A Genetic Screen for Novel Components of the Ras/Mitogen-Activated Protein Kinase Signaling Pathway That Interact With the *yan* Gene of Drosophila Identifies *split ends*, a New RNA Recognition Motif-Containing Protein

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ABSTRACT

The receptor tyrosine kinase (RTK) signaling pathway is used reiteratively during the development of all multicellular organisms. While the core RTK/Ras/MAPK signaling cassette has been studied extensively, little is known about the nature of the downstream targets of the pathway or how these effectors regulate the specificity of cellular responses. Drosophila *yan* is one of a few downstream components identified to date, functioning as an antagonist of the RTK/Ras/MAPK pathway. Previously, we have shown that ectopic expression of a constitutively active protein (yan^{ACT}) inhibits the differentiation of multiple cell types. In an effort to identify new genes functioning downstream in the Ras/MAPK/yan pathway, we have performed a genetic screen to isolate dominant modifiers of the rough eye phenotype associated with eye-specific expression of yan^{ACT}. Approximately 190,000 mutagenized flies were screened, and 260 enhancers and 90 suppressors were obtained. Among the previously known genes we recovered are four RTK pathway components, *rolled* (MAPK), *son-of-sevenless*, *Star*, and *pointed*, and two genes, *eyes absent* and *string*, that have not been implicated previously in RTK signaling events. We also isolated mutations in five previously uncharacterized genes, one of which, *split ends*, we have characterized molecularly and have shown to encode a member of the RRM family of RNA-binding proteins.

DURING development, multicellular organisms must coordinate the growth, differentiation, and maintenance of many different cell types. To achieve this, each cell must continually integrate a complex array of external signals, including both inductive and inhibitory cues, and then translate these instructions into spatially and temporally appropriate developmental responses. Many of the signaling mechanisms regulating these decisions are used repeatedly, generating different cellular responses in different developmental contexts.

The Drosophila compound eye is an ideal tissue in which to study the molecular mechanisms underlying cell-cell communication (Rubin 1988; Zipursky and Rubin 1994). The adult eye is composed of a regular array of ~800 ommatidial units, each one containing ~20 cells recruited in a stereotyped sequence of inductive interactions that involves extensive networks of intercellular signaling events (Tomlinson and Ready 1986). The same evolutionarily conserved signaling mechanisms used reiteratively during the development of all multicellular organisms, including receptor tyrosine kinase (RTK), Notch, hedgehog, wingless, and transforming growth factor-β (TGF-β) pathways, play essential roles in establishing the architecture of the fly eye (Cagan and Ready 1989; Heberlein *et al.* 1993; Dickson and Hafen 1994; Treisman and Rubin 1995; Pignoni and Zipursky 1997). Because the Drosophila eye is not required for viability or fertility of the adult, these essential signaling pathways can be perturbed and manipulated in an eye-specific manner without affecting the development or viability of the animal as a whole.

We have been focusing our investigations on the RTK-mediated signaling pathway that regulates a broad range of developmental events including mitogenesis, cell fate specification, and differentiation (Marshall 1994; Maruta and Burgess 1994; van der Geer *et al.* 1994). Extensive investigations from numerous laboratories have shown that RTKs signal through an evolutionarily conserved pathway that involves the GTPase Ras and the mitogen-activated protein kinase (MAPK) cascade of serine/threonine kinases (Marshall 1994; Zipursky and Rubin 1994). In Drosophila, the RTK/Ras/MAPK cascade acts inductively to promote cell fate specification and differentiation of numerous embryonic and adult tissues including the central nervous system

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(CNS), muscles, tracheal system, wing veins, neuronal and nonneuronal cells in the eye, and oogenesis (Shilo 1992; Dickson and Hafen 1994; Duffy and Perrimon 1994; Schnorr and Berg 1996; Engstrom *et al.* 1997; Lee and Montell 1997; Schweitzer and Shilo 1997; Buff *et al.* 1998). While genetic and biochemical screens have identified many of the components required for Ras activation and for transmission of this information through the MAPK signaling cascade, the downstream effector molecules that coordinate different physiological responses, and thereby impart specificity to the pathway, have remained largely elusive.

A small number of genes have been identified as downstream targets of activated MAPK in Drosophila. These include two Ets-domain transcription factors, *pointed* and *yan*, the AP-1 transcription factor *D-jun*, and the cell death gene *hid* (O'Neill *et al.* 1994; Kockel *et al.* 1997; Bergmann *et al.* 1998). However, little is known about how these proteins regulate gene expression in response to RTK-initiated signals or whether the Ras/MAPK signal alone regulates their activity. We have been investigating the function of one of these genes, *yan*, in an effort to understand more about how RTK-mediated signals are transduced to the nucleus.

yan was originally identified as a negative regulator of R7 photoreceptor neuron differentiation in the developing eye, acting as an antagonist to the proneural signal mediated by the *sevenless* RTK signaling pathway (Lai and Rubin 1992). Subsequent studies have shown that *yan* functions as a fairly general inhibitor of differentiation, preventing both neuronal and nonneuronal cell types from responding inappropriately to external signals in multiple developmental contexts (Rebay and Rubin 1995). Experiments in cultured cells suggest yan functions as a transcriptional repressor, and that phosphorylation of yan by MAPK negatively regulates this repressor activity (O'Neill *et al.* 1994).

Mutation of the phosphoacceptor residues in all eight of the MAPK phosphorylation consensus sites in *yan* produces a stable protein that cannot be properly "turned off" (Rebay and Rubin 1995). This allele is referred to as *yan*^{ACT} for "activated" *yan*. Overexpression of *yan*^{ACT} inhibits differentiation of multiple neuronal and nonneuronal cell types throughout development, indicating that *yan* function is not restricted to the *sevenless (sev)* RTK pathway, which operates only in a discrete subset of cells in the developing eye (Rebay and Rubin 1995). Work from several labs has confirmed that in addition to its role in antagonizing the *sev*-mediated signal, *yan* also functions downstream of and negatively regulates at least two other known RTK pathways, the epidermal growth factor receptor and the fibroblast growth factor receptor pathways (Shilo 1992; Rebay and Rubin 1995; Samakovlis *et al.* 1996). Thus, because yan functions as a transcriptional repressor downstream of multiple RTK pathways, it offers an ideal tool with which to address the issue of how specificity of response to RTK-mediated signaling events is generated in different developmental contexts.

We have undertaken an extensive genetic screen to identify dominant modifiers of the rough eye phenotype associated with eye-specific overexpression of the *yan*^{ACT} transgene. Our screen was modeled on previous successful screening efforts that have been pivotal in identifying components of the RTK/Ras/MAPK cascade. Each screen has taken a slightly different starting point, beginning with the RTK and progressing down the pathway (for example, Simon *et al.* 1991; Dickson *et al.* 1996; Karim *et al.* 1996). While there has been some overlap in the genes identified, each new screen has also isolated unique genes, and thereby has made significant contributions to our understanding of how the signal is transduced from the RTK, to Ras, Raf, and the MAPK cascade. Thus, by approaching the pathway from various angles, new information has been discovered at each step. Centering the screen around one of the most downstream components in this conserved pathway should facilitate identification of some of the elusive downstream effectors and regulators of the RTK pathway.

In this article we describe the results of a genetic modifier screen in which we isolated 15 complementation groups that dominantly enhance the *yan*^{ACT} rough eye phenotype and 6 complementation groups that dominantly suppress it. These genes include known components of the pathway such as *rolled*/MAPK, the guanine nucleotide exchange factor *son of sevenless*, and the Ets family transcription factor *pointed*, as well as several novel genes, which, based on genetic interaction data, are likely to encode relevant new components of the Ras/MAPK pathway. We report preliminary molecular analysis of one of these novel genes, called *split ends*, that suggests it encodes a member of the RNA recognition motif (RRM) family of proteins.

MATERIALS AND METHODS

***yan*^{ACT} modifier screen:** To facilitate chromosomal linkage analysis and establishment of balanced stocks, the GMR-*yan*^{ACT} and sev-*yan*^{ACT} transgenes (Rebay and Rubin 1995) were mobilized onto *CyO* and *TM3Sb* balancer chromosomes. Independent insertion events in which the *yan*^{ACT} phenotype cosegregated with the balancer were screened for strength, dose sensitivity, and variability of phenotype. Based on these criteria, two lines, Sev-*yan*^{ACT} on *CyO* (*SCY*) and GMR-*yan*^{ACT} on *CyO* (*GCY*), were selected as suitable for use in the modifier screen. Two other lines, sev-*yan*^{ACT} on *TM3Sb* (*STY*) and GMR-*yan*^{ACT} on *TM3Sb* (*GTY*), were selected for use in determining chromosomal linkage.

Male w^{1118} flies isogenic for the second and third chromosomes were fed 25 mm EMS or treated with 4000 R of X rays (1000 sec at 115 kEV, 5 mA), and then mated to w^{1118};*Sco*/*SCY* or w^{1118};*Sco*/*GCY* females. The eyes of F_1 progeny were examined under a dissecting microscope for enhancement or suppression of the *SCY* or *GCY* phenotype. Potential modifiers were backcrossed to w^{1118};*Sco*/*SCY* or w^{1118};*Sco*/*GCY* as appropriate, and the F_2 progeny were rescored for suppression or enhancement. In the F_2 generation, linkage of the mutation

to the second chromosome could be determined if all non-*Sco* flies bearing the *SCY* or *GCY* chromosome showed the appropriate modification; in this case a balanced stock, w^{1118};modifier/*SCY* or *GCY*, was established. If the modifier segregated randomly with respect to the *SCY* or *GCY* chromosome, this indicated linkage of the mutation to the third chromosome. To establish a balanced stock, flies of the genotype w^{1118};+/*SCY* or *GCY*;modifier/+ were crossed to w^{1118};*CxD*/*STY* or *GTY*. In the next generation, a balanced stock of w^{1118};modifier/*STY* or *GTY* was established by selecting *STY* or *GTY* flies showing the appropriate enhancement or suppression. x-chromosome-linked modifiers were too difficult to maintain in the *yan*^{ACT} backgrounds, which on their own exhibit reduced fertility, and were lost. Therefore, only the second and third chromosomes have been screened for modification of the *yan*^{ACT} phenotype.

Complementation tests based on lethality were performed among all mutations mapping to the same chromosome. Single hits are those mutations that complement all other mutations on that chromosome. A number of mutations are viable; these have not been mapped, and therefore it is not possible to know how many different genes they represent. Two alleles for each defined complementation group were mapped meiotically relative to *b pr c px sp* on the second chromosome and *ru h th st cu sr e ca* on the third. Both the lethality and enhancement or suppression of *yan*^{ACT} were mapped. Lethal complementation tests with deficiencies, *P*-element insertions, and other mutations in the region were used to further refine the map position. Noncomplementation was as follows (refer to Table 1): EY2-1 and *clift(eya1)*; EY2-2 and *rolled*^{S135} (Karim *et al.* 1996); EY2-3 and *son of sevenless*^{e2H} (Simon *et al.* 1991); EY2-4 and *Star*$^{ES2\text{-}4e220}$ (Neufeld *et al.* 1998); EY2-5 and *Df(2L)ast1*; EY2-7 and *P* elements *l(2)03350, l(2)k06805, l(2)k07612, l(2)k07721, l(2)k08102, l(2)k13233, l(2)k13624,l(2)k15612, l(2)k06703, l(2)k10325, l(2)k13601*; EY2-8 and *Df(2L)CX1*; EY2-9 and *sin3*$^{l(2)7401}$ (Neufeld *et al.* 1998); EY2-10 and *dead ringer*^{1} (Gregory *et al.* 1996); EY3-1 and *pointed*$^{7825\Delta 78}$ (O'Neill *et al.* 1994); EY3-2 and *Df(3R)e-R1*; EY3-3 and *Df(3R)e-R1*; SY2-1 and *SS2-1*^{s35} (Neufeld *et al.* 1998); SY3-1 and *SR3-4a*$^{S\text{-}192}$ (Karim *et al.* 1996); SY3-2 and *TAF110*^{S117} (Karim *et al.* 1996); SY3-3 and *glass*^{1}; SY3-4 and *string*^{eAS7a} (Verheyen *et al.* 1996).

Histology: Flies were prepared for scanning electron microscopy by fixation for 2 hr in 1% glutaraldehyde, 1% paraformaldehyde in 1 m cacodylate buffer followed by dehydration through an ethanol series and critical point drying. Fixation and sectioning of adult eyes was as described by Tomlinson *et al.* (1987).

Molecular analysis of EY2-7: Plasmid rescue (Pirrotta 1986) was used to isolate genomic DNA flanking *l(2)7721* and *l(2)7612*. An ~20-kb *Bgl*II fragment on one side of the *P*-element insertion site and an ~25-kb *Sac*II site on the other were recovered. Cytological examination of X-ray-induced EY2-7 alleles revealed one allele, designated *xLS1000*, to be a cytologically visible inversion. To determine whether either of the two plasmid rescue DNA fragments crossed the *xLS1000* breakpoint, chromosomal *in situ* hybridization on *xLS1000* chromosomes was performed. The *Sac*II fragment crossed the *xLS1000* breakpoint, while the *Bgl*II fragment was entirely distal (data not shown). The entire *Sac*II genomic fragment and various smaller subclones of this fragment were used as probes to screen cDNA libraries prepared from eye-antennal imaginal discs (made by A. Cowman), adult heads (obtained from the lab of Y. N. Jan), and embryos (made by L. Hong and G. M. Rubin). To obtain cDNAs spanning the entire coding sequence, four successive rounds of screening were performed, each using a probe taken from the most 3′ sequence of the longest cDNA at hand. The cDNAs were sonicated. Random ~1-kb fragments were subcloned into Bluescript and then sequenced with Universal and Reverse primers on an ABI 373 sequencer. An ~2-kb gap in the coding sequence (nucleotide coordinates 12018–14125), apparent from comparison of our sequence to the Berkeley Drosophila Genome Project (BDGP) genomic sequence (GenBank accession no. AC005334), was filled in by RT-PCR using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Primer pairs used to amplify two overlapping fragments covering the region were: primer pair 1: 5′ ATGGTAAGCCAAC CATCACC 3′ and 5′ TCTGGTTACAGGAGCTGTTACGGG-3′; primer pair 2: 5′ ATCATGATACAGAGGACGAAACGG 3′ and 5′ AGTATCCGTGAGCAACATGTGACCG 3′. Sequencing primers were designed based on the BDGP genomic sequence, and both strands of the RT-PCR products were sequenced directly without subcloning. To confirm the existence of the noncoding cDNAs j1 and 2A1, RT-PCR was performed from adult heads and embryos. Primers were designed to span the introns in both cDNAs: for cDNA j1: primers 5′ CGAAACGG CAAGTGCTTAAGCGAAAG 3′ and 5′ AACAGTTCGTTGTG GATAGCAGCACC 3′. For cDNA 2A1: primers 5′ TGGTAAGC TATCAAGAACATATCTGC 3′ and 5′ TACCTTCCCACACA GATCGTACTCC3′. For negative control, primers were designed in the predicted introns: for cDNA j1: intron primers 5′ AGTGAAAGGCGTGTGGTAGATCACG 3′ and 5′ TACC TTCCCACACAGATCGTACTCC 3′; for cDNA 2A1: intron primers 5′ AATACGACTTCAAGATGAGTCGTCG 3′ and 5′ TTTCGGAAGTCTGTGAATCGATAGC 3′. If our cDNAs were artifactual, no PCR product would be recovered in the case of j1, which has an ~12-kb predicted intron, and a larger than expected product would be amplified for 2A1, which has a much smaller predicted intron. For both j1 and 2A1, bands of the expected sizes were obtained, indicating that these two cDNAs represent *bona fide* spliced mRNA species (data not shown). No bands were amplified with the intronic primers. The approximate position of the introns was determined by comparing our cDNA sequence to BDGP genomic sequence.

RESULTS

A genetic screen for modifiers of *yan*^{ACT}: Overexpression of a *yan* cDNA in which all eight putative MAPK phosphorylation consensus sites were mutated to a nonphosphorylatable form, referred to as *yan*^{ACT}, inhibits the differentiation of multiple neuronal and nonneuronal cell types (Rebay and Rubin 1995). Previously, we have described the effects of expressing the *yan*^{ACT} transgene in the developing Drosophila eye using the sevenless (sev) and GMR promoters (Figure 1, A–C; Fortini *et al.* 1992; Hay *et al.* 1994; Rebay and Rubin 1995). sev-*yan*^{ACT} flies have rough eyes characterized by the loss of the R3, R4, and R7 photoreceptor neurons and the nonneuronal cone cells. GMR-*yan*^{ACT} flies have more severely disrupted eyes due to inhibition of differentiation of photoreceptors R1–R8, the cone cells, and other accessory cells of the eye. Both sev-*yan*^{ACT} and GMR-*yan*^{ACT} rough eye phenotypes are sensitive to the copy number of the transgene (data not shown). That is, flies carrying two copies of the transgene have more severely disrupted eyes than flies with only one copy. The dose sensitivity of the phenotype suggested that both sev-*yan*^{ACT} and GMR-*yan*^{ACT} genetic backgrounds might represent good starting points for a genetic

TABLE 1

Summary of genetic interactions

Groups	No. of alleles	Map position	Alleles	GMRYan^{ACT} (*GCY*)	SevYan^{ACT} (*SCY*)	Yan^{S2382}	Ras^{V12} (*T2B*)	Ras^{N17}	Raf^{HM7}18°
EY2-1	10	26E1-4	G130	E++	E++	E+	−	E+	E++
eyes absent			A188	E++	E++	E+	S++	E+	Lethal
EY2-2	10	h38-h41	A0648	E++	E++	E+++	S++	E+++	Lethal
rolled/MAPK			AL528	E++	E++	E+	S++	E++	Lethal
EY2-3	12	34D4	D120	E+	E+	E++	S++	E+++	Lethal
son of sevenless			xB10	E++	E+++	E++	−	E+++	Lethal
EY2-4	35	21E2	BA651	E++++	E++++	E++++	−	E++++	Lethal
Star			U132	E++++	E++++	E++++	−	E++++	Lethal
*EY2-5	2	21C7-22B1	DW1080	E++	−	−	−	−	−
			AH425	−	E++	−	E++	E+	E+
EY2-6	3	2nd	AQR618	−	E++	E+	−	E+	Lethal
			AC403	E+++	E++	E++	E++	E++	Lethal
EY2-7	37	21A1-B6	AH393	E++	E+++	E+	−	E+	Lethal
			BU720	E+	E++	E+	S+	E+	Lethal
EY2-8	2	49C1-50D2	xCB204	E++	−	−	−	−	Lethal
			EP1160	E++	−	−	−	E−	Lethal
EY2-9	4	49B3-6	DW1046	E++	−	−	−	−	Lethal
sin3			CY886	E++	−	−	S+	−	Lethal
EY2-10	2	59F3-4	B142	−	++	−	E+++	−	−
deadringer			x1N793	−	+++	−	E++	−	Lethal
EY3-1	44	94E	AP553	E+++	E+++	E+++	−	E++	Lethal
pointed			AF397	E+++	E+++	E++	−	E+++	Lethal
EY3-2	3	93B3-D4	xAZ145	E++	−	E+	−	−	Lethal
			xCF301	E++	−	−	−	−	Lethal
			xDF411	E++	−	−	S+++	−	Lethal
EY3-3	2	93B3-D4	xBE183	E++	−	−	E+++	−	−
			xF6	E++	−	−	−	−	Lethal
EY3-4	2	3rd	xII790	−	E++	−	E++	E++	Lethal
			x1S805	−	E++	E+	−	E+++	Lethal
EY3-5	2	61A-B	xZB970	E+	E++++	E++	−	E++	Lethal
			xKR845	E+	E++	E+	−	E+	Lethal
SY2-1	22	23C1-2	DR999	+++	−	−	−	S+	−
			DE884	+++	−	−	−	−	−
SY2-2	2	2nd	xEX552	S+++	S+++	S+++	E++++	S++	S+++
			CS874	S+++	S+++	S+++	E++++	S+++	S+++
SY3-1	7	76C-D	DI998	S++	−	−	−	−	−
			EZ1217	S++	−	−	−	−	−
SY3-2	3	72D5	I53	−	S+++	−	S++	−	S++
TAF110			AG456	−	S+++	−	S+++	−	−
SY3-3	8	91A1-2	ER1085	S++++	−	−	−	−	−
glass			DZ1053	S++++	−	−	−	−	−
*SY3-4	3	99A5-6	D115	S+	S+++	−	−	−	−
string			DD937	S+	S+	S+++	E+++	−	−

Genetic interactions with lethal enhancer and suppressor groups. The strength of suppression or enhancement scored in each genetic test was scaled relative to the w^{1118} parental strain that was judged to have no effect. The complementation groups that interact with both sev and GMR-driven *yan*^{ACT} are underlined. Of the underlined groups, only two, indicated with an asterisk, do not also interact with the gain-of-function allele *yan*^{S2382}. The map positions indicated are those of noncomplementing *P*-element alleles that have been cytologically mapped by the BDGP, reported breakpoints of noncomplementing deficiencies, or published cytological locations of known genes (see materials and methods).

−, little or no effect; E, enhances; S, suppresses; +, refers to the strength of interaction with a single + indicating a mild interaction and ++++ indicating a very strong interaction.

screen designed to identify potential regulators or targets of *yan* activity.

An important feature of this type of genetic screen is that it is designed to isolate dominant modifiers of a specific phenotype. The expectation is that a twofold reduction in activity of a pathway relevant gene, achieved by mutating one of the two copies present in the diploid genome, will dominantly enhance or suppress the starting background phenotype. In contrast, in a wild-type background, a twofold reduction in gene

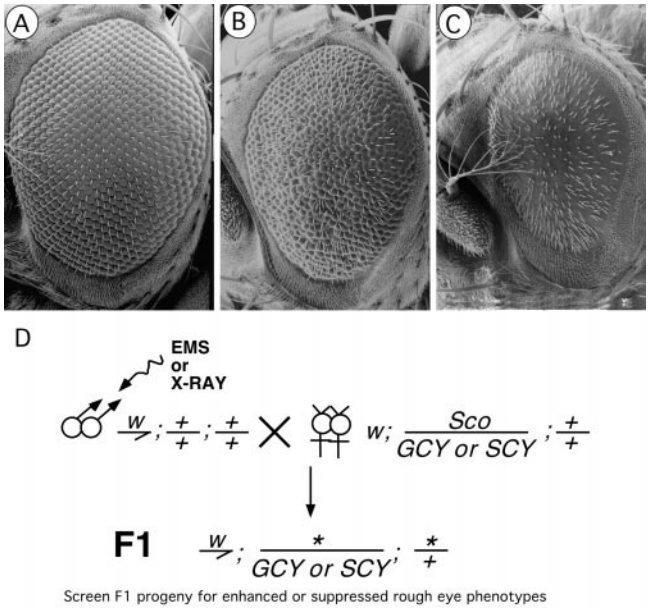


Figure 1.—The *yan*^{ACT}-based genetic screen. Scanning electron micrographs (SEM) of the starting phenotypes used in the genetic modifier screen. (A) Wildtype, (B) sev-*yan*^{ACT}/+, and (C) GMR-*yan*^{ACT}/+. (D) Schematic representation of the strategy for the screen. w^{1118} males were mutagenized with either EMS or X rays and were mated to virgin females carrying the *yan*^{ACT} transgene either under sevenless or GMR promoter control (*SCY* and *GCY*, respectively). In the subsequent (F_1) generation, progeny were scored for enhancement or suppression of the rough eye phenotype. An asterisk indicates a mutagenized chromosome.

activity will produce dominant phenotypes only rarely. Thus in a wild-type background, screens designed to study the phenotypic consequences of reduction in gene activity will require at least two generations to produce a homozygous mutant and a visible phenotype. However, many of the genes involved in signal transduction pathways are essential for viability, necessitating either analysis of lethal embryonic phenotypes or clonal analysis in particular tissues. While such F_2 screens have been extremely successful at isolating genes functioning in specific developmental processes (for example, Nusslein-Volhard *et al.* 1984; Duffy *et al.* 1998) the genetic modifier screen has proven to be a highly efficient method of dissecting signaling pathways in genetically manipulable organisms (for example, in Drosophila, Simon *et al.* 1991; Dickson *et al.* 1996; Karim *et al.* 1996; Verheyen *et al.* 1996). In addition, the ease of a dominant F_1 screen allows large numbers of flies to be screened, thereby approaching saturation.

In our screen, because *yan* is a negatively acting factor in the RTK pathway, expression of the stable *yan*^{ACT} product reduces overall signaling output by the pathway. Thus in the *yan*^{ACT} background, a twofold reduction in activity of positively acting genes elsewhere in the pathway will further reduce signaling output, thereby exacerbating the eye phenotype. Such mutations will be recovered as enhancers. Conversely, halving the activity of negatively acting components of the pathway will increase the overall output of the pathway. Such mutations will be recovered as suppressors of *yan*^{ACT} or as flies with less severely disrupted eyes.

Approximately 80,000 progeny from EMS-mutagenized flies and 110,000 progeny from X-ray-mutagenized flies were screened for ability to dominantly modify the *yan*^{ACT} phenotype. The scheme of the screen is diagrammed in Figure 1D. Approximately half of the screen was performed in the sev-*yan*^{ACT} background and half in the GMR-*yan*^{ACT} background (Figure 2, A and D). Both enhancers and suppressors of the rough eye phenotype were recovered from each portion of the screen (Figure 2, B, C, E, and F). A total of 260 enhancer mutations was recovered, 177 on the second chromosome and 83 on the third chromosome. Also, 90 suppressor mutations were recovered, 49 on the second chromosome and 21 on the third chromosome. Complementation analysis placed 215 of the mutations into 21 complementation groups (Table 1). The remaining 135 mutations include both "single hit" alleles that do not fall into any of the complementation groups and viable mutations. These 135 mutations have not been mapped or characterized further.

Genetic tests to determine which are the "pathway relevant" groups: One of the most important aspects of a genetic interaction screen is to design suitable secondary screens that will distinguish pathway relevant mutations from inevitable background. These secondary tests serve as a sort of "genetic triage" that allows rapid classification of the isolated mutations and prioritization of further efforts to understand the role of the genes in the particular context being studied. Our secondary screens were designed to identify those mutations that specifically affect the RTK/Ras/MAPK/yan pathway. All alleles from the 21 complementation groups were tested, and the results for two alleles representative of each group are shown in Table 1. The results of these genetic tests, together with the identification of several known components of the RTK signaling pathway among the candidate genes isolated, indicate that the *yan*^{ACT} interaction screen appears to have worked as designed.

Test 1: To distinguish potential pathway relevant mutations from those that affect the strength of the promoters driving yan^{ACT} *expression:* Because our screen involves expressing the *yan*^{ACT} transgene under the control of eye-specific promoter elements, one class of modifiers we expected to isolate and wanted to eliminate was that affecting the strength of the promoters. For example, because the *yan*^{ACT} phenotypes are dose sensitive, if we recover a mutation that increases the strength of the promoter element, this will result in increased *yan*^{ACT} expression and an enhancement of the rough eye phenotype. Such a mutation does not affect *yan* or Ras pathway activity

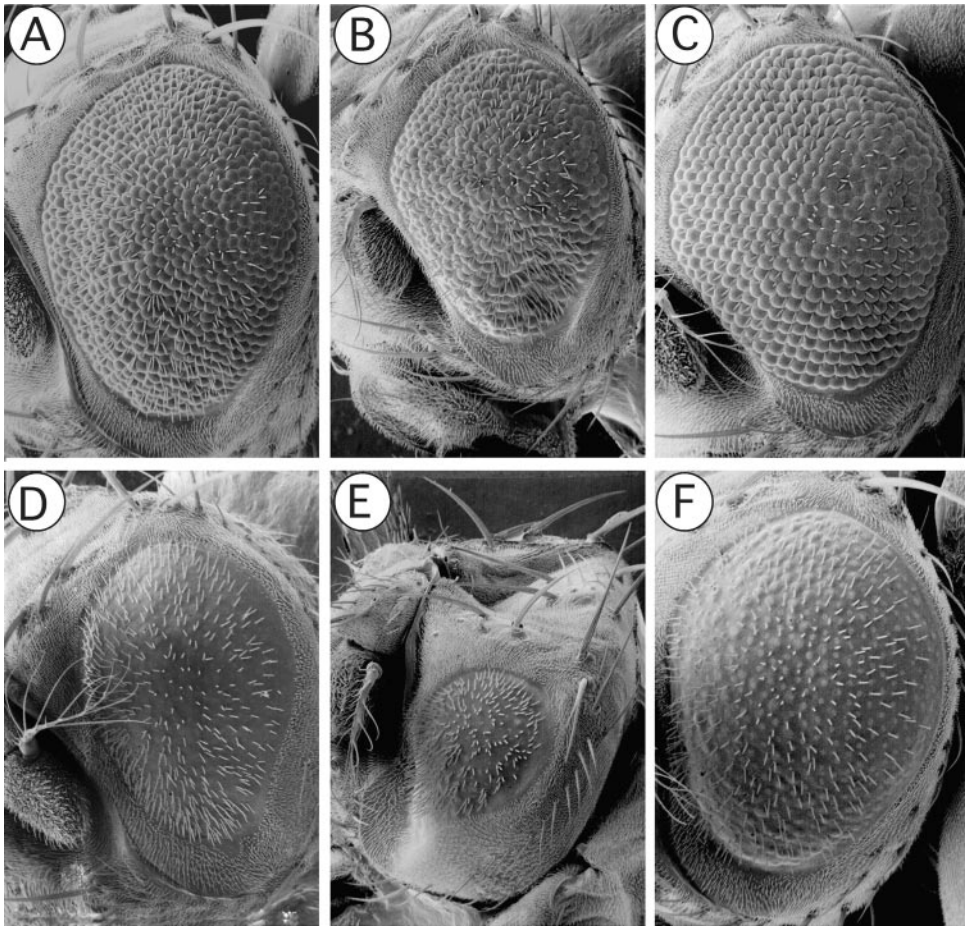


Figure 2.—Isolation of enhancers and suppressors of *yan*^{ACT}. SEMs of both the starting *yan*^{ACT} phenotypes and examples of recovered modifiers determined by the genetic tests as likely to encode RTK pathway relevant products. (A) *SCY*/+; (B) EY2-3/*SCY*; (C) SY2-2/*SCY*; (D) *GCY*/+; (E) EY2-3/*GCY*; (F) SY2-2/*GCY*. For enhancer mutations, the eye is smaller and more disorganized than the parental strain. For suppressor mutations, the eye is larger and the ommatidial lattice is more regular in appearance. EY, enhancer of *yan*^{ACT}; SY, suppressor of *yan*^{ACT}.

directly, and is unlikely to be relevant to the signaling pathway. Thus, the first genetic test was designed to separate the pathway relevant mutants from the promoter-specific mutants. We reasoned that pathway relevant mutants will modify both the GMR-*yan*^{ACT} and sev-*yan*^{ACT} rough eye phenotypes, whereas promoter-specific mutations will modify one background only. Therefore, all modifiers obtained from the GMR-*yan*^{ACT} half of the screen were crossed to the sev-*yan*^{ACT} background, and conversely all modifiers obtained from the sev-*yan*^{ACT} half of the screen were crossed to the GMR-*yan*^{ACT} background.

On the basis of this test, 11 of the 21 complementation groups appear relevant to Ras/MAPK/yan signaling (these are underlined in Table 1). In fact, for the complementation groups that passed test 1, alleles were isolated both from the sev-*yan*^{ACT} and the GMR-*yan*^{ACT} halves of the screen, confirming these results in an unbiased manner. Further confirming that this test was working as planned, among the 11 pathway relevant groups are known components of the RTK pathway including *rolled*/MAPK, *son of sevenless*, *Star*, and *pointed* (Rogge *et al.* 1991; Simon *et al.* 1991; Klambt 1993; Biggs *et al.* 1994; O'Neill *et al.* 1994). Isolation of these genes as enhancers of the *yan*^{ACT} phenotype is consistent with their known roles as positive factors in the RTK pathway.

Two of the 11 pathway relevant groups were found to be allelic to previously characterized genes whose role, if any, in the RTK signaling pathway has not yet been determined. EY2-1 is allelic to *eyes absent* (*eya*), a gene known to function within a hierarchy of genes essential for determining eye fate during development (Bonini *et al.* 1993, 1997). SY3-4 is allelic to *string*, the Drosophila homologue of the cdc25 phosphatase cell cycle regulator (Edgar and O'Farrell 1989). The remaining RTK pathway candidate groups, EY2-5, EY2-6, EY2-7, and EY3-5, appear to be uncharacterized genes.

Among the known genes classified as promoter specific are *TAF110*, a coactivating factor in PolII transcription (Hoey *et al.* 1993), *glass*, a transcription factor essential for expression of the GMR element (Ellis *et al.* 1993; Hay *et al.* 1994), *dead ringer*, a homeodomain-related protein (Gregory *et al.* 1996), and *sin3A*, a transcriptional repressor (Wang *et al.* 1990). Other genes in this category that have been isolated in previous screens are SY2-1, which is allelic to SS2-1, and SY3-1, which is allelic to SR3-4a and SS3-4 (Karim *et al.* 1996; Neufeld *et al.* 1998). The genes corresponding to the EY2-8, EY3-2, EY3-3, and EY3-4 complementation groups have to our knowledge not been identified previously.

Test 2: To determine which groups interact with an endogenous gain-of-function yan *allele:* In the second genetic test

to determine which were the relevant *yan*-interacting genes, a gain-of-function *yan* allele (*yan*^{S2382}; Karim *et al.* 1996) was crossed to all alleles of the 21 complementation groups (Table 1). The phenotypes of *yan*^{S2382} animals are similar to, although milder than, those seen with the *yan*^{ACT} transgene. Homozygous *yan*^{S2382} flies have slightly roughened eyes, reflecting an increase in stability of the *yan*^{S2382} protein product that inhibits differentiation of the photoreceptor neurons. Sequence determination of the molecular lesion associated with the mutation revealed the predicted protein to be a truncated form of *yan* that lacks the C-terminal 162 amino acids (Rebay and Rubin 1995). The sequence deleted in *yan*^{S2382} covers one of the PEST-rich regions of the protein that is postulated to be critical for normal downregulation of *yan* in response to Ras/MAPK activation. Thus *yan*^{ACT}, a gain-of-function allele generated by mutating the phosphoacceptor residue in all eight putative MAPK sites to alanine, and *yan*^{S2382}, a truncation allele, although they each cause very different alterations in the protein, both result in similar phenotypes in the developing eye. Modifiers that interact strongly with both gain-of-function *yan* backgrounds are likely to encode proteins relevant to *yan* function.

The results of this second test overlapped almost perfectly with the first test, further supporting our logic that those groups "passing" the tests represent likely RTK/*yan* pathway relevant genes. We found that 9/11 of the genes determined to be pathway relevant by test 1 interacted strongly with *yan*^{S2382}, whereas none of the promoter-specific groups affected *yan*^{S2382}. The two groups (indicated in Table 1 with an asterisk) that did not modify the *yan*^{S2382} background but are not promoter specific are EY2-5 and SY3-4 (*string*). One possibility is that these two groups are relevant to the Ras/MAPK/*yan* pathway, but that functional differences between *yan*^{ACT} and the *yan*^{S2382} gain-of-function allele create differences in sensitivity to second site modifiers. An alternative possibility is that these two genes act at the level of promoter strength, but act similarly on both the GMR and sev elements. Interestingly, EY2-5, a group of two alleles, was placed in the category of genes passing test 1 because one allele was isolated from the sev-*yan*^{ACT} part of the screen and the other from the GMR-*yan*^{ACT} part. However, neither EY2-5 allele modifies the eye phenotype in the other promoter background.

Test 3: To determine which groups interact with other RTK pathway components: The third test was based on the assumption that if a modifier of *yan*^{ACT} is involved in the RTK signaling pathway, it is likely to interact with other components of the pathway in addition to *yan*. We therefore tested whether the modifiers of *yan*^{ACT} interacted with two eye-specific transgenes, *sev* promoter-driven dominant negative Ras (Ras^{N17}) and *sev* promoter-driven activated Ras (Ras^{V12}), as well as with a hypomorphic Raf alleles which at 18° produces occasional hemizygous males with rough eyes (Raf^{HM7}; Karim *et al.* 1996). Furthermore, the direction of interaction, whether enhancement or suppression, should be consistent with the modifier acting as a positive or negative factor in the pathway. For example, the expectation for an allele that enhances *yan*^{ACT} is that it should enhance dominant negative Ras^{N17} and hypomorphic Raf^{HM7}, but suppress activated Ras^{V12}. All 11 groups identified as RTK pathway relevant showed consistent interactions with additional components of the pathway, whereas the promoter-specific groups did not (Table 1).

The alleles we isolated of known components of the RTK pathway behaved generally as expected or as shown previously by others. For example, EY2-2 (*rolled*/MAPK) alleles, which were isolated as enhancers of *yan*^{ACT}, strongly suppress Ras^{V12}, enhance Ras^{N17}, and are synthetic lethal with Raf^{HM7}. The direction of modification is in each case consistent with *rolled* functioning as a positively acting component of the RTK pathway. Several complementation groups exhibited similar patterns of interactions in these crosses, strongly suggesting an involvement as positively acting factors in the pathway. For example, most alleles of EY2-1 (*eyes absent*) suppress Ras^{V12} (although several, including G130, show no interaction), enhance Ras^{N17}, and are either synthetic lethal with Raf^{HM7} or else produce hemizygous "escaper" males with an enhanced rough eye phenotype (data not shown). EY2-7 exhibited a similar pattern of interactions except all alleles were synthetic lethal with Raf^{HM7}.

Other groups exhibited less consistent patterns of interactions in the various Ras pathway backgrounds. For complementation groups consisting of small numbers of alleles, there were allele-specific differences in behavior that made classification difficult. With large complementation groups of 10 or more alleles, it was easier to establish the general pattern of interaction, despite an occasional allele-specific inconsistency. SY3-4 (*string*) alleles showed no interaction with Ras^{N17} or Raf^{HM7}, but 1 of the 2 alleles strongly enhanced Ras^{V12} while the other showed no interaction. EY2-5 and EY2-6 also showed some contradictory patterns of interaction. One allele of EY2-5 and 1 allele of EY2-6 were found to enhance both Ras^{N17} and Ras^{V12}, while the other allele showed no interaction. Other groups, such as EY2-3 (*sos*), EY3-1 (*pointed*), and EY3-5, enhanced Ras^{N17} but showed little or no interaction with Ras^{V12}. These inconsistencies could simply be indicative of the different sensitivities of a particular genetic background to modification by reduction of dosage of a specific gene, or could suggest that the interaction may be more complex, possibly involving both positive and negative regulatory feedback loops. It should be stressed that these initial tests are used to prioritize future efforts as to which genes to investigate in detail. Definitive proof of involvement in RTK/*yan*-mediated signaling events must await further genetic and biochemical characterization of the function of the genes.

Molecular analysis of EY2-7: EY2-7 comprises a group

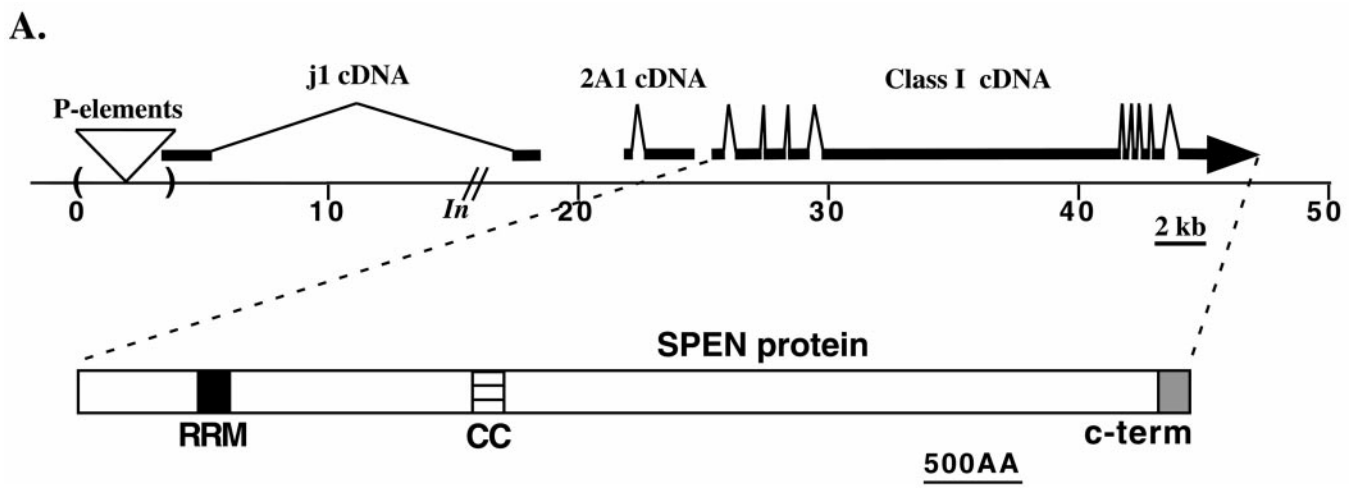
of 37 alleles, of which 18 are EMS induced and 19 are X-ray induced. Meiotic mapping localized the gene to the distal end of the left arm of the second chromosome. To define the genomic location of the gene more precisely, we screened deficiencies and lethal *P*-element insertions in the region for failure to complement EY2-7. *Df(2L)PMF47c* and 11 lethal *P*-element insertions fail to complement EY2-7 and also enhance *yan*^{ACT} (data not shown).

To determine whether the *P*-element insertions were responsible for the lethal noncomplementation, the *P* elements were excised from all 11 lines. For all *P* alleles, both viable and lethal excision events were recovered. The viable lines, presumably precise excision events in which the *P* elements were removed without causing chromosomal deletions, complemented EY2-7 alleles, and no longer enhanced the *yan*^{ACT} rough eye phenotype, indicating that they no longer carried an EY2-7 mutation (data not shown). The lethal lines, presumably imprecise excisions in which the chromosomal region surrounding the original *P*-element insertion site was disrupted, failed to complement EY2-7 alleles and enhanced *yan*^{ACT}(data not shown). These results suggested that the *P* alleles were inserted in or near the EY2-7 gene and could be used as molecular probes with which to clone the gene.

We isolated ~45 kb of genomic DNA flanking the insertion sites of the two *P* elements showing the strongest enhancement of *yan*^{ACT} (see materials and methods). We have subsequently determined that all 11 *P*-element lines are inserted within the same ~4-kb region (Figure 3A; data not shown). A 25-kb fragment of genomic DNA flanking the *P*-element insertion sites was found to cross the inversion breakpoint of a cytologically visible X-ray-induced EY2-7 allele (Figure 3A; data not shown). Southern blot restriction fragment polymorphism (RFLP) analyses using this genomic fragment as a probe revealed polymorphisms in multiple EY2-7 alleles (data not shown). Together, these data suggested the EY2-7 gene would reside within the 25 kb of genomic DNA. This genomic fragment was therefore used as a probe to screen an embryonic cDNA library. A single class of cDNAs was isolated (referred to as class I; Figure 3A), the largest of which was ~6.5 kb in length. Restriction mapping placed the cDNA ~20 kb away from the site of *P*-element insertion. *In situ* hybridization showed that the cDNA crossed the EY2-7 inversion allele breakpoint (data not shown). Sequence analysis revealed that the class I cDNA contained a 6-kb open reading frame (ORF) but lacked a stop codon. To obtain the full-length cDNA, several additional rounds of screening were needed to compile an ~18-kb contig.

Because the 5′ end of our cDNA was so far away from the *P*-element insertion site, we wanted to rule out the possibility that the true EY2-7 gene lay within the intervening ~20 kb of genomic DNA. To test whether there were additional transcripts in this region, we subdivided the intervening ~20 kb into five different subclones, and screened several cDNA libraries with each fragment. Two cDNA clones were isolated, one from an eye imaginal disc library (2A1) and the other from an adult head library (j1; Figure 3A). When sequenced, both were found to lack ORFs greater than ~250 amino acids in any reading frame. Because we isolated so many EMS alleles of EY2-7, we reasoned the gene was unlikely to encode a short polypeptide, and did not consider these noncoding cDNAs likely candidates to encode EY2-7. We have used RT-PCR to confirm that the non-

Figure 3.—Molecular characterization of EY2-7 (spen). (A) The 50-kb genomic region encompassing the *spen* gene is depicted as a black line. The numbers underneath indicate the size of the region in kilobases. The scale of the drawing is indicated with a short line representing 2 kb. The *P*-element lines that fail to complement EY2-7 mutations are inserted over an ~4-kb region. The position of the inversion allele, xLS1000, is indicated with a pair of lines at ~15 kb along the genomic sequence, and is marked as *In* for inversion. Three cDNAs, j1, 2A1, and class I, are indicated as thick black lines above the genomic line. j1 and 2A1 are noncoding cDNAs; class I corresponds to the *spen* gene. Introns are depicted as open triangles above the cDNAs. The predicted spen protein product derived from the class I cDNA is depicted underneath. Three structural domains, RRM, black box, coiled-coil (CC, striped box), and C-terminal conserved region (c-term, gray box) are indicated. The scale bar beneath indicates 500 amino acids. (B) The spen RRM motif is shown aligned with RRMs from several predicted proteins. The positions of conserved residues that define the RRM consensus sequence are indicated with an asterisk above the amino acid. The amino acid consensus sequence defining the RRM motif is indicated above the asterisk for the first RRM of spen. The RNP1 hexapeptide and RNP2 octapeptide regions for each RRM are indicated with a line above the amino acid sequence. The three spen RRMs are indicated with circled numbers 1, 2, and 3. In the alignment, identical amino acids are boxed and highlighted in dark gray. Similar amino acids are boxed and highlighted in light gray. Alignment was done using MacVector's ClustalW program. Accession numbers for all GenBank sequences are as follows: Dm 44a, AC005448; Hs spen, AL096858; Mm RRM, CAB01562 (the aligned sequence for Mm RRM is a composite of the GenBank sequence and sequence from a partial cDNA isolated from a mouse teratocarcinoma library; I. Rebay, unpublished results; sequence available upon request); Ce RRM, AF067616. (C) The conserved spen C terminus is shown aligned with C-terminal domains from several predicted proteins. Identical amino acids are boxed and highlighted in dark gray. Similar amino acids are boxed and highlighted in light gray. Alignment was done using MacVector's ClustalW program. Accession numbers for all GenBank sequences are as follows: Hs spen, AL096858; Dm 44a, AC005448; Ce CAA91320, Z66511. (D) Sequence of the composite full-length spen cDNA. Nucleotide sequence is indicated above; conceptual translation of the ORF is indicated below. The putative RRM region is underlined with a solid line. The predicted coiled-coil region is underlined with a dashed line. The conserved C-terminal region is highlighted in gray. Two putative potential polyadenylation signal sequences are highlighted in bold. GenBank accession no. AF184612.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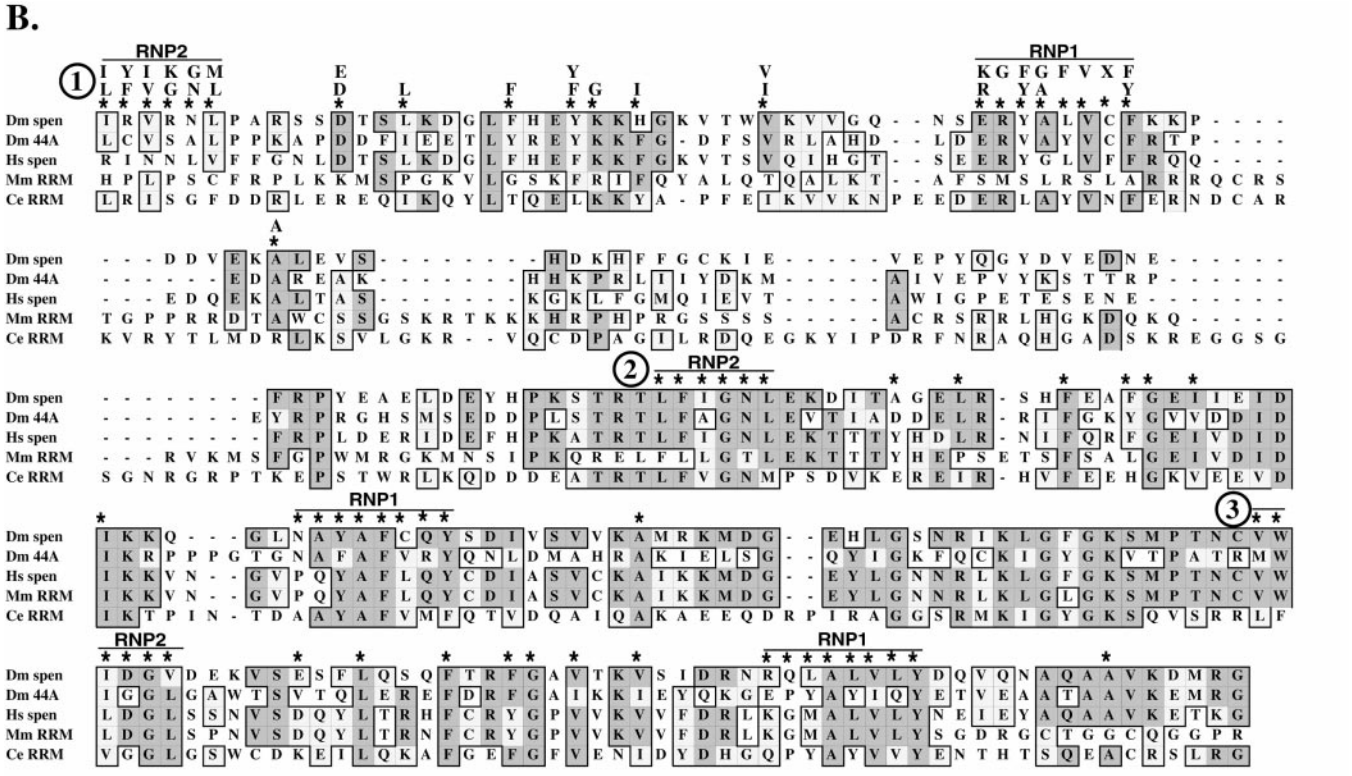
2A1 cDNA
Class I cDNA
P-elements
0
10
In
20
30
40
50
2 kb
SPEN protein
RRM
CC
c-term
500AA


B.
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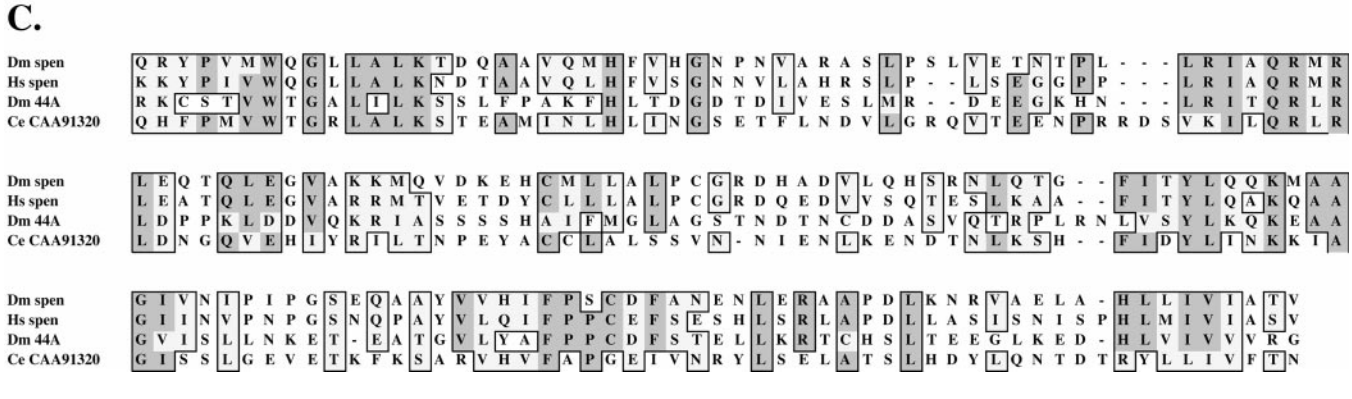
RNP1
Dm spen
Dm 44A
Hs spen
Mm RRM
Ce RRM


C.
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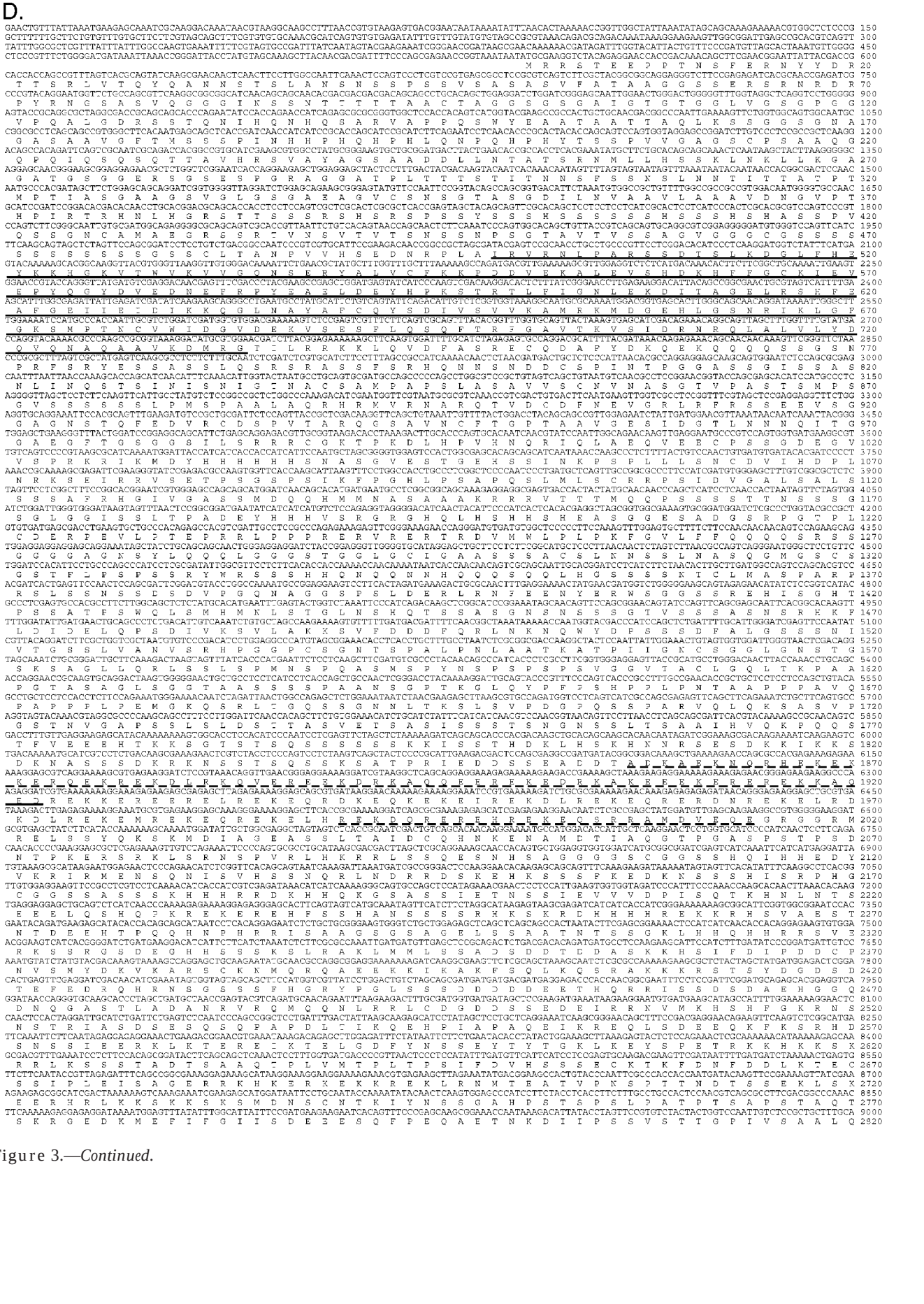

Figure 3.—*Continued.*

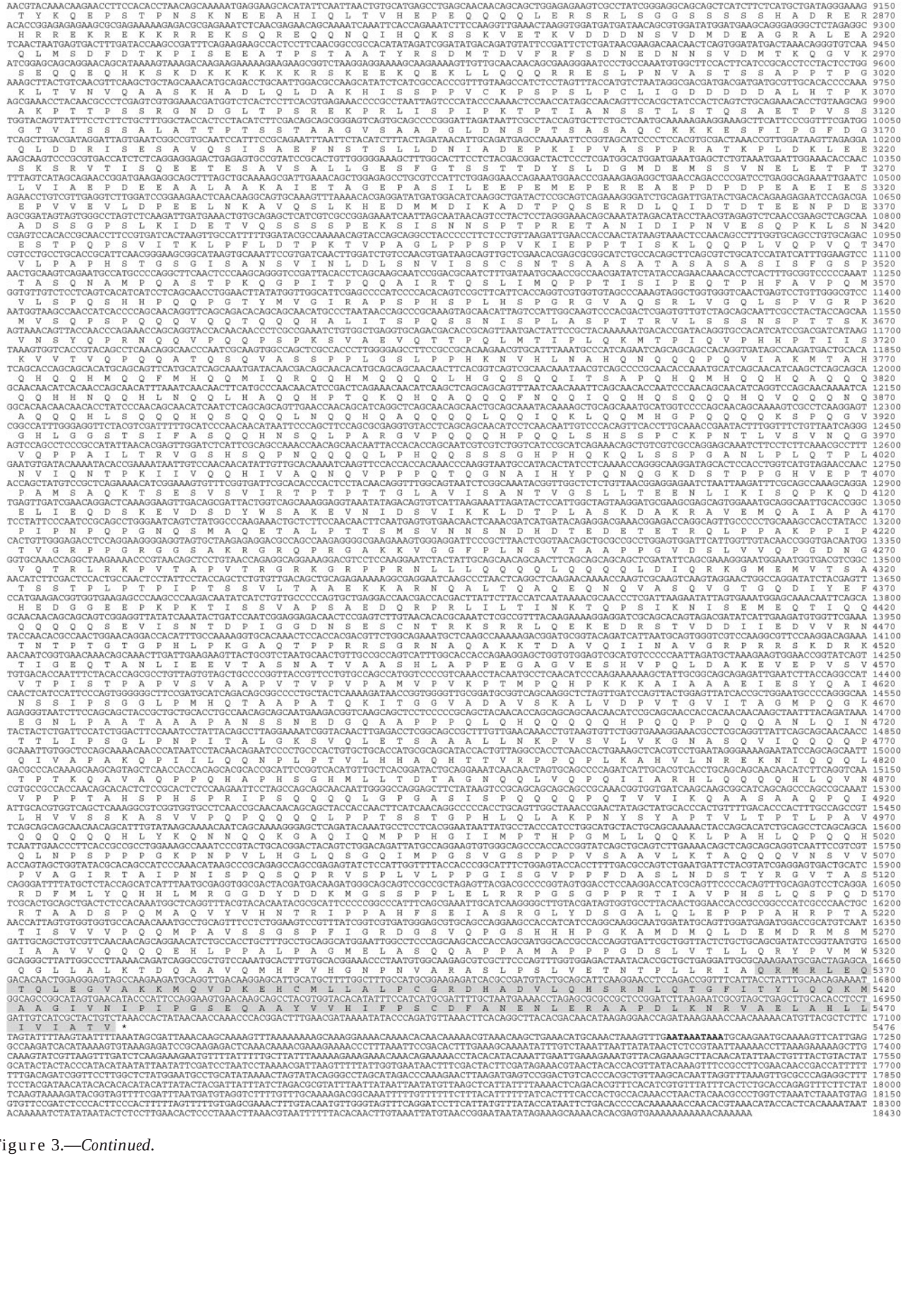

Figure 3.—*Continued.*

coding cDNAs we isolated reflect actual transcripts rather than possible genomic contaminants in the libraries (see materials and methods).

Another possibility was that the EY2-7 transcript was within this ~20-kb genomic region, but was of such low abundance that even extensive screening of the libraries would fail to detect it. However, confirming that our lack of success in finding a candidate cDNA within the ~20-kb genomic region was not simply due to its being an extremely low abundance message, but rather due to the lack of coding transcripts within this region, the BDGP has submitted complete genomic sequence covering this region (GenBank accession no. AC005334). Conceptual translation of this ~20-kb genomic DNA confirms the absence of any sizeable ORFs (data not shown), suggesting that there are no additional coding sequences residing in between the *P*-element insertion sites and our class I cDNA.

On the basis of this information, our best cDNA candidate for EY2-7 resides ~20 kb away from the *P*-element insertion sites. We think it is likely that the noncoding cDNAs we isolated may represent parts of a large and complex 5′ untranslated region (5′UTR) or possibly alternatively spliced 5′ ends of EY2-7. Supporting this idea, the insertion sites for several of the noncomplementing *P*-element alleles fall within one of these cDNAs (j1; Figure 3A).

Complementation tests show that EY2-7 is allelic to the gene *split ends* (*spen*) (Kolodziej *et al.* 1995). Mutations in EY2-7 fail to complement two alleles of *spen* that have been molecularly characterized and shown to have lesions within the coding sequence of the EY2-7/*spen* cDNA that result in predicted truncated protein products (B. Kuang and P. Kolodziej, unpublished results). This indicates that our class I cDNA is indeed encoded by the EY2-7 gene. From now on we will refer to EY2-7 as *spen*.

spen encodes a predicted protein of 5476 amino acids. Database searches indicate *spen* belongs to a family of RRM proteins. The RRM is a loosely conserved RNA binding domain of ~22 conserved amino acids spread over an 80–100-amino-acid-long region (for review see Burd and Dreyfuss 1994). The most highly conserved sequences within the RRM motif are two ribonucleoprotein (RNP) domains designated RNP2 (a hexapeptide) and RNP1 (an octapeptide). Other conserved residues are scattered throughout the domain and include primarily hydrophobic amino acids.

Spen contains three RRM motifs in tandem toward the N terminus of the protein (amino acids ~500–750). The spen RRMs are most similar to RRMs in several novel proteins of unknown function. These include proteins predicted from conceptual translation of the BDGP Drosophila genomic sequence and the *Caenorhabditis elegans* genomic sequence, and proteins predicted from conceptual translation of human and mouse EST sequences (Figure 3B). The first RRM of spen is most divergent from the RRM consensus, and is also more loosely conserved among other spen-like proteins. Homology between spen and other known RRM proteins in Drosophila or other species is less striking and strictly limited to residues defining the RRM consensus sequence (not shown). Thus, spen may define a new subclass of RRM proteins.

Other motifs in spen include a predicted coiled coil region over amino acids 1857–1922 (probability 1.0) and amino acids 1979–2014 (probability 0.589) that could be suggestive of protein-protein interactions (Berger *et al.* 1995; Wolf *et al.* 1997) and a highly conserved C-terminal domain of unknown function that is found in proteins from worms to humans (Figure 3, A and C). Otherwise, the spen protein sequence appears novel. Because some of the sequences identified by the database searches correspond to short EST sequences (for example, Mm RRM), it will be necessary to isolate full-length cDNA clones in order to determine whether these proteins contain both the RRM and the C-terminal domain. However, both motifs are found in a second Drosophila protein and in a human protein (referred to as Dm 44A and Hs spen, respectively, in Figure 3, B and C), suggesting that Drosophila spen is a member of a novel family of proteins defined by both the RRM and C-terminal motifs.

DISCUSSION

We have performed a dominant modifier screen for enhancers and suppressors of the rough eye phenotype associated with expression of the *yan*^{ACT} transgene in the developing Drosophila eye with the goal of isolating novel components of the RTK/Ras/MAPK pathway. Genetic tests suggest 11 of the 21 complementation groups recovered are Ras pathway relevant genes. We have found that 4 of these 11 groups are allelic to known RTK/Ras pathway genes. Isolation of known Ras pathway signaling components confirms that both the screen and the genetic tests are working as designed. That is, known Ras pathway genes are isolated as modifiers of *yan*^{ACT}, and these genes can be functionally distinguished from the promoter-specific factors that were also recovered in the screen.

In addition to known pathway components, we have also isolated a set of seven genes that have not been implicated previously in RTK signaling events, but appear likely to play important roles in this pathway, based on the genetic tests. Thus, by initiating a genetic modifier screen based on one of the most downstream components of the RTK pathway identified to date, we have successfully isolated new candidate signaling molecules that were not recovered in previous RTK pathway-based screens. These seven genes include two genes with known functions in other developmental contexts, *eyes absent* and *string*, as well as five previously uncharacterized genes. We have initiated the molecular character-

ization of one of these novel genes, called *split ends*, and show it encodes a member of the RRM family of RNA-binding proteins.

Isolation of known RTK pathway genes: Among the mutations in known RTK pathway genes isolated in our screen, 44 alleles of the Ets domain transcription factor *pointed* were recovered as strong enhancers of *yan*^{ACT}. Genetically, *pointed* is a positive regulator of RTK/Ras-mediated signals, functioning downstream of MAPK in multiple developmental contexts including the developing oocyte, the embryonic CNS, and the photoreceptors of the eye (Klambt 1993; Brunner *et al.* 1994; O'Neill *et al.* 1994; Scholz *et al.* 1997). Experiments in cultured cells have demonstrated that *pointed* is a transcriptional activator whose activity is stimulated by the Ras/MAPK pathway (O'Neill *et al.* 1994). Because pointed and yan are members of the same family of transcription factors and will bind to the same DNA sequences *in vitro*, it has been postulated that cellular responses to RTK signaling may involve a competition between yan and pointed for access to certain promoters. An alternate model is that yan and pointed represent two different branches of gene regulation downstream of MAPK. Efforts to distinguish between these hypotheses have been thwarted by our lack of knowledge of the identities of these downstream targets. In addition, other than homology and parallel placement in the Ras pathway, there was no evidence supporting a functional interaction between yan and pointed. Our isolation of *pointed* alleles as dominant enhancers of *yan*^{ACT} is a strong indication of an intimate interaction between yan and pointed. Future genetic and biochemical investigations will be required to understand more fully the mechanisms underlying the genetic relationship between these two transcription factors in RTK signaling events.

Our screen also identified a group of 35 enhancers allelic to *Star*. *Star* encodes a membrane protein of unknown biochemical function that is thought to assist in processing of the epidermal growth factor receptor (Egfr) ligand Spitz, thereby promoting signaling (Kolodkin *et al.* 1994; Pickup and Banerjee 1999). *Star* mutations exhibit a dominant rough eye phenotype, suggesting the enhancement of *yan*^{ACT} could simply represent an additive phenotype rather than a synergistic interaction. On the other hand, the enhancement of *yan*^{ACT} is extremely strong, reducing the eye to a tiny slit (data not shown). This phenotype is stronger than would be expected if the phenotypes were simply additive, and this suggests that there may be a synergistic aspect to the interaction. Mutations in *Star* have been isolated in numerous, but not all, genetic screens based on rough eye phenotypes, again suggesting there may be meaningful specificity to the interaction (Dickson *et al.* 1996; Karim *et al.* 1996; Verheyen *et al.* 1996; Neufeld *et al.* 1998). Assuming Star functions at the level of regulating the production of an activated ligand for Egfr (Pickup and Banerjee 1999), the interaction with yan is likely to be indirect unless regulatory feedback loops or direct transcriptional control is involved.

The third known RTK pathway gene isolated in our screen is *rolled*. *rolled* encodes the MAPK that functions downstream of multiple RTKs in Drosophila, and has been shown to phosphorylate yan directly *in vitro* (Biggs *et al.* 1994; Brunner *et al.* 1994). Because the yan^{ACT} product lacks the eight MAPK consensus phosphorylation sites and can thus presumably no longer be phosphorylated by MAPK, it is likely that enhancement of the yan^{ACT} eye phenotype by mutations in *rolled* reflects either an interaction with the endogenous wild-type yan protein or with other pathway components such as pointed. For example, reducing the activity of MAPK would increase the stability of the endogenous pool of yan protein, thereby exacerbating the rough eye phenotype associated with yan^{ACT}. Reduction in MAPK activity would also result in a reduction in levels of pointed activation, which, based on our isolation of *pointed* mutations in the screen, causes a strong enhancement of the yan^{ACT} rough eye phenotype.

The final known RTK component we isolated is *son of sevenless* (*sos*), the guanine nucleotide exchange factor (GEF) for Ras (Rogge *et al.* 1991; Simon *et al.* 1991; Chardin *et al.* 1993). We were somewhat surprised to isolate alleles of *sos* because prior to initiating the genetic screen, we had tested all available RTK pathway mutations, including *sos* null mutations, for interaction with our yan^{ACT} background (I. Rebay and G. M. Rubin, unpublished results). We found that mutations in *sos* exhibited a barely detectable enhancement of the rough eye phenotype, and thus we did not expect to isolate *sos* in our screen. However, mapping and complementation tests showed that EY2-3, a complementation group of 10 alleles that includes several extremely strong enhancers of yan^{ACT} (Figure 2, B and E), is allelic to *sos*. In addition, *sos* null alleles are strong suppressors of the RasV12 background, whereas our EY2-3 alleles show little or no suppression (F. Chen and I. Rebay, unpublished observations). On the basis of the difference in interaction patterns between null *sos* alleles and our EY2-3 alleles, we think it is likely that our screen has selected for a particular class of *sos* alleles. In addition to the Ras GEF domain, sos also contains a so-called Dbl homology domain that is thought to serve as a GEF for the Rho family of small GTPases that included Ras, Rho, and Cdc42 (Nimnual *et al.* 1998; Soisson *et al.* 1998). Thus, one possibility is that our EY2-3 alleles have normal Ras GEF function, but have altered Rho GEF function. If correct, this hypothesis implies a role for yan downstream of the Rho family GTPases in addition to its role downstream of the Ras pathway.

Consistent with the idea that our *yan*-based screen may be identifying genes that function in pathways other than the Ras/MAPK cascade and that yan itself may be a downstream target of multiple signaling pathways, yan

has been shown previously to be a downstream target of Jun kinase (Jnk; Riesgo-Escovar and Hafen 1997). Jnk is a member of the MAPK family, and is thought to function downstream of the Rac/Rho/Cdc42 GTPases (Noselli 1998). *In vitro*, Jnk can directly phosphorylate yan on the same sites phosphorylated by rolled/MAPK, and based on genetic interactions between *yan* and *basket* (Jnk), this phosphorylation presumably destabilizes and inactivates yan (Riesgo-Escovar and Hafen 1997). It is possible that *sos*, being capable of serving as a GEF for both Ras and Rho family GTPases, may be a key mediator of cross-talk between these signaling cascades, and that yan, a direct target of at least two MAPK family members, may represent an additional downstream integration point for the different signals. Further investigation of points of intersection between the Ras, and the Rac, Rho, and Cdc42 signaling pathways will be required to elucidate the mechanisms underlying these interactions.

Isolation of *string* and *eyes absent*, two known genes that have not been implicated previously in RTK signaling events: Among the remaining seven pathway relevant candidates are two genes of known function that have not been implicated previously in RTK/Ras-mediated signaling events. One of these groups, SY3-4, is allelic to the phosphatase *string*, a Drosophila homologue of the yeast cell cycle gene *cdc25* (Edgar and O'Farrell 1989). *string* regulates the G2-M transition in dividing cells by dephosphorylating and activating the cdc2 kinase, thereby allowing formation of cyclin/cdc2 complexes that promote S phase (Edgar and O'Farrell 1990). On the basis of the direction of interaction with yan^{ACT}, *string* would be postulated to be an antagonist of Ras signaling. Previous suggestions of a possible antagonistic relationship between *string* and Ras signaling came from a screen for modifiers of *roughex*, a negative regulator of G1 progression in the developing eye, which identified *string* as a suppressor and *ras1* as an enhancer (Thomas *et al.* 1994).

yan itself has been implicated in cell cycle control (Rogge *et al.* 1995). Whereas hypomorphic yan mutations are semi-viable and have an extra photoreceptor phenotype (Lai and Rubin 1992), null mutations in *yan* are embryonic lethal, with the embryos dying as a result of overproliferation of cells in the dorsal neuroectoderm (Rogge *et al.* 1995). Thus, depending on the developmental context, *yan* regulates not only the transition between undifferentiated and differentiated cell types, but also the choice between differentiation and cell division. Recovery of *string* alleles in our screen could reflect cross-talk between cell cycle and differentiation pathways that occurs in part at the level of transcriptional regulation. Thus, it is possible that the downstream targets of *yan* will include cell cycle regulators such as *string* or that yan expression and stability may be linked to cell cycle controls. Alternatively, string could have postmitotic functions essential to differentiation.

To our knowledge, *string* has not been isolated in any other Ras pathway screens; however, it was isolated, again as a suppressor, in a screen for modifiers of activated Notch (Verheyen *et al.* 1996). The direction of interaction suggests *string* acts as a positive regulator of Notch signaling. Expression of N^{ACT} and yan^{ACT} have similar developmental consequences in that both inhibit or delay differentiation of the cell types in which they are expressed (Fortini *et al.* 1993; Rebay and Rubin 1995). Isolation of *string* in both screens could indicate a point of cross-talk between the Notch and the RTK/Ras pathway. Alternatively, isolation of *string* as a suppressor of both $Notch^{ACT}$ and yan^{ACT} could have more to do with the similar terminal phenotype of these two backgrounds rather than reflecting direct interactions with the two pathways. Supporting the first hypothesis, cross-talk between the Notch and RTK pathways has been reported by numerous labs (Cagan and Ready 1989; Rogge *et al.* 1995; Karim *et al.* 1996; Verheyen *et al.* 1996; Price *et al.* 1997). Despite all the genetic interaction data, the mechanisms whereby the Notch and RTK pathways intersect remain to be determined. Experiments designed to study signaling by both pathways *in vivo* have suggested an antagonistic relationship (de Celis and Bray 1997; Price *et al.* 1997; Greenwald 1998; Miller and Cagan 1998), which would be consistent with *string* acting as a negative regulator of Ras signal transduction and a positive regulator of Notch signal transduction.

The second gene with a previously defined function that may be relevant to the RTK pathway on the basis of our genetic tests is *eyes absent* (*eya*; Bonini *et al.* 1993). *eya* encodes a novel nuclear protein of unknown function that has been shown recently by several labs to function in a hierarchy of "master eye regulatory genes" that are required to specify and promote differentiation of eye tissue (Chen *et al.* 1997; Pignoni *et al.* 1997; Halder *et al.* 1998). However, on the basis of expression pattern and phenotypes, it is possible that *eya* plays additional roles in development independent of its role in determining competence to become eye tissue (Bonini *et al.* 1998; Leiserson *et al.* 1998). One possibility is that *eya* could be directly complexed with *yan*, and could direct its transcriptional repressor activity in certain tissues. However, preliminary yeast two-hybrid experiments have failed to indicate yan-eya protein-protein interactions (A. Williams and I. Rebay, unpublished results). An alternate possibility to be investigated is transcriptional regulation of eya by yan. Given the genetic interactions we have observed between *eya* and yan^{ACT}, it will be interesting to investigate the possible role of eya in RTK/yan-mediated signaling events in the embryo and developing eye. It could be that in order to differentiate as eye tissue, a developing cell must receive both a "general" differentiation signal from the RTK pathway and a more specific eye fate specification signal.

Isolation of potential new components of the RTK/

Ras/Yan signaling pathway: Four of the remaining five pathway relevant complementation groups, EY2-5, EY2-6, EY3-5, and SY2-2, are in the initial stages of characterization. Potential roles for these genes include downstream transcriptional targets of yan, upstream kinases or phosphatases, proteins directly complexed with yan that regulate its activity, or elements of intersecting signaling pathways that cross-talk with the Ras pathway at the level of yan (Figure 4). We are particularly interested in SY2-2 as it is one of the few suppressor groups recovered in the screen that passed both genetic tests, strongly suppressing GMR-*yan*^{ACT}, Sev-*yan*^{ACT}, and *yan*^{S2382} (Figure 2, C and D; Table 1). Based on the direction of our screen, suppressor mutations are expected to be negative regulators of Ras signal transduction. In addition, both alleles of SY2-2 interact very strongly, in the expected direction, with all Ras pathway backgrounds tested except GMR-*sina* and GMR-*ttk* (Table 1). Therefore, because of the unique nature of SY2-2 in the screen and because of the strength and consistency of its genetic behavior, a primary focus in the future will be to determine what it encodes. To date, complementation analyses with available Ras pathway and other candidate genes have been uninformative.

spen encodes a novel member of the RRM family of RNA-binding proteins: We have cloned the fifth pathway relevant complementation group, *spen* (EY2-7), and found that it encodes a member of the RRM family of RNA-binding proteins (Burd and Dreyfuss 1994). The RRM sequence is an 80–100-amino-acid stretch of sequence that forms an RNA-binding domain (RBD). This motif is characterized by a bipartite RNP consensus sequence that forms the RNA-binding surface of the domain, as well as several predominantly hydrophobic amino acids dispersed throughout the RRM that are essential for the overall structure of the domain. RRM-containing proteins mediate a variety of post-transcriptional RNA-processing reactions including mRNA splicing, stability, and transport. In Drosophila, RNA-processing reactions and the RRM-containing proteins that mediate them are critical to multiple developmental processes. For example, in sex determination, sex-specific alternate splicing reactions are regulated by the RRM-containing proteins tra2 and Sxl (Bell *et al.* 1988; Amrein *et al.* 1994). Establishment of dorsoventral polarity in the oocyte requires input from the RRM protein squid to localize the mRNA encoding the Egfr ligand gurken (Matunis *et al.* 1994). Another RRM protein, elav, is expressed in all Drosophila neurons and is required for determination and maintenance of the neuronal fate, although the precise mechanism of function is not clear (Koushika *et al.* 1996; Yannoni and White 1997).

spen has three RRM motifs in tandem within the first 1000 amino acids of the protein. Database searches identify a number of predicted proteins from either genomic sequencing efforts or EST projects that have very similar RRMs. For these proteins, the homology within the RRM region extends beyond the conserved residues that define the motif. Homology between spen and other RRM-containing proteins is less striking and is limited to the residues that comprise the motif (data not shown). Because the RRM domain is thought to mediate specificity of RNA-binding interaction, it is possible that the spen class of RRM proteins interacts with similar substrates (Burd and Dreyfuss 1994). Further experiments will be required to determine whether spen actually binds RNA, what its *in vivo* targets are, and what the function of this activity is in the context of RTK signaling events during development.

Two additional structural motifs of note in the spen protein are the C-terminal ~170 amino acids and a region of predicted coiled-coil near the middle of the protein. The coiled-coil domain is likely to mediate pro-

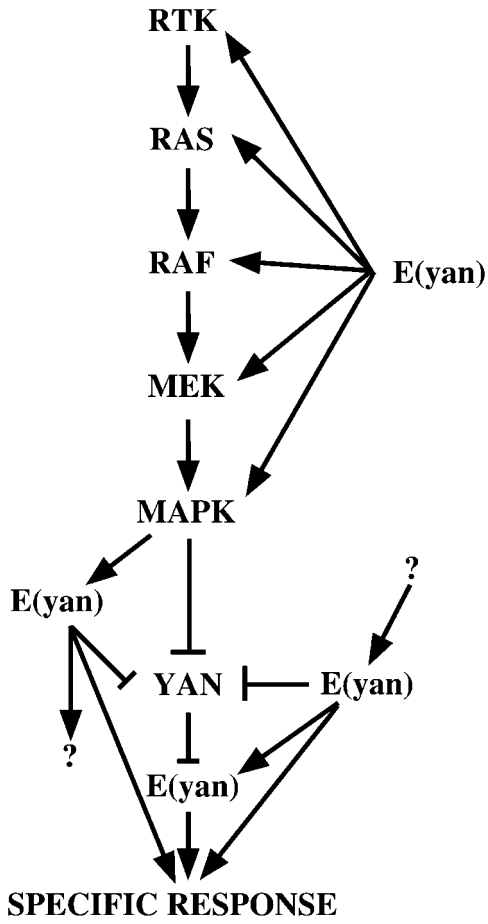

Figure 4.—Potential functions for yan-interacting genes isolated in the screen. A simplified RTK pathway is depicted. Arrows indicate a positive direction of interaction, and blunt arrows indicate a negative direction of interaction. Enhancers of *yan*^{ACT} [E(yan)], predicted to be positively acting components of the pathway, could potentially function as downstream targets of yan's transcriptional activity, as cofactors binding to yan that regulate its transcriptional activity, as components of other intersecting signaling pathways that feed into the RTK pathway at the level of yan, as other downstream targets of MAPK that function in parallel or in conjunction with yan, or as regulators of the RTK/Ras/MAPK cascade upstream of yan.

tein-protein interactions, either with spen itself or with other proteins (Berger *et al.* 1995; Wolf *et al.* 1997). One possibility is that spen interacts directly with yan via this domain. Alternatively, the interaction between spen and yan could be less direct. Homology searches using the C terminus of the spen detect several proteins, several of which, including a Drosophila protein predicted from the BDGP genomic sequence and a human protein compiled from multiple overlapping ESTs, also have spen-class RRMs. Although the structure of the C terminus of spen is not homologous to any protein domain of known function, the high degree of conservation of this region between fly, worm, and mammalian proteins suggests it is likely to have a conserved function. Apart from the RRM, coiled-coil, and C-terminal domains, the rest of the spen protein is novel, and to date it is uninformative in terms of providing clues as to function. Because the protein is so large (5476 amino acids), structure-function analyses and determination of the molecular lesions associated with the various spen alleles will be required to determine whether there are other important functional domains not detected by sequence homology. Investigation of the function of spen homologues in other species may also be informative.

Our isolation of *spen* as an enhancer of *yan*^{ACT} suggests it may play a role as a positive regulator of the RTK/Ras pathway. Preliminary results indicate spen is a nuclear protein broadly expressed in most tissues and enriched in neuronal lineages (F. Chen and I. Rebay, unpublished results). We currently do not know whether *spen* functions upstream or downstream of *yan*. One possibility is that *spen* might regulate the stability of the *yan* transcript. It has been postulated that the mechanism for downregulating *yan* activity involves post-translational modifications of the protein, namely phosphorylation by activated MAPK, that subsequently targets *yan* for degradation. Such post-translational regulation of *yan* would presumably need to be reinforced at the transcriptional and/or translational level. Thus, *spen* might play a role in destabilizing *yan* mRNA in response to Ras signaling. This would be consistent with our isolating mutations in *spen* as enhancers of *yan*^{ACT}. Alternatively, spen could be transcriptionally regulated by *yan*, and could play a role in splicing, stability, or transport of other downstream effector genes. Future phenotypic, genetic, and biochemical characterization of *spen* will be necessary to understand its role in Ras/yan signaling events.

In conclusion, our genetic screen has identified several genes that are capable of modulating Ras/MAPK/yan-mediated signaling events. Among these genes are known RTK pathway elements, thereby validating the screening approach and suggesting that those genes that had not been implicated previously in RTK signal transduction may also be relevant to the pathway. Our hope is that further analysis of these new genes will improve our understanding of RTK pathway function in different contexts throughout development, and will shed light on the mechanisms whereby the RTK-mediated signals are integrated with other developmental signals to effect coordinated and context-appropriate cellular responses.

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Note added in proof: The isolation and sequence of *spen* was recently reported by E. L. Wiellette, K. W. Harding, K. A. Mace, M. R. Ronshaugen, F. Y. Wang and W. McGinnis (1999, spen encodes an RNP motif protein that interacts with Hox pathways to repress the development of head-like sclerites in the Drosophila trunk. Development **126:** 5373–5385).

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