; Geyer 1997; Scott et al. 1999, Bell and Felsenfeld 1999; Dorsett 1999; Sun and Elgin 1999; Udvardy 1999; Bell et al. 2001, Gerasimova and Corces 2001). In our work we have identified a novel insulator that may specify a regulatory domain for the $yan$ gene enhancer region.
The best studied insulator is the gypsy insulator which is a 340-bp retroposon element that contains 12 direct DNA binding repeats for the Suppressor of Hairywing [Su(Hw)] protein. These repeats contain a 12 bp consensus YRTTGCATACCY with a 6 bp invariant core sequence TGCATA (Y = C or T, R = A or G). The Su(Hw) protein is essential for gypsy insulator function. Loss-of-function mutations in Su(Hw) abolish the enhancer blocking activity of the gypsy insulator. It has been shown that Su(Hw) does not act alone and requires Modifier of mdg4 [Mod(mdg4)] to form the complex required for insulator function (Gerasimova et al. 1995, Geyer 1997, Dorsett 1999, Gerasimova and Corces, 2001, Gause et al. 2001, Ghosh et al. 2001).

In our 122 bp yan enhancer we identified an insulator activity and correlate its function to two putative insulator binding sites (TATGCA GAAG TGCATA). These sites contain the core sequence identified for Su(Hw) and are located in the 5’ region of our enhancer (figure 31a). Initially, when trying to isolate a yan eye-specific module we noticed that the A-fragment which ultimately contained the eye-specific enhancer was orientation-dependent and only functioned in the endogenous orientation. When the A-fragment was reversed in transgenic flies reporter expression was lost in embryos, larval CNS and eye discs (data not shown). Because of this orientation dependence all of our subsequent analyses for enhancer elements were done in the endogenous orientation. For the 122 bp eye-specific enhancer the endogenous orientation confers eye-specific expression but upon reversing this orientation (yanOR-lacZ; R = reverse) reporter gene expression is abolished (figure 31c). Knowing this information we made cluster mutations of the two putative Su(Hw) binding sites to determine insulator activity. When we mutated the insulator binding sites in the endogenous orientation reporter expression
Figure 31. Insulator Function Within yan Enhancer
(a) shows enhancer in the endogenous orientation with insulator sites in green. (b) shows enhancer in endogenous orientation with cluster mutations of insulator sites. The mutation in the insulator site are from (TATGCAGAAGTCATA) to (CCGATCGAAGCTGTAC). (c) shows reverse orientation of enhancer with insulator sites between regulatory elements and promoter. In this orientation no activity is detected. In (d) we regained activity when insulator sites were mutated in the reverse orientation.

is still detected (figure 31b). This was positive in that it demonstrated that the mutated sequences do not convey regulatory function for the yan gene as do other elements in this enhancer. To further test insulator activity we reversed the enhancer with the mutated insulator site and were able to detect reporter activity (figure 31d). This provided evidence that we have identified a bona fide insulator.
Our next step was to determine if our insulator was regulated by Su(Hw) or Mod(mdg4). In genetic assays using yanOR-lacZ transgenic flies loss-of-function analysis using Su(Hw) and Mod(mdg4) mutant backgrounds \([Su(Hw)^v/Su(Hw)^f, Su(Hw)^2/Su(Hw)^f, Su(Hw)^{p[w+RPH215]}, Mod(mdg4)^u1}\) showed no genetic interaction could be detected. Our yanOR reporter constructs did not regain reporter activity. If this experiment would have conveyed a genetic interaction this would have been the first instance for an endogenous insulator activity with Su(Hw) in a non-gypsy retroposon manner. From literature searches, in all instances tested, Su(Hw) insulation only occurs with gypsy retroposons containing 12 binding sites for Su(Hw). Four Su(H) binding sites are the minimal number needed to show insulator activity (Scott et al. 1999). Anything less than four has no function. In using the Flyenhancer search program (flyenhancer.org) (Markstein et al. 2002) we found that the Su(Hw) sites do not cluster in a gypsy like fashion within the genome. Fewer than seven cases exist where three or more cluster within a 1 kb stretch even with a more degenerate consensus for Su(Hw) binding (YRYTGCAAYYY). Less than one thousand cases exist where there are three or more binding sites clustered together within 1 kb using just the core sequence (TGCATA). However, there are approximately 7,400 instances where two of these core sites are found together. Our work indicates that these sites may play a significant role in transcriptional regulation. From our data we show that as few as two TGCATA sites are enough to maintain insulator function. What we do not know is what role the orientation and spacing between these sequences plays. We believe that these endogenous insulator sites may prove to be involved in a more global genomic organization of the chromosome. In our analysis our insulator is located between the third and fourth SacI
sites on the yan locus (figure 7). Upstream of this element is the start of another gene. It is then possible to imagine that these insulator sites are preventing the yan enhancer from regulating the upstream gene or vice versa.

From personal communication with the Geyer lab I have learned that they have identified endogenous DNA elements that bind Su(Hw) in vitro but none of these sites show any insulator activity. This information validates our data and underscores that perhaps Su(Hw) is not the protein conveying insulator activity and that any insulator activity it does convey is a by product from its endogenous function. Still, Su(Hw) appears to have an endogenous function because in antibody staining of polytene chromosomes squashes, Su(Hw) is found on over 200 sites.

Insulator activity for our site may be provided by another element that has a chromatin boundary effect. One candidate providing this function is the Boundary Element-Associated Factor (BEAF) which was identified by a cDNA screen looking for elements that bound the scs’ boundary element GCATA from Drosophila melanogaster 87A7 hsp70 locus (Zhao et al. 1995; Hart et al. 1998; Cuvier et al. 1998). In our binding site our sequence shares some characteristics to the insulator binding site GCATA of BEAF. Our element is CGATA. Currently, genetic analysis of BEAF remains to be conducted so little is known about its role in vivo. Recently, from personal communication with C. Hart, in overexpression analysis of a dominate-negative form of BEAF (UAS-BDN) driven by a ubiquitous driver (daughterless-Gal4) embryo and larval lethality were seen. This data differs from that seen with null mutations of Su(Hw) which only have a fertility phenotype. The severity of the BEAF phenotype indicates that in fact it may be playing a greater role than Su(Hw) in preventing communication between
module elements. If insulators are required throughout the genome, loss of this function would cause numerous problems early in development. Therefore, one would expect that mutations of such an element would lead to developmental defects and possibly lethality.
Chapter 4

Conclusion

4.1 Synopsis

During my time here at Penn State my studies have focused on two questions “What promoter and/or enhancer elements are required for eye-specific expression of the yan gene?” and “What is the mechanism by which these elements regulate yan gene expression?” To answer our first question, we undertook a project where we characterized regulatory sequences of the yan locus. To this end, we generated constructs that fuse the bacterial lacZ gene with various Drosophila melanogaster genomic sequences surrounding the yan locus and generated transgenic flies through germline transformation. From this information, we identified module elements with the capacity to regulate yan gene expression throughout Drosophila development. In particular interest to us was the identification of a 122 bp eye-specific enhancer that mimicked endogenous yan expression. Further studies using this eye-specific enhancer identified key regulatory molecules and pathways required for proper yan expression within the developing eye. Significantly, this work demonstrated that the yan enhancer provides a point of interaction for the Notch and RTK signaling pathways.

4.2 Module Regulation of the yan Gene

In this thesis we describe the impact of cis-acting regulatory modules on yan gene expression. We have identified module elements for the yan gene that convey expression
in a temporal and spatial specific pattern during *Drosophila* development. Through *in vivo* analysis, we demonstrate that the genomic region surrounding the *yan* first exon contains regulatory modules sufficient to drive gene expression during embryogenesis, larval CNS and eye imaginal discs.

The complex *yan* pattern seen during embryogenesis is manifested likewise by a complex array of regulatory modules. Although fragments A through E all contain information necessary for *yan* expression in embryos, none are sufficient to fully replicate *yan*’s expression pattern. Different fragments of our genomic analysis communicate different subportions of the overall *yan* expression seen during embryogenesis. In contrast, fragments *B* and *J-lacZ* include all the information necessary for expression within the CNS. For eye development, a minimal module of 122 bp contains all the information necessary for replicating *yan* gene expression. Together this information highlights the high degree of complexity required within and between cis-acting regulatory elements for the proper timing and patterning of *yan* gene transcription.

### 4.3 A Model for *yan* Regulation in the Eye

Knowing that the *yan* module was capable of faithfully replicating *yan* expression in the eye we analyzed this sequence to identify potential DNA binding motifs (figure 32). We identified three Su(H) binding sites (S1, S2 and S3) (blue), two ETS DNA binding motifs (purple), an overlapping N-box and Runt motif (pink and grey respectively), a structural insulator element that provides a boundary function (green) and a repetitive hexamer element (yellow) that acts as a positive regulator.
In this model, we propose that Notch signaling acts in undifferentiated cells to promote yan expression and that Su(H) is directly involved in this process. We also propose that potentially a cofactor that binds the Yan hexamer motifs, Yan hexamer binding protein (YHBP), is involved to promote yan expression. To a lesser extent we also think that Lozenge binding to the Runt domain positively modulates yan expression. In an inhibitory feedback mechanism E(spl) and Yan proteins negatively regulate yan transcription. Upon induction of RTK instructive signaling for differentiation, Yan protein is targeted for degradation (Rebay and Rubin 1995) and from our data Pnt induction blocks yan transcription. Another element of interest is the yan insulator motif that may provide protection from upstream enhancer elements activating yan transcription. In the above model the Su(H) binding sites are in blue, the Yan hexamer sites are in yellow, the Runt domain is grey, the E(spl) protein bind site (N-box motif) is pink, the ETS binding sites are purple and the Insulator binding sites are green.

Based on our findings we have proposed the following mechanistic model for yan expression in undifferentiated cells during eye development. Our data indicates that in the eye yan transcription is turned on by the activation of the Notch pathway. Su(H) with the help of activated Notch (NICD) binds the yan enhancer to turn on transcription. Potentially this activation may use the cooperative efforts of a putative Yan hexamer binding protein (YHBP) and to a lesser extent the Runt domain binding protein Lozenge (Lz) for transcriptional activation. Together Su(H), YHBP and Lz positively regulate yan
transcription. At the same time Su(H) is activating *yan* transcription it is also turning on members of the *E(spl)*-complex in the eye. These genes produce transcription factors that are known to be involved in blocking photoreceptor specification and may interact with the *yan* enhancer to modulate *yan* transcription by binding to the N-box repressor site. With activation of the *yan* transcript, Yan protein is made and is able to prevent differentiation occurring until proper signaling cues via the RTK pathway are detected.

It has been well established that the RTK pathway phosphorylates MAPK which subsequently phosphorylates Yan protein targeting it for degradation. This targeted degradation then allows for other signaling cues to induce differentiation. From our data we show that not only is the RTK pathway targeting Yan protein for degradation but it is also involved in down regulating *yan* transcription. We show that the ETS protein Pnt is able to bind and compete with Su(H) for binding to the S2 site and that Yan protein itself may autoregulate its own transcription. The Notch and RTK pathway data illustrates a mechanism whereby these two major signaling pathways interact in an opposing manner. This is a key point because few examples have been found where these two major pathways interact directly at the transcriptional level. In particular, we provide an example where these two pathways appear to physically compete for the same DNA binding site which has not previously been documented.

Finally we identified a novel insulator element located in the *yan* gene locus. We have tested for insulator activity and all tests indicate an authentic insulator. Through genetic data we know that Su(Hw) and Mod(mdg4) have no effect in the regulation of this element. Future experiments should clarify what regulates this insulator function and hopefully provide significant insight to understanding the role of insulators throughout
the Drosophila genome. Collectively, this thesis, by using the yan gene as our model, highlights the high degree of complexity and constant communication involved for proper regulation of gene expression. Such information can serve to better understand developmental mechanisms for turning on gene expression during developmental processes.
Appendix A

Conserved yan Enhancer Sequence Between *D. melanogaster* and *D. pseudoobscura*
Appendix A

Conserved yan Enhancer Sequence Between D. melanogaster and D. pseudoobscura

With the release of the D. pseudoobscura sequence it has become possible to compare genomic sequences between species to aid in the identification of cis-regulatory regions. With the data we have for the D. melanogaster yan eye-specific enhancer we set out to find its counterpart in D. pseudoobscura. To our delight we find a 91% identity between the two sites:

\[
\begin{align*}
\text{D. melanogaster} & : & \text{atgaaaccgcgaacctttcaccgtcatttgcact-gcgttcttgtggtaaggtgccctcg} \\
\text{D. pseudoobscura} & : & \text{atgaaatctgaaacctttcaccgtcatttgctctggcgttcttgcggtaaaggtgcctctcg}
\end{align*}
\]

The conservation begins right after the S2 site and continues beyond the 122 bp we identified for specifying eye-specific expression (figure 18). It does include information for the S1 binding site, the N-box/Runt domain and the Hexamer sequence all of which we show are essential for yan eye expression. Below is an analysis between the two Drosophila species to identify conservation of cis-regulatory sequences (figure 33). We show a schematic diagram indicating the relative size and positions of exons for yan loci. The analysis for conserved cis-regulatory regions is only presented for the region between the CG15383 gene and the yan first exon.
Figure 33. The yan locus of D. melanogaster and D. pseudoobscura

The yan locus of D. melanogaster and D. pseudoobscura are presented above with the relative size and distance between and within exons (a). A table with actual bp sizes is shown below (b). In (c) we show a schematic diagram of conserved regions between D. melanogaster and D. pseudoobscura for the genomic sequence between CG15383 and the yan first exon. The relative position for each conserved sequence is shown and color coded. Grey regions depict areas with non-conserved sequence. A detail list of the conserved regions is listed in the text. The color for the conserved regions was chosen at random but is repetitive from orange, yellow, purple, green, blue and red.
For the sequence analysis above the corresponding color clusters are listed below with sequence identity.

#1 1st Orange 93% Identity
ggcataaagattcccgagatgtgctgcttcctctct D.p.
ggcattcccgattcccgagatgtgctgcttcctctct D.m.

#2 1st Yellow 97% Identity
tagattatgtaagccagcttggtgctgtgaqtc D.p.
tagattatgtaagccagcttggtgctgtgagtc D.m.

#3 1st Purple 96% Identity
gacggtagcgtgctgtaacaactttt D.p.
gacggtagcgtgctgtaacaactttt D.m.

#4 1st Green 98% Identity
gaggaagttatataatcagacaacatcgcagataatgcctcaatcaat D.p.
gaggaagttatataatcagacaacatcgcagataatgcctcaatcaat D.m.

#5 1st Blue 97% Identity
ggattatggtggtggtggcaatcaggttgggagcatcg D.p.
ggattatggtggtggtggcaatcaggttgggagcatcg D.m.

#6 1st Red 79% Identity
cctgagattccctactaggggtgatcgtgatacgagttac setSupportActionBar
ctgagattccctactaggggtgatcgtgatacgagttac setSupportActionBar
aggatcgtgataac D.p.
aggagctgatcaac D.m.

#7 2nd Orange 100% Identity
aaagtcgataagccatgtgcta D.p.
aaagtcgataagccatgtgcta D.m.

#8 2nd Yellow 100% Identity
gtaattaatccagacagcattttcatttgga D.p.
gtaattaatccagacagcattttcatttgga D.m.
#9 2nd Purple 86% Identity
tctcatttgctgccatttccacgcttcgaaacgttcacattg-tttgtgtg
tttgt-gttctgttttaacgatattcccagaaagctgcatcagatcgag D.p.
tttgtgttctgttttaacgatattcccagaaagctgcatcagatcgag D.m.

#10 2nd Green 100% Identity
gcgattatattaattgataaagatccacattcc D.p.
gcgattatattaattgataaagatccacattcc D.m.

#11 2nd Blue 91% Identity
atgaaatctgaaacctttcaccgtcatttgctctggcgttcttgcggtaaggttgctctcg D.p.
atgaaatctgaaacctttcaccgtcatttgctctggcgttcttgcggtaaggttgctctcg D.m.
acacgcacac D.p.
acacgcacac D.m.

#12 2nd Red 94% Identity
catcccttaagtgcactcttgggttttcataccca D.p.
catcccttaagtgcactcttgggttttcataccca D.m.

#13 3rd Orange 91% Identity
ccttgtctgtgctcactttgctttccctttcataccca D.p.
ccttgtctgtgctcactttgctttccctttcataccca D.m.

#14 3rd Yellow 93% Identity
ttcgatcagtttttccttgcgtgtgcttcttc D.p.
ttcgatcagtttttccttgcgtgtgcttcttc D.m.

#15 3rd Purple 90% Identity
gcgagttttccacggttttctttccccctttttgcaggtgtcatttccatcattcagac D.p.
gcgagttttccacggttttctttccccctttttgcaggtgtcatttccatcattcagac D.m.
#16 3\textsuperscript{rd} Green 90% Identity
tccttaatatgcgcagtggcggcaaaaatcttacaattaccctgttgccakaagaggaa
tgtgtaacaattagccagggac D.p.
tgtgtaacaattagccagggac D.m.

#17 3\textsuperscript{rd} Blue 95% Identity
cgcgtcttggaatacttccgccatcgggagaagactttgtcggg D.p.
cgcgtcttggaatacttccgccatcgggagaagactttgtcggg D.m.

ttgtaacaattagccagggac D.p.
ttgtaacaattagccagggac D.m.

#18 3\textsuperscript{rd} Red 100% Identity
atatagtgcagggcatatatcaaatcgctggc D.p.
atatagtgcagggcatatatcaaatcgctggc D.m.

#19 4\textsuperscript{th} Orange 92% Identity
gaccttgaggcatatcttacttatataatgtgcactttggcatcgaacctaa
aacataagac D.p.
acataaacaac D.m.

tgattggctcaattggacgactatttgaactctgaaccccg-accatgctcatcgcagctc D.p.
tgattggctcaattggacgactatttgaactctgaaccccg-accatgctcatcgcagctc D.m.

#20 4\textsuperscript{th} Yellow 90% Identity
tgattggctcaattggacgactatttgaactctgaaccccg-accatgctcatcgcagctc D.p.
tgattggctcaattggacgactatttgaactctgaaccccg-accatgctcatcgcagctc D.m.

#21 4\textsuperscript{th} Purple 96% Identity
cccgatggcgcagcactgccatattttgactattttgggccaggtgcgtcg D.p.
cccgatggcgcagcactgccatattttgactattttgggccaggtgcgtcg D.m.

#22 4\textsuperscript{th} Green 96% Identity
gccgtttatggcatttgactggaatgtcaac D.p.
gccgtttatggcatttgactggaatgtcaac D.m.

#23 4\textsuperscript{th} Blue 100% Identity
caaaagggagtgcagtgcactgacacgcatcttt D.p.
caaaagggagtgcagtgcactgacacgcatcttt D.m.
From the sequence information above, we were able to identify 27 clusters of high sequence identity. Sequence alignment was done, using the NCBI BLAST 2 sequence program, a pairwise alignment tool. Using this pairwise blast, no significant similarity was found for sequences between conserved clusters. With this sequence similarity information we were able to correlate functional module information with cluster conservation (figure 34 and 35). In figure 34 the A, G and J fragment all drive expression in embryos, LPCs and in the eye disc. For the J fragment the embryo expression is a subset of the expression seen with either A or G. For fragments F, H and I expression of lacZ reporter gene is not detected in a yan like pattern. For fragment J it contains information encoded in the 2nd blue, 2nd red, 3rd orange and 3rd yellow clusters (figure 34 and 35). These clusters must then contain the information necessary for
Figure 34. Correspondence between functional yan locus module elements and conserved sequences identified from comparative analysis between D. melanogaster and D. pseudoobscura.

A schematic diagram for the D. melanogaster yan locus is shown in (a) with the following corresponding restriction enzyme digest sites: Bm=BamHI, RI=EcoRI and S=SacI. In (b) we show module elements with the corresponding conserved sequence. The legend provided gives information to corresponding tissue expression.
Figure 35. Continued correspondence between functional yan locus module elements and conserved sequences identified from comparative analysis between D. melanogaster and D. pseudoobscura. A schematic diagram for the D. melanogaster yan locus is shown in (a) with the following corresponding restriction enzyme digest sites: Bm=BamHI, RI=EcoRI and S=SacI. In (b) we show module elements with the corresponding conserved sequence. The legend provided gives information to corresponding tissue expression.
expression in the eye and in the LPCs of the brain as well as some embryo expression.

Fragments L and O are eye-specific and only include information located in the 2\textsuperscript{nd} blue cluster (figure 35). No other expression is seen with these two fragments. Fragment L contains the entire 2\textsuperscript{nd} blue cluster while O contains the majority of the cluster and is still able to drive expression. For fragments K, M, N or P expression was not seen in embryos or LPCs. This indicates that these fragments alone are not sufficient to drive reporter expression. For fragment B it is able to drive expression within embryos, in cells within the brain and ventral ganglion and glial cells in the eye. From this information, we can say that for some expression, as in the eye, is contained in a single conserved sequence cluster. For other expressions, as in the LPCs, expression may be regulated by multiple clusters. As for the complex expression in embryos it may be regulated by combination of multiple elements. We know that fragments A, B, C, D and E all contain information for expression in embryos and that each of these fragments alone can not completely replicate endogenous \emph{yan} embryo expression. As for similarity in spacing between conserved clusters, I can only guess that they may have a function, somehow necessary for the regulation of the conserved sequences or that the evolution time between these two species is not so great, that distances between regulatory elements is not drastically changed. What can definitively be said is that the unraveling of \emph{cis}-regulation elements is far from trivial and studies identifying regulatory elements have just begun to take hold. The \emph{D. melanogaster} sequence was obtained from the genome annotation release 3. The sequence for \emph{D. pseudoobscura} is from the whole genome assembly February 02, 2003.
Appendix B

Notch Activation of yan Expression is Antagonized by RTK/Pointed Signaling in the Drosophila Eye
Notch Activation of yan Expression Is Antagonized by RTK/Pointed Signaling in the Drosophila Eye

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Summary

Receptor tyrosine kinase (RTK) signaling plays an instructive role in cell fate decisions, whereas Notch signaling is often involved in restricting cellular competence for differentiation. Genetic interactions between these two evolutionarily conserved pathways have been extensively documented. The underlying molecular mechanisms, however, are not well understood. Here, we show that Yan, an Ets transcriptional repressor that blocks cellular potential for specification and differentiation [1, 2], is a target of Notch signaling during Drosophila eye development. The Suppressor of Hairless (Su(H)) protein of the Notch pathway is required for activating yan expression, and Su(H) binds directly to an eye-specific yan enhancer in vitro. In contrast, yan expression is repressed by Pointed (Pnt), which is a key component of the RTK pathway. Pnt binds specifically to the yan enhancer and competes with Su(H) for DNA binding. This competition illustrates a potential mechanism for RTK and Notch signals to oppose each other. Thus, yan serves as a common target of Notch/Su(H) and RTK/Pointed signaling pathways during cell fate specification.

Results and Discussion

Notch-mediated lateral inhibition blocks cellular competence for differentiation [3, 4]. The Ets protein Yan plays a similar role in the Drosophila eye [1, 2]. In a sensitized assay, the eye phenotypes of yan mutants can be dominantly enhanced by the reduction of a key component of the Notch pathway, Su(H). yan homozygotes exhibited 20% mutant ommatidia [1], while yan/yian; Su(H)013/013 flies had 44% (n = 380) mutant ommatidia. This result suggests that the yan function or expression might require the Su(H) activity. To test a hypothesis that yan is a downstream target of Notch signaling, the level of Yan protein was examined in eye discs mutant for Su(H). Normally, Yan is highly expressed in all undifferentiated cells posterior to the morphogenetic furrow (MF) (Figures 1A–1C). In the absence of Su(H) function, Yan expression was greatly reduced or eliminated (Figures 1D–1I). A similar phenotype was also observed in N0 eye discs (Figures 1J and 1K). Thus, Notch/Su(H) signaling is required for yan expression.

To investigate how yan expression is regulated, DNA fragments comprising a 20-kb genomic sequence surrounding the first exon of yan were tested for regulatory potential in corresponding transgenic flies (M.P, E.R., and Z.-C.L., unpublished data). Through this approach, a 122-bp eye-specific enhancer located approximately 3.5 kb upstream of the first exon was identified (Figure 1M) [1]. In eight out of nine transgenic lines, this enhancer activated expression of a bacterial lacZ reporter gene within posterior undifferentiated cells of eye discs. This recapitulates the endogenous yan gene expression in eye discs with the exception of the MF region (Figure 2A) [1]. The enhancer was unable to drive the reporter expression in the morphogenetic furrow. Moreover, the reporter gene expression was not detected in other larval tissues or embryos (data not shown). Three putative Su(H) binding sites were found in the yan enhancer (Figure 1M). When tested through an in vitro electrophoretic mobility shift assay (EMSA), the Su(H) protein was shown to specifically bind to these sequences (Figure 1N). Further, the yan enhancer became inactive in most of the posterior undifferentiated retinal cells when the Su(H) function was removed (Figure 2B). It is unclear at the moment why two small patches of cells located in the dorsal and ventral margins were still positive for the reporter expression (Figure 2B) but the endogenous yan gene was inactive throughout the eye disc in this same Su(H) mutant background (Figure 1E). All together, these loss-of-function and DNA binding analyses support the notion that Su(H) is required to promote yan transcription and that yan is a target gene of Su(H) in the eye.

To test the functional significance of the Su(H) binding sites, clustered point mutations were introduced through site-directed mutagenesis. Mutations in S1 (six out of seven transgenic lines) or S2 (all nine lines) abolished the enhancer function (Figures 2C and 2D), while mutations in S3 (five out of six lines) had little effect (Figure 2E). These results indicate that the Su(H) binding sites S1 and S2 are essential for the yan enhancer function while the S3 site plays a minor role.

Overexpression analysis was carried out to further address how Su(H) is involved in regulating yan expression. When Su(H) was overexpressed in heat-treated hs-Su(H) eye discs, the yan enhancer was downregulated, contrary to expected results based on Su(H) loss-of-function analysis (data not shown). This effect might be simply caused by the transcriptional repressor activity of Su(H) [4, 5]. However, the following evidence indicates that the repression of the yan enhancer may not be directly mediated by Su(H). First, overexpression of a fusion protein Su(H)GFP, which is a transcriptional activator, also resulted in repression of the yan enhancer in GMR-Gal4/UAS-Su(H)GFP eye discs (data not shown). Second, similar results were observed in GMR-Gal4/UAS-Nras or heat-treated hs-Nras eye discs (data not shown). In the presence of Nras, Su(H) can be converted from a repressor to an activator of transcription [4, 5].

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Figure 1. Notch/Su(H) Signaling Is Required for yan Expression

(A–F) (A–C) Wild-type and (D–F) Su(H)$^{AR9}$/Su(H)$^{SF8}$ eye discs were stained with Elav, a neural-specific protein [32] (red, [A] and [D]), and Yan (green, [B] and [E]) antibodies. (C) was merged from (A) and (B), and (F) was merged from (D) and (E).

(G–I) A Su(H)$^{PCT}$ clone produced in Su(H)$^{PCT}$/H11001 flies. The lack of GFP staining (G) defines the boundary of the clone, within which Yan expression (red) is eliminated (H). (I) was merged from (G) and (H).

(J–L) Eye discs derived from (J) wild-type, (K) N$^{ts}$ (3rd instar eye discs targets of N signaling, could be responsible for repress-were treated at 32°C/H11034 for 7 hr before being used for antibody staining), and (L) GMR-Gal4/UAS-PntP1 were stained with Yan antibodies.

(M) The sequence of the yan enhancer was marked by S1-3 [Su(H)] binding sites, an Ets binding site (Ets), a basic helix-loop-helix protein binding site (N box), and an Lz/Runt binding site.

(N) The mobility-shift assay shows that Su(H) specifically binds to S1, S2, and S3. The GST-Su(H) protein [33] was used in the assay, which was carried out as described [34].

Figure 2. Regulation of the Eye-Specific yan Enhancer

(A and B) Expression of yanO-lacZ examined in (A) wild-type and (B) Su(H)$^{AR9}$/Su(H)$^{SF8}$ eye discs.

(C) mS1 mutant version of yanO-lacZ.

(D) mS2 mutant version of yanO-lacZ.

(E) mS3 mutant version of yanO-lacZ.

(F) mHEX mutant version of yanO-lacZ.

(G) yanQ-lacZ.

(H) yan6Q-lacZ.

(I) mN/RBS mutant version of yanO-lacZ.

(J–L) Expression of yanO-lacZ examined in (J) Iz$^{ts}$/Y, (K) GMR-Gal4/UAS-E(spl)m7 UAS-E(spl)m8, and (L) GMR-Gal4/UAS-yan$^{PCT}$ eye discs.

One copy of yanO-lacZ was used in (A), (B), (J), and (K). A 22-bp sequence derived from yanO (yanQ) was used in (G) and (H). yan6Q contains six copies of the yanQ sequence. Two copies of the transgene were present in eye discs shown in (C)–(I). Arrows indicate the morphogenetic furrow, and anterior is oriented toward the left in all panels.

One explanation for the discrepancy between N/Su(H) loss-of-function and gain-of-function results is that Enhancer of split (E[spl]) genes, which are downstream targets of N signaling, could be responsible for repress-
transcriptional repressors. Secondly, it is also possible that N^{ins} and Su(H) might require a cofactor for yan activation, and the availability of this factor is limited in eye discs. Evidence indicates that Su(H) binding sites alone can play a very limited role in regulating transcription in the absence of other factors [6]. Thirdly, yan overexpression experiments indicate that Yan is able to negatively regulate its own transcription. Taken together, these results suggest that regulation of the yan enhancer involves only an activating but not repressing function for Su(H). When N^{ins} or Su(H)VP16 was overexpressed in eye discs, the Yan protein level was not apparently changed (data not shown). Again, this could be due to a balanced effect of N^{ins}/Su(H) and E(spl) proteins, which play opposite roles to regulate yan transcription.

E(spl) proteins are basic helix-loop-helix (bHLH) repressors, and most of them (m7, m8, m9, m10, and m11) are expressed in the posterior undifferentiated cells in eye discs [7, 8]. When E(spl) proteins (e.g., m7 and m8) were overproduced in eye discs, the yan enhancer activity was strongly reduced (Figure 2K). Similarly, the level of Yan protein was also reduced (data not shown). These results show that yan expression can be negatively regulated by E(spl) proteins. E(spl) proteins might act through an N box (5'-CACAAG-3') [9] in the enhancer (Figure 1M). Interestingly, mutations of the N box didn’t cause upregulation of the reporter gene, but, instead, the reporter expression was abolished in all three transgenic lines (Figure 2l). One explanation for this result is that the N box sequence might be shared by an activation element located in the region. Indeed, a Runt domain binding site (RBS) (5'-RACCRCA-3', R = purine) [10] overlaps with the N box (Figure 1M), which could mediate an effect by the Runt domain protein Lozenge (Lz), which has previously been shown to act as a transcriptional activator in the developing eye [11]. Supporting this idea, the yan enhancer was completely inactivated in lz^{iz} mutant eye discs (Figure 2J). However, the level of Yan protein was not apparently affected by the lz mutation (data not shown). This result suggests that Lz is not essential for the expression of the endogenous yan gene and that the loss of lz function could be compensated by other molecules so that yan expression is unaffected in lz mutants.

A candidate factor that may be involved in this compensation and could cooperate with N^{ins}/Su(H) proteins might be a DNA binding protein capable of interacting with a 5'-GAAACC/A-3' sequence. Two direct repeats of a 5'-GAAACC-3' sequence (hexamer, HEX) were found between S1 and S2 (Figure 1M). The second half of the S2 site might be considered as a third HEX, since there is only one variant base (as 5'-GAAACA-3'). When clustered point mutations were introduced into the first and second HEX, expression of the reporter gene was abolished in all six transgenic lines (Figure 2F). Therefore, the HEX element is essential for the yan enhancer activity. Expression analysis of the HEX repeats provided further evidence supporting the finding that the hexamer is an activation element. The reporter gene expression can be detected over the entire eye disc of all six lines when a six-copy concatemer of a 22-bp sequence containing three HEX repeats was used (Figure 2H); although, one copy of this 22-bp oligonucleotide was not sufficient to

Figure 3. RTK Signaling Blocks yan Expression

One copy of yanO-lacZ was present in (A) and (C)–(F), and two copies were present in (B). GMR-Gal4 was used as a driver. (A and B) Wild-type. (C) UAS-to \^{\mu}_{2}\-DER. (D) UAS-Ras^{1\mu}_{3}. (E) UAS-pntP1 (but UAS-pntP2 did not cause any apparent effect, data not shown). (F) pnt^{iz}/pnt^{iz} (the same effect was also observed in the pnt^{iz}/pnt^{iz} eye disc, data not shown). (G) yanS1, yanS2, and yanS3 were labeled and used as probes for binding with GST-YanC [1] and GST-PntC [35] fusion proteins. (H) The yanS2 probe was used for binding with GST-Su(H) and GST-PntC proteins. Arrows indicate the morphogenetic furrow, and anterior is oriented toward the left in panels (A)–(F).
induce gene expression in eye discs (five out of six lines) (Figure 2G). We propose that a putative HEX binding protein functions together with Su(H) and N\textsuperscript{Mito} to activate the yan enhancer. The nature of the HEX binding factor remains to be investigated.

An Ets domain binding site (EBS, 5'-GGAA/T-3') [12] was found within the S2 site (Figure 1M). As Yan is an Ets domain protein and a transcriptional repressor, we examined if Yan could be involved in autoregulation. When a constitutively activated Yan (Yan\textsuperscript{Act}) [2] was overproduced in eye discs, the reporter gene expression was strongly reduced (Figure 2L). As Yan is capable of negatively regulating yan transcription, this autoinhibitory mechanism could be used to prevent overproduction of Yan in undifferentiated cells. DNA binding data suggests that Yan can be directly involved in this negative regulation (Figure 3G). However, this Yan-mediated autoinhibitory feedback appears to play a minor role in regulating yan expression, because the yan enhancer activity was not apparently affected in yan mutant clones produced in eye discs (data not shown).

A role for RTK signaling in regulating yan transcription was investigated. When the RTK pathway was constitutively activated by tor\textsuperscript{D} or Ras V12, the yan enhancer activity was greatly reduced (Figures 3C and 3D). Thus, RTK signaling appears to negatively regulate yan transcription, in addition to its effect on Yan protein stability [13, 14]. The following evidence supports a view that the inhibitory effect of RTK/Ras signaling on yan expression is mediated through the pointed (pnt) gene. First, overexpression of pnt caused a reduction in the yan gene activity (Figures 1L and 3E). Second, loss of pnt function resulted in an upregulation of the yan enhancer (Figure 3F). Third, the yan enhancer activity was dominantly suppressed by the reduction of pnt function in a sensitized assay. There were only about 1.3% (n = 1516) mutant ommatidia in the eyes of yan\textsuperscript{+/+}, pnt\textsuperscript{+/+}/ files. In comparison, 20% of the ommatidial structures were abnormal in yan\textsuperscript{hid} homozygotes [1]. Finally, gel retardation analysis demonstrated that Pnt, also an Ets domain protein, specifically binds to the S2 site but also in some Yan expression was reduced not only in ungutated eyen mutant clones produced in eye discs (data not shown).

In this model, we propose that Notch signaling acts in undifferentiated cells to prevent yan expression and that Su(H) is directly involved in this process. Both E(spil) and Yan can negatively regulate yan transcription, which provides an inhibitory feedback mechanism. Once inductive RTK signals are available to initiate cellular differentiation, the Ets domain protein Pnt blocks yan transcription, while activated MAP kinase targets the Yan protein for phosphorylation and degradation [13, 14]. Therefore, opposition between Notch and RTK signals can be mediated at the level of enhancer through action of nuclear factors such as Su(H) and Pnt.

Figure 4. A Model for Crosstalk between Notch and RTK Signaling Pathways

In this model, we propose that Notch signaling acts in undifferentiated cells to promote yan expression and that Su(H) is directly involved in this process. Both E(spil) and Yan can negatively regulate yan transcription, which provides an inhibitory feedback mechanism. Once inductive RTK signals are available to initiate cellular differentiation, the Ets domain protein Pnt blocks yan transcription, while activated MAP kinase targets the Yan protein for phosphorylation and degradation [13, 14]. Therefore, opposition between Notch and RTK signals can be mediated at the level of enhancer through action of nuclear factors such as Su(H) and Pnt.

The nesting of an Ets binding site within the S2 site suggests a possible mechanism whereby the binding of Pnt could interfere with Su(H)'s DNA binding activity. Indeed, increasing the amount of Pnt effectively prevented Su(H) from DNA binding (Figure 3H). Such competition provides a mechanism by which RTK/Pnt signaling directly antagonizes Notch-mediated lateral inhibition at the transcriptional level. As Ets binding sites are nested in many Su(H) binding sites, competitive occupancy of the common sequence could be a general mechanism for regulating expression of genes targeted by both Notch and RTK pathways.

We propose that spatially restricted yan expression in the developing eye is coordinated by actions of multiple regulatory factors that include Su(H) and Pnt (Figure 4). Consequently, the yan enhancer provides an interface for Notch and RTK signals to oppose each other. Our DNA binding analysis and mutagenesis of yan enhancer sites provide evidence that supports a cell-autonomous role of Notch and RTK signaling in the regulation of yan expression. Interestingly, we observed that Yan expression was reduced not only in Su(H)\textsuperscript{hid} clones but also in some Su(H)\textsuperscript{+/} cells that surround the mutant clones in eyen discs (Figures 1G–1I). This result implies that loss-of-Su(H) function might also cause a cell-non-autonomous effect on yan expression, possibly due to upregulation of RTK signaling in those Su(H)\textsuperscript{+/} cells. This upregulation may occur via an increase of a diffusible activator of the RTK pathway due to the loss of Su(H). In Caenorhabditis elegans, the RTK pathway is negatively regulated by Notch signaling through transcriptional activation of a MAPK (mitogen-activated protein kinase)-phosphatase LIP-1 [18]. Interestingly, cooperation between Notch and RTK signals can also be mediated at the enhancer level [11]. The model presented here illustrates a mechanism that should help explain how progenitor cells are maintained in an undifferentiated state by Notch-mediated inhibitory signals and how they can be effectively induced for cellular differentiation by RTK-mediated inductive signals.

Experimental Procedures

Molecular Analysis and Germline Transformation

Standard methods for DNA analysis were used [19]. Point mutations were generated by polymerase chain reaction (PCR) and the Quick-
Genetics and Immunocytochemistry

Mutant strains used in this study include Su(H)neo and Su(H)res (M. Ashburner); Su(H)res [22]; N (The Bloomington Drosophila Stock Center); I(3) [23]; pnt1/2, pnt3, and pnt6 [13, 14]; hs-N (N. Baker); hs-Su(H) and UAS-N (J. Treisman); UAS-Su(H)res [24]; UAS-E(spl)m7 and UAS-E(spl)m8 [25]; UAS-yanres [2]; UAS-torΔ-DER [26]; UAS-Ras110 [27]; UAS-pnp1 and UAS-pnp2 [28]; and GMER-Gal4-4 [29]. The Gal4/UAS system was used for overexpression analysis [30]. Clonal analysis was done as described [31]. Rat anti-Elav (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-Yan [1], and rabbit anti-GFP (Santa Cruz Biotechnology) antibodies were used for immunostaining. Texas red anti-rat and anti-mouse, fluorescein anti-rabbit and anti-mouse, and biotinylated anti-mouse IgG were the secondary antibodies (Vector Laboratories). Images were collected on a Bio-Rad MRC-1024 Confocal Laser Scanning System. The I-galactosidase activities were monitored by X-gal staining, which was done at 37°C for 15 hr, except for the experiments depicted in Figure 2H (37°C, 4 hr) and Figure 3F (37°C, 5 hr).

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References

Appendix C

Identifying Functional Cis-Acting Regulatory Modules of the yan Gene in

_Drosophila melanogaster_
SHORT COMMUNICATION

Edward Ramos · Mitch Price · Margaret Rohrbaugh · Zhi-Chun Lai

Identifying functional cis-acting regulatory modules of the yan gene in Drosophila melanogaster

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Abstract Yan is a nuclear DNA-binding protein that acts as a general inhibitor of cellular differentiation and proliferation in Drosophila melanogaster. The genetic and biochemical mechanisms required for regulating Yan protein function are well understood, however, the molecular mechanism of yan gene transcriptional regulation has not been fully elucidated. Here we show that the dynamic expression of the yan gene is specified by distinct spatial and temporal cis-acting regulatory elements in embryos and larval tissues. Each of these distinct elements is thus capable of replicating vital aspects of endogenous yan gene expression.

Keywords Drosophila melanogaster · yan · Regulatory modules · Enhancer

Introduction

Genes contain predominantly two types of DNA sequences, those sequences that are transcribed and those required for the instruction of transcription. Throughout development the transcriptional regulatory regions within the genomic DNA carry out their function by activating or inactivating transcription in a time-, space- and sometimes lineage-dependent manner. In general, studies of cis-regulatory regions show that separate cis-regulatory subelements or modules carry out different parts of the overall regulatory function (review in Arnone and Davidson 1997). Studies from these modules have given some insight into a better understanding of the structural organization presented in the noncoding regions of eukaryotic genomes. Still, the regulatory elements from relatively few genes have been studied despite their vital role in the developmental process. To address this issue, we report characterization of cis-acting elements that are responsible for driving Drosophila melanogaster yan gene expression in the developing embryo and imaginal tissues.

The yan gene encodes a member of the E twenty six (ETS) DNA-binding family of proteins composed of a broad array of molecules involved in many diverse processes including proliferation, differentiation, transformation and apoptosis (Yordy and Muise-Helmericks 2000). Yan protein is known to be a general inhibitor of differentiation and proliferation in embryo and larval tissues. Studies have shown that the subcellular nuclear localization and stability of the Yan protein are regulated through phosphorylation by Ras-mediated mitogen-activated protein kinase (MAPK) signaling (reviewed in Schweitzer and Shilo 1997; Treisman and Heberlein 1998; Hsu and Schulz 2000; Rebay 2002). In the above studies the major regulatory components of the Yan protein are detailed but they do not address the transcriptional regulatory elements of the yan gene. For this study, genomic fragments encompassing a 20-kb region surrounding the first exon of the yan locus were tested for their potential to drive expression of a lacZ reporter gene in transgenic flies. We show that specific sections of this 20-kb region are sufficient to drive expression of the lacZ reporter in a spatial and temporal pattern similar to wild-type yan expression.

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Materials and methods

Drosophila stocks

All stocks were maintained on standard yeast-agar medium at 18 or 25°C.

Generation of reporter constructs

Genomic DNA encompassing the 5' yan locus was subcloned from λfix genomic clones (Fig. 1a; Lai and Rubin 1992). Constructs were cloned into the pCaspERE-AUG-βgal, pwnβE (vector containing a nuclear localization signal) or pBluescript KSII+ vectors (Stratagene). Constructs K to P were generated by PCR and standard molecular techniques (Fig. 1b). DNA isolated from positive clones was examined for proper orientation by either restriction enzyme digests and/or by DNA sequencing. All P[ LacZ, w+] transgenes were introduced into w1118 and/or w; Drf/TM3, SbΔ2–3 embryos as described in Rubin and Spradling (1982) and mapped to chromosomes following standard protocols. For each reporter construct multiple (at least three) independent insertions were obtained. Further information about construct design can be supplied upon request.

Immunocytochemistry and histology

Embryos were fixed and stained using a mouse anti-β-galactosidase antibody (Price and Lai 1999). Eye discs from third instar larvae were immunostained with mouse α-Yan antibody (1:50 dilution) fixed with 4% formaldehyde as described by Wolff (2000). In both embryo and eye tissues primary antibodies were detected using a biotinylated goat anti-mouse antibody (1:200) and Vecta staining. Staining to biotinylated goat anti-mouse antibody (1:200) and Vecta staining embryo and eye tissues primary antibodies were detected using a fixed with 4% formaldehyde as described by Wolff (2000). In both eye discs from third instar larvae were immunostained with mouse α-Yan antibody (1:50 dilution) fixed with 4% formaldehyde as described by Wolff (2000). In both embryo and eye tissues primary antibodies were detected using a biotinylated goat anti-mouse antibody (1:200) and Vecta staining.

Results and discussion

The yan gene plays a critical role in regulating proliferation and differentiation throughout development (reviewed in Schweitzer and Shilo 1997; Treisman and Heberlein 1998; Hsu and Schulz 2000). It is detected in ovaries and throughout embryogenesis. Previous studies during larval development have demonstrated that Yan is only expressed in the CNS and developing eye. It is not detected in any other larval tissues (Lai and Rubin 1992). Thus, yan’s expression pattern shows a high degree of complexity and specificity that correlates well with Yan function. To better understand how these complex expression patterns are generated a transgenic approach was undertaken to identify the cis-regulatory elements or modules necessary for yan expression. Through this approach, we identified key module elements capable of instructing gene expression in a manner that mimics the endogenous yan gene. Figure 1a depicts a schematic diagram representative of the yan locus and Fig. 1b shows

In situ hybridization and whole-mount immunostaining

Yan antibody and β-gal RNA double staining was done as described by Cadigan et al. (1998). Yan antibody was used at the same concentration as in the previous section. LacZ DNA to be transcribed by RNA polymerases was made by using the Lig’n Scribe No-Cloning Promoter Addition Kit (Ambion). Digoxigenin-labeled single stranded RNA probe was used for hybridization. Binding of the probe was detected using an alkaline phosphatase-(AP) conjugated anti-Dig-AP Fab fragment antibody (1:200) and color visualized using NBT/BCIP color detection (Roche Molecular Biochemicals). Images were photographed on a Zeiss Axiophot compound microscope.

Fig. 1a, b A schematic diagram of the Drosophila melanogaster yan locus and genomic fragments used in making lacZ reporter constructs. a A restriction map of the genomic yan locus showing BamH I (Bm), EcoRI (R), SacI (S), and XhoI (X) restriction enzyme sites. The positions of exons in the yan locus are represented by boxes with the coding region in black. Also, in black is the predicted gene CG15383 of unknown function located upstream of the yan locus. Arrows indicate direction of gene transcription. As a marker for yan gene expression, the yan enhancer trap line was used. This line contains a P-element insertion in the first intron and expresses β-galactosidase (β-gal) in tissues and stages in which Yan protein and mRNA are detected. b Genomic fragments used to make lacZ reporter constructs. Multiple independent transgenic lines for each construct were generated through germ line transformation. Solid black lines (A, B, C, D, E, G, J, L and O) denote fragments that drive lacZ expression while gray lines (F, H, I, K, M, N, and P) do not drive any expression in the embryos or larval tissues examined.
the area represented by the genomic fragments used in making the lacZ reporter constructs. Each filled box (black) represents a module region that mimics, in part, the yan gene expression pattern throughout fly development. As can be seen in Fig. 1b the transcriptional regulation of the yan gene requires a broad range of genomic information spanning over 20 kb. However, we will also demonstrate that although complete yan expression requires a large section of genomic information smaller fragments are capable of regulating individual, temporal and spatial components of yan’s expression pattern.

**Fig. 2a–o** yan-lacZ expression in the developing eye and central nervous system (CNS). a–j Anterior is to the left and the red arrow demarcates the morphogenetic furrow (MF). a–f β-Gal detection in the eye discs. a β-Gal detection for the yanP line; b–f detection for different transgenic lines. g–h’ Double-labeled eye discs with anti-yan antibody (brown) and β-gal (blue) show a delay of β-gal protein expression based on activity. Inserts (g’, h’) are enlargements of boxed regions in g and h. i, j Antibody/in situ double staining for Yan protein (brown) and β-gal transcript (purple) within and posterior to the MF. i A control with the lacZ sense probe; j Co-localization with the lacZ antisense probe. k–o β-gal detection in the third instar larval CNS. k yanP drives β-gal expression in the laminar precursor cells (LPCs) of the optic lobes in the CNS. In addition to expression in LPCs, yanP expression is seen in a number of cells throughout the brain and ventral ganglion (k). A-lacZ (l) drives expression in the LPCs of the optic lobes while B-lacZ (m) drives transcription in distinct cells within the optic lobes and ventral ganglion. G- and J-lacZ (n, o) both show LPC expression similar to A-lacZ.

In the developing third instar eye, Yan protein is expressed in all undifferentiating cells within and posterior to the morphogenetic furrow (MF). Its role in these cells is to maintain their undifferentiated state until the proper signaling, via the RTK pathway, induces them to differentiate into photoreceptors or other cells of the ommatidium. In our initial analysis of the large yan genomic fragments A to E, only A and B are able to drive gene expression in the eye imaginal discs. As a control for β-gal expression we used yanP, an enhancer trap line within intron 1 of the yan gene (Figs. 1a, 2a), that has been demonstrated to recapitulate yan gene endogenous expression (Lai and Rubin 1992). Fragment A drives a pattern similar to yanP expression (Fig. 2a, b) while B drives expression in subretinal cells likely corresponding...
to glial cells (Fig. 2c). This glial cell expression has not been detected in previous experiments looking for Yan protein (Lai and Rubin 1992; Price and Lai 1999). Therefore, it is possible that fragment B lacks a repressor element normally present in the surrounding genomic DNA.

Analysis of the A fragment demonstrated that increasingly smaller fractions of this DNA are sufficient to drive lacZ expression within the eye (G-, J- and L-lacZ; Fig. 2d, e, and data not shown). A minimum regulatory sequence, O-lacZ (Fig. 2f), consisting of 122 bp of the original A fragment was able to drive gene expression in the larval eye disc (Rohrbaugh et. al. 2002). Also, O-lacZ was eye-specific driving expression in no other tissue (data not shown). To determine if Yan protein and reporter expression were overlapping we double-labeled eye discs with an anti-yan antibody (brown) followed by a β-gal detection assay (blue). Again, yanp was used as a control. As shown in Fig. 2, a low level of β-gal activity for yanp is detected within cells in the MF and a high level in cells posterior to it (Fig. 2g, g’). Similar results were seen in a double-labeling experiment using anti-Yan and anti-β-gal antibodies (data not shown). Fragments A-, G-, J-, L- and O-lacZ (only O shown; Fig. 2h, h') also show limited activity within the MF. However, all drive reporter expression in all Yan-positive cells posterior to the MF.

To directly address whether the yan-O fragment is sufficient to drive transcription within the MF we looked at lacZ transcript by in situ analysis. Figure 2j shows a double-labeling of Yan antibody and lacZ mRNA while Fig. 2i shows a negative control for lacZ mRNA detection. As can be seen, lacZ transcript is present within the MF in a pattern corresponding to Yan protein. This result is in contrast to Fig. 2h' which indicates that the majority of β-gal protein expression is posterior to the MF. Combining the results of these two experiments demonstrates a delay between the detection of lacZ transcript and β-gal protein expression. This may be due to a delay in translation of the lacZ mRNA or a need for an accumulation of the β-gal protein to sufficient detectable levels. Thus, this data demonstrates that the yan-O fragment is sufficient to regulate the complete yan expression pattern in the eye-imaginal disc within and posterior to the MF. This is contrary to our previous report based on β-gal activity and Yan protein expression that indicated that the yan-O fragment was insufficient to drive expression within the MF (Rohrbaugh et. al. 2002).

yan cis-acting regulatory modules in the larval CNS

In the larval CNS protein data demonstrates yan expression in the laminar precursor cells (LPCs) of the optic lobe (Price and Lai 1999), which suggests a role for yan in CNS development. However, Yan protein function at this stage has yet to be defined. In contrast, yanp is detected in the LPCs and in various cells throughout the brain and ventral ganglion (Fig. 2k). In our reporter analysis expression in the brain seems to be very compartmental in its organization. Fragments A (Fig. 2l) and B (Fig. 2m) together completely regenerate yanp expression. Fragment A expression mimics Yan protein in LPCs while fragment B drives expression in the ventral ganglion and in cells throughout the brain similar to yanp.

Due to the similarity of fragment A and Yan expression data, further analysis of the A fragment was conducted. A smaller fragment, J-lacZ (Fig. 2o), was capable of recapitulating A fragment expression. When the J-lacZ fragment was divided even further expression in the LPCs was eliminated. The smaller fragments derived from J were not able to mimic even a portion of the J-lacZ expression within the CNS. Therefore, the 800-bp J-lacZ fragment is the smallest module able to replicate Yan expression within the CNS. Together, regulatory sequences from J and B fragments appear to be sufficient to drive gene expression similar to that of the endogenous yan gene.

Complex yan cis-acting regulatory modules in embryos

Previously, yan mRNA has been detected in embryos as early as stage 5 in the neurogenic region and dorsal ectoderm. During gastrulation (stage 6), yan mRNA and protein expression become restricted to the head region and ectoderm layer, and are absent along the ventral midline (Gabay et al. 1996; Price and Lai 1999). Later in embryonic development (stage 11), Yan protein is detected in cells near the tracheal pits and in cells close to the ventral midline in the epidermal layer. Finally, in late embryonic development yan expression becomes confined to head and midline developing regions (Rogge et al. 1995; Scholz et al. 1997; Dumstrei et al. 1998; Price and Lai 1999).

In our analysis, β-gal expression is first seen during germ band elongation. In stage-10 embryos, A-lacZ (Fig. 3c) drives expression in the ventral ectoderm and head in a manner resembling Yan protein pattern (Fig. 3a; Price and Lai 1999). Nevertheless, some differences in expression are obvious. A-lacZ drives strong expression in cells close to the midline where yanp (Fig. 3b) and Yan protein expression are not detected. This aberrant expression is possibly due to the absence of an inhibitory or repression element positioned outside of A that may regulate yan expression in midline cells. This element might prevent or minimize yan transcription in cells near the midline under normal developmental conditions thus precluding detectable expression.

Other fragments that drive expression during stage 10 are D- and E-lacZ located within the first intron (Fig. 1). D-lacZ drives a fairly general pattern of expression in the ventral ectoderm (Fig. 3d) and head while E-lacZ drives expression in lateral stripes in the ectoderm and at low levels in the head (Fig. 3e). Interestingly, cells positive for A-lacZ and E-lacZ expression in stage 9–10 embryos together regenerate a pattern in the ventral ectoderm similar to Yan protein staining (data not shown).
Fig. 3a–w  Complex embryonic reporter gene expression from constructs A to E. a, f, l, r, u Stage-specific anti-yan antibody detection for embryos. a–e A ventral view of stage-10 embryos. f–q A dorsolateral view of stage-11 and 13 embryos are shown. a–e A-, D-, and E-lacZ stage-10 embryos drive β-gal expression in a subset of the ectoderm in a similar pattern to Yan immunostaining. Detection can be seen for each construct in subset regions in the head and ectoderm but none completely reiterates endogenous expression. One aberration is seen with A-lacZ which shows a strong expression in cells near the midline whereas yanP and Yan protein have no detectable staining. At this stage B- and C-lacZ do not express. In a dorsolateral view of stage-11 embryos (f–k) Yan protein and yanP expression is still seen in the ectoderm in addition to cells near the tracheal pits. A striped pattern of expression is also seen in the ectoderm of stage-11 embryos from A-lacZ, D-lacZ, and E-lacZ. For the first time B-lacZ drives expression and it can be seen in a small number of cells in each segment near the tracheal pits. In stage-13 embryos (l–q) expression is limited to the ectoderm and tracheal system. β-Gal expression is detected in the ectoderm of stage-13 embryos from A-lacZ, D-lacZ and E-lacZ. Only a small number of cells in each segment express β-gal in stage-13 embryos from B-lacZ flies. In lateral (r–t) and dorsal (u–w) views of stage-15 embryos, C-lacZ and D-lacZ, expression is detected in cells along the dorsal midline and in the developing head, a pattern not seen with Yan antibody.
Later in germ band extension (stage 11), constructs A-, D-, and E-lacZ continue to contribute to expression in the epidermis with A- (Fig. 3h) and D-lacZ (Fig. 3j) having patterns most closely resembling Yan and yanP (Fig. 3f, g). Expression in the head region at this stage is still evident in both A-lacZ and D-lacZ, but not in E-lacZ lines (Fig. 3k). At this stage, for the first time, B-lacZ (Fig. 3i) begins to drive reporter expression. B-lacZ drives a very restricted pattern of expression in cells that are consistent with the clusters of Yan-positive cells located behind and beneath the tracheal pits (Gabay et al. 1996; Price and Lai 1999).

Throughout stage-13 embryos, β-gal expression in yanP (Fig. 3m) correlates with the Yan immunostaining pattern (Fig. 3l) where expression is detected at a lower level in the epidermal cells and at a higher level in the tracheal system. At this stage of embryonic development, constructs A-, D- and E-lacZ (Fig. 3n, p, q) drive expression in a pattern consistent with yanP and Yan protein. B-lacZ (Fig. 3o) continues to drive a very restricted pattern in the cells beneath the tracheal pits.

In embryos undergoing dorsal closure (stages 14–16), C- and D-lacZ drive reporter expression within the head region in cells consistent with forming the optic lobes (Fig. 3r–w). Specific expression is also detected in rows of cells along the dorsal midline, a pattern not seen with Yan antibody staining (Fig. 3r, u). This midline expression can be seen as early as stage 13 in both C- and D-lacZ embryos (data not shown). These cells along the midline are known to play a role in the circulatory system (reviewed in Cripps and Olson 2002) and may indicate a greater function than previously thought for yan in cardiac development. Data from Halfon et al. 2000 supports the observation that yan is involved in cardiac development by showing that an increased number of Even-skipped (Eve) positive pericardial progenitor cells can be detected in a yan mutant background. Thus, with the similarity in the expression pattern of our reporter constructs within midline cell development and the known involvement of yan in cardiac progenitor cells, there is a likely role for yan in cardiac development that has yet to be fully understood. This midline expression is unlikely to be due to positional effects because it can be detected with multiple independent insertions with two different transgenes.

One concern regarding the midline expression from our reporter constructs is that this pattern is not seen with yanP or Yan protein staining. In the case of yanP, it may be due to the location of its P-element insertion. Sequence analysis indicates that the enhancer trap line insertion lies within the genomic region mutual to both C- and D-lacZ transgenic lines. This insertion may be disrupting an endogenous enhancer element preventing the yanP line from exhibiting midline expression. However, there may be a repression element in the endogenous situation that is missing in C- and D-lacZ transgenic lines allowing for easier disclosure of β-gal. As for the low level of Yan protein in midline cells, perhaps this results from a dynamic turnover of Yan protein leading to decreased immunodetection.

yan cis-acting regulatory modules in ovaries

Genetic studies demonstrate that flies homozygous mutant for yan have a fertility phenotype indicating a requirement for Yan in ovaries (Lai and Rubin 1992). Indeed, Yan protein is expressed in follicle and nurse cells within ovaries (Price and Lai 1999). However, none of our yan genomic fragments are able to drive gene expression within these cells. Based on this data, we assume that the genomic requirement for regulating expression within ovaries may be mediated either by regulatory regions that lie outside the area covered in this analysis or by a combination of elements from different regions within the yan locus area studied.

Synopsis

In this paper we have described how cis-acting module elements can impact yan gene expression. Also, we have identified new potential areas of yan expression relating to function not detectable with previous methods. Through in vivo analysis, we have demonstrated that the genomic region surrounding the yan first exon contains regulatory modules sufficient to drive gene expression in embryos, larval CNS and eye imaginal discs. Fragments A to E contain most of the information necessary for yan expression in embryos. In the larval CNS, B- and J-lacZ can replicate previous expression data and in the developing eye the 122-bp O module contains the minimal information needed to specify gene expression mimicking yanP and the Yan protein pattern. Taken in combination, this data indicates that the yan-lacZ transgenes recapitulate most of the expression patterns of the endogenous yan gene. Thus, this data highlights the high degree of complexity required within and between cis-acting regulatory elements for the proper timing and patterning of yan transcription.

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References

Reference List


7. BDGP [www.fruitfly.org]


18. de **Celis, J.F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C., Bray, S.** (1996). Functional relationships between Notch, Su(H) and the bHLH genes of the E(spl) complex; the E(spl) genes mediate only a subset of Notch activities during imaginal development. Development 122, 2719-2728.


25. **Drosophila Genome Project** [www.hgsc.bcm.tmc.edu/projects/drosophila]


Enhancer of split m gamma and m delta expression. Mol.Cell Biol. 17, 5620-5628.


29. FlyBase [www.flybase.org]


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