Homology of the *eyeless* Gene of *Drosophila* to the *Small eye* Gene in Mice and *Aniridia* in Humans

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A *Drosophila* gene that contains both a paired box and a homeobox and has extensive sequence homology to the mouse *Pax-6* (*Small eye*) gene was isolated and mapped to chromosome IV in a region close to the *eyeless* locus. Two spontaneous mutations, ey^2 and ey^R, contain transposable element insertions into the cloned gene and affect gene expression, particularly in the eye primordia. This indicates that the cloned gene encodes *ey*. The finding that *ey* of *Drosophila*, *Small eye* of the mouse, and human *Aniridia* are encoded by homologous genes suggests that eye morphogenesis is under similar genetic control in both vertebrates and insects, in spite of the large differences in eye morphology and mode of development.

A small multigene family of paired box–containing genes (*Pax* genes) was first identified in *Drosophila* and subsequently found in vertebrates from zebrafish to humans (*1*). *Pax* genes encode sequence-specific DNA-binding transcription factors that play an important role in embryonic development, particularly in the nervous system. Pax proteins are characterized by a 130–amino acid paired domain, which functions as a sequence-specific DNA-binding domain. In addition, some Pax proteins contain a second DNA-binding domain, a homeodomain, which in some cases is truncated, or a specific octapeptide (or both). These multiple combinations of protein domains illustrate evolutionary tinkering at the molecular level (*2*). In contrast to *Hox* genes, which in mammals are arranged in four repeated homologous clusters, the *Pax* genes are dispersed and are present in single copies. Mutants are known for at least three of the nine known *Pax* genes in mammals. *Pax-1* is mutated in *undulated* mice (*3*); *Pax-3* is affected in *Splotch* mice (*4*), which corresponds to Waardenburg's syndrome in humans; and the *Small eye* mutations in mice (*5*) and rats (*6*) and the *Aniridia* mutation in humans (*7*) affect the *Pax-6* gene. The degree of evolutionary conservation among *Pax* genes is higher than it is for *Hox* genes. For example, the coding regions of the *Pax-6* genes of mice and zebrafish (*8*) are 80% identical in their nucleotides and 97% identical in their amino acids (*8*). A single amino acid difference is found in the respective paired boxes, and the two homeodomains are identical. There is also a strong conservation of splice sites, which indicates that the two genes are homologous.

We have isolated the *Drosophila* homolog of *Pax-6*, which shows 94% amino acid sequence identity to *Pax-6* of humans, mice, and quail (*9*) and 93% identity to zebrafish in the paired domain. The homeodomains of *Pax-6* show 90% sequence identity between *Drosophila* and vertebrates. Loss-of-function mutations in *Pax-6* primarily affect eye development. A reduction in eye size and the absence of the iris are observed in heterozygous carriers of the human *Aniridia* syndrome, and the eyes are reduced in heterozygous *Small eye* mutant mice and rats, whereas homozygous mutant embryos lack eyes completely and die. We found that the *Pax-6* homolog of *Drosophila* is encoded by the *eyeless* (*ey*) gene, which is also involved in eye morphogenesis. These findings indicate that not only the amino acid sequences have been conserved in evolution but also the function of the gene in the developmental pathway leading to eye morphogenesis. Because *Pax-6* is involved in the genetic control of eye morphogenesis in both mammals and insects, the traditional view (*10*) that the vertebrate eye and the compound eye of insects evolved independently has to be reconsidered.

The *Drosophila* homolog of *Pax-6* was isolated from an expression library in a screen in which an oligonucleotide corresponding to a homeodomain binding site was used as a probe (*11*). One clone, 8321, was isolated that bound the oligonucleotide probe particularly strongly. Sequence data revealed that the clone contained both a paired box and a homeobox, and a computer search through the European Molecular Biology Laboratory database indicated that the clone had homology to mouse *Pax-6* and human *Aniridia* sequences. In situ hybridization of 8321 phage DNA to giant polytene chromosomes gave two signals, one located at 102D on chromosome IV and another located at 67B on chromosome II. A 1.1-kb Eco RI fragment containing the paired box and homeobox sequences hybridized only to 102D, a region close to the *ey* locus.

In order to isolate more complete complementary DNA (cDNA) clones, a λ gt10 library prepared from 3- to 12-hour *Drosophila* embryos (*12*) was screened by plaque hybridization at high stringency with the 1.1-kb Eco RI fragment from phage 8321. The longest cDNA clone isolated, E10, had an insert of 2.8 kb. The complete nucleotide sequence (*13*) and the deduced amino acid sequence of E10 are shown (Fig. 1). The E10 cDNA insert is 2850 base pairs (bp) long, and the open reading frame starts at position 89 and terminates at position 2603, which suggests that it encodes a protein of 838 amino acids with a predicted molecular weight of 82,490 daltons and an isoelectric point (pI) of 7.9. All three reading frames upstream of the ATG codon at position 89 are closed. The sequence preceeding this ATG, CAACTATG, corresponds to the consensus translation initiation sequence C-AAAC-AATG for *Drosophila* (*14*), except that an additional T residue is inserted in front of the ATG as it is in the zebrafish *Pax-6* (*8*) and in the *Drosophila paired* genes (*15*). This suggests that the ATG at position 89 represents the initiation codon. A termination codon TGA is found at position 839, followed by a putative polyadenylation signal (AATAAA) and a 17-nucleotide polyadenylate [poly(A)] tract (Fig. 1). Near the NH_2-terminus of the deduced protein sequence, a 130–amino acid paired domain was found, which in terms of its amino acids is 94% identical to the paired domains of *Pax-6* in mice, humans, and quail and 93% identical to that of zebrafish (Fig. 2). Amino acids differing from those found in vertebrates are indicated (Figs. 1 and 2). Two of these substitutions are in positions that so far had been found to be invariant. Position 14, occupied by asparagine in all previously known paired boxes, is replaced by glycine, whereas the invariant proline at position 78 is replaced by alanine. Twenty-four out of 28 amino acids characteristic of *Pax-6* in vertebrates are also found in *Drosophila*.

The paired box is separated from a paired-type homeobox by a linker region that is considerably larger in *Drosophila* than in vertebrates. Nevertheless, there is also scattered sequence homology in the linker region (Fig. 1). The homeodomains of *Drosophila* and of vertebrate *Pax-6* homologs differ at only 6 out of 60 positions (Fig. 2): four in α helix I, one in α helix II, and one in the turn of the helix-turn-helix motif (aspartate → glycine). A characteristic feature of the homeodomains of the paired class is an extended homology of 18 amino acids at the NH_2-terminus (*1*). However, in the *Pax-6* homologs, this extended homology is confined to six amino acids immediately preceding the homeodomain. These six amino acids, LILKRK in *Drosoph-*

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ila and LQLKRK in vertebrates (*16*), are completely conserved in at least 11 proteins, including the *Pax*-6 homologs of humans, mice, rats, quail, and zebrafish and also in the *Drosophila* genes *paired* (*15*), *gooseberry*, *gooseberry-neuro* (*17*), and *aristaless* (*18*). The three basic residues in this sequence suggest that it may possibly represent a nuclear targeting signal. With the exception of *aristaless*, which contains a homeodomain only, all these proteins have a paired domain. At the COOH-terminus, another seven amino acids immediately adjacent to the homeodomain (KLRNQRR) (*16*) are completely conserved in all presently known *Pax*-6 homologs. The 47 amino acids located COOH-terminally of the homeodomain show 45% sequence identity between mice and *Drosophila*. The COOH-terminal region includes 368 amino acids and is more than twice as long as the corresponding region in vertebrates. It is rich in alanine (11%) and glycine (8%), in serine (20%) and threonine (6%), and also in proline (11%). Another region of limited homology is found between amino acids 629 and 660, in which 35% identity is found between the *Drosophila* gene and mouse *Pax*-6. These sequence data indicate that the cloned *Drosophila* gene is homologous to the *Pax*-6 gene in vertebrates.

The sequence of a larval transcript isolated from an imaginal disk cDNA library (*19*) is represented in the upper part of Fig. 1. The 5′ sequence of the larval cDNA differs from that of the embryonic transcript because it contains a different first exon that is spliced directly to exon 3, which contains the paired box. The proteins encoded by the two types of transcripts differ only with respect to the NH_2-terminal sequences up to the first amino acid in the paired domain.

The genomic organization of the locus was determined by the isolation and analysis of a number of overlapping clones from genomic libraries of *Drosophila melanogaster* with the 1.1-kb Eco RI fragment of phage 8321 and a 1.4-kb Eco RI fragment of the cDNA clone E10 as probes (*20*). The transcription unit spans approximately 18 kb (Fig. 3), but the transcription initiation site has not been determined. The gene encodes two transcripts, which differ with respect to their first exons, that are spliced to exon 3, which is shared. The splice junctions were determined by sequencing the genomic clones and defining the exon-intron boundaries. The paired domain is encoded by three exons. The splice site in the first codon of the paired box and the third splice site between codons 116 and 117 are at exactly the same position as reported for *Pax*-6 in humans, mice, and quail, which suggests that the *Drosophila* gene is a true homolog of the vertebrate *Pax*-6 gene. The second splice site in the paired box differs from that in vertebrates, which has a characteristic position between codons 44 and 45. This site serves for differential splicing, because it can accommodate an additional exon of 42 bp in some

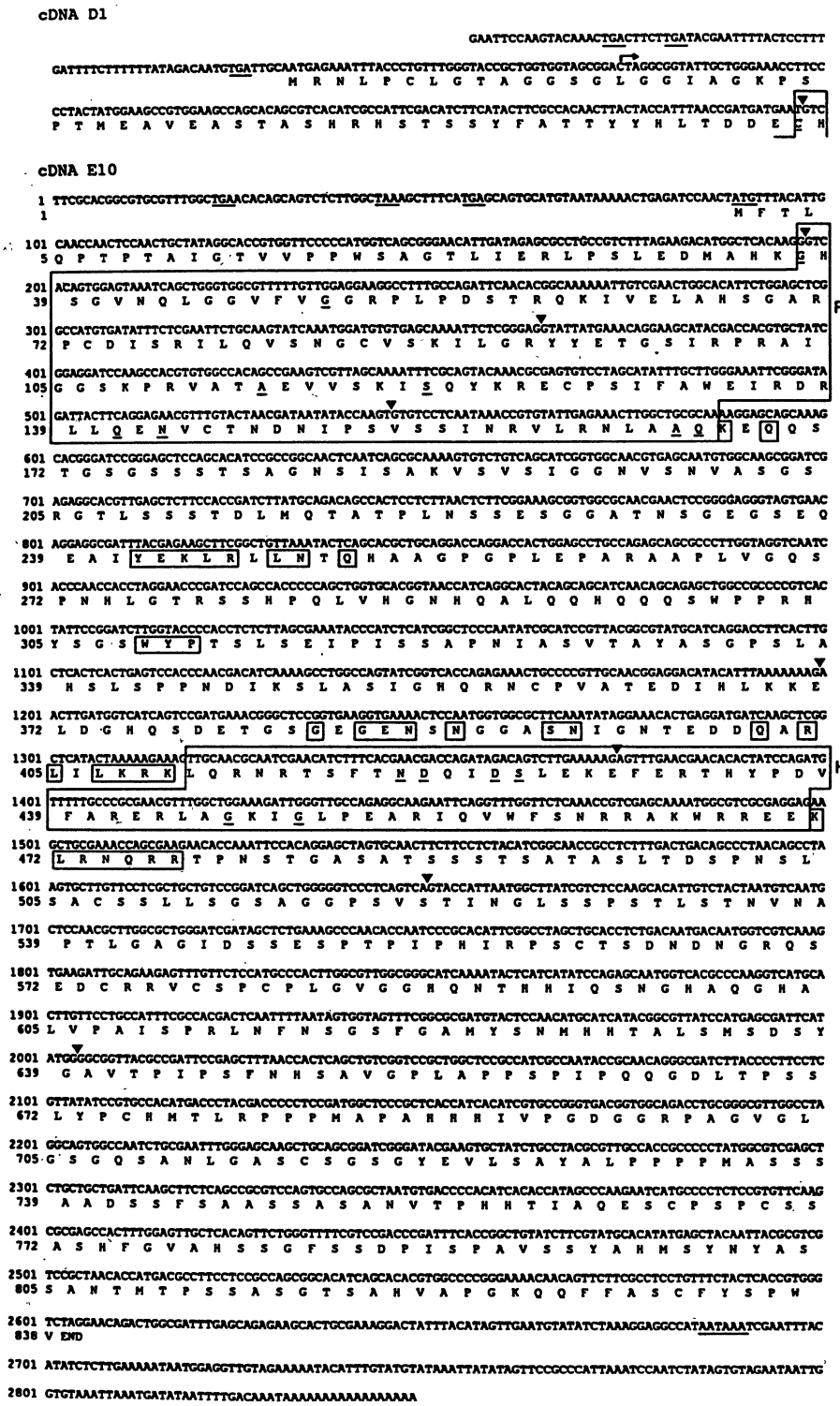

Fig. 1. Nucleotide and deduced amino acid sequences of a larval (D1) and an embryonic (E10) cDNA clone of the *Drosophila Pax-6* homolog, which corresponds to the *ey* gene (*16*). The D1 sequences differ from those of E10 with respect to the first exon preceding the paired box. The paired domain (PRD) and the homeodomain (HD) are boxed in. The stop codons preceding the putative translation initiation sites are underlined. An arrow indicates the 5′ end of D1. Amino acids differing from those found in *Pax-6* of vertebrates are underlined; identical amino acids outside of the paired domains and homeodomains are framed in. The splice sites are indicated by arrowheads. The putative polyadenylation signal is underlined.

transcripts, which encode a 14–amino acid insertion into the paired domain, that are found in humans, mice, quail, and zebrafish (*5–8*). In *Drosophila*, this site is missing, and the second splice site in the paired box is located further downstream in codon 58, which is the same position as in the *Pax* genes 2, 5, and 8 of the mouse. One of the splice sites in the homeodomain, in codon 19, is also conserved in evolution, whereas the second splice site found in *Pax-6* of humans and quail is absent in *Drosophila*.

By in situ hybridization to polytene chromosomes, the *Drosophila Pax-6* homolog was mapped to position 102D on chromosome IV, a position close to the *ey* locus (Fig. 3B). A number of different *ey* mutations have been isolated, some of which are lethal. The viable hypomorphic alleles show a characteristic reduction in the size of the compound eyes, which in some individuals may be missing completely (*21*). To find out whether *Drosophila Pax-6* corresponds to *ey*, we analyzed the available *ey* mutants by whole-genome Southern (DNA) blots. DNA from the two spontaneous alleles ey^2 and ey^R showed an alteration in the pattern of restriction fragments that hybridize to the corresponding wild-type probe. In both mutants, different restriction fragments hybridized to the 1.7-kb Sal I fragment of ey^+ (Fig. 4). This suggests that these two mutations are caused by insertions of transposable elements near the 5′ end of the cloned gene (Fig. 3), a common phenomenon in spontaneous *Drosophila* mutants. The size and restriction pattern of the insertion in ey^2 resembled the *doc* transposable element (*22*), which we confirmed by cloning a hybrid phage carrying this region from a genomic ey^2 library (*23*) and determining the DNA sequences around the point of insertion. The presence of a *doc* element in ey^2 at 102D has also been confirmed by in situ hybridization (Fig. 3C). A corresponding phage was isolated from an ey^R genomic library and analyzed (*23*). The partial DNA sequence obtained from this phage allowed us to identify the inserted element as *blastopia*, a retrotransposon identified recently by its spatially restricted expression in the prospective head region of the blastoderm-stage embryo (*24*).

To find out whether the two insertions affect the expression of the cloned gene, whole-mount in situ hybridizations to embryos and larvae of wild-type, ey^2, and ey^R homozygous mutants were carried out. Transcripts were detected in a bilaterally symmetrical pattern in the brain and in every segment of the ventral nervous system for ey^+ embryos at the germ-band stage (Fig. 5, A and B). Anterior to the brain, transcripts accumulated in the primordia of the eye imaginal discs (Fig. 5B) (*25*). This site of expression was not detected in ey^2 mutants (Fig. 5C). Later in embryogenesis, the transcripts became confined to the brain and the primordia of the eye disks. In the eye-antennal disks of third instar wild-type larvae, a band of expression at the anterior edge of the eye disk was detected (Fig. 5D), whereas hardly any transcripts were detectable in ey^2 (Fig. 5E) and ey^R (Fig. 5F) eye disks (*26*). The lack of expression in the eye primordia of the ey^2 and ey^R mutants indicates that the transposon insertions affect the expression of the cloned gene and that the *Drosophila Pax-6* homolog is indeed the *ey* gene. Our preliminary analysis of the distribution of *ey* transcripts showed that the expression was confined to the eye imaginal disks and was not detectable in leg or wing disks. The transcripts accumulated in the cells at the anterior

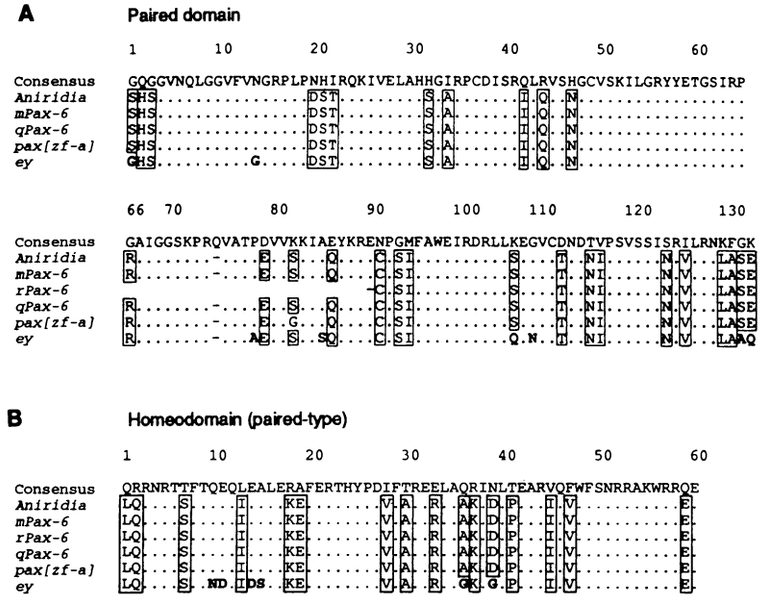

Fig. 2. Comparison of the amino acid sequences between the paired domains (**A**) and the homeodomains (**B**) of *Pax-6* homologs of vertebrates and *Drosophila* (*16*). The consensus sequences for all genes of the paired family (*33*) are compared to human *Aniridia*, *Pax-6* from mice (*mPax-6*), rats (*rPax-6*), quail (*qPax-6*), zebrafish [*pax*(*zf-a*)], and *Drosophila ey*. Shared amino acids (characteristic for *Pax-6*) are boxed in; amino acids differing between *Drosophila* and vertebrates are in bold.

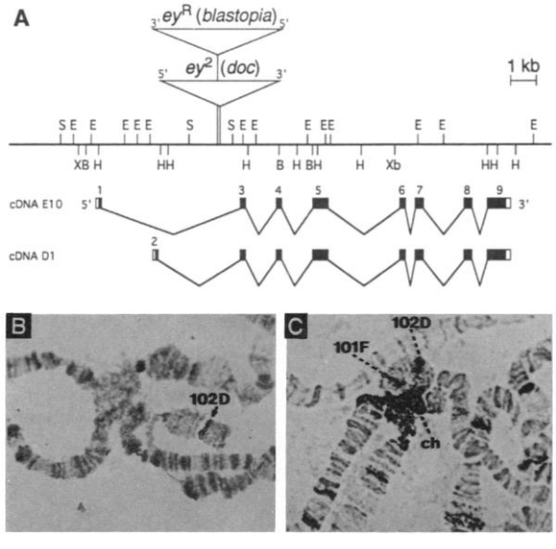

Fig. 3. Structural organization of the *ey* locus. (**A**) Genomic organization of *ey*. The restriction map of the *ey* locus is shown within the region covered by the isolated genomic phages. The two different types of cDNAs are shown below this map. Noncoding regions are indicated by white boxes, coding regions by black boxes. The exon-intron structure was determined by sequencing of the corresponding genomic regions. The positions of two transposon insertions causing mutations in the *ey* gene (ey^2 and ey^R) were determined by DNA sequence analysis (*23*) and are indicated by triangles. B, Bam HI; E, Eco RI; H, Hind III; S, Sal I; Xb, Xba I; X, Xho I. (**B**) In situ hybridization of a 1.1-kb Eco RI cDNA fragment from phage 8321 to polytene salivary gland chromosomes of ey^+ larvae (*34*). A single band of hybridization is detected at section 102D close to the *ey* locus on chromosome IV. (**C**) In situ hybridization of a *doc* transposable element (*22*) probe (pDoc) to polytene chromosomes of ey^2 larvae (*34*). Bands of hybridization are detected at 102D and 101F on chromosome IV, in the chromocenter (ch), and at several additional sites on other chromosomes.

margin of the eye disk and extended posteriorly to the morphogenetic furrow and to a few rows of cells beyond the furrow. The ey^+ transcripts also accumulated in parts of the brain and the ventral ganglion, as well as in the salivary glands. The finding that both of the insertions of transposable elements in ey^2 and ey^R, which affect gene expression in the eye primordia, occurred within 75 bp suggested to us that the transposable elements might disrupt an eye-specific gene regulatory element. Therefore, the sequences flanking the points of insertion were inserted into an enhancer detection vector containing a minimal promoter and the gene encoding the lacZ reporter. Transgenic flies carrying this construct selectively express β-galactosidase in the eye primordia (*27*). This finding supports the conclusion that the eyeless phenotype in ey^2 and ey^R mutants is caused by the insertion of transposons into genomic regulatory elements required for expression in the eye primordia.

The pattern of expression of the mouse *Pax-6* gene has been studied extensively in the embryo (*5*). It is first expressed in the forebrain and the hindbrain, followed by expression in the neural tube along the entire anteroposterior axis. At day 8.5 after conception, expression is first detected in the optic sulcus, followed by expression in the optic cup and the neural retina. The overlying ectoderm, which subsequently gives rise to the lens and later the cornea, also expresses *Pax-6*, which suggests that *Pax-6* is involved in eye induction. The nasal epithelium and Rathke's pouch show expression 1 and 2 days later in embryogenesis, respectively. This pattern of expression resembles the one found in *Drosophila*: The ey^+ transcripts are detected first in the central nervous system, in the brain, and the ventral nerve cord. The first signs of expression in the developing eyes are found in the embryonic anlagen of the eye imaginal disks. During larval stages, the anteriormost part of the eye disk expresses ey^+ transcripts. This is compatible with an early determinative role of the gene in eye morphogenesis, because the differentiation of the ommatidia proceeds in a posterior to anterior direction, as reflected by the gradual movement of the morphogenetic furrow in the anterior direction (*28*). The cells immediately anterior to the furrow are still undifferentiated and express the *eyes-absent* (*eya*) gene (*29*). Because *ey* is also expressed anteriorly to *eya*, *ey* seems to control an even earlier step in eye differentiation than *eya*. These observations suggest the hypothesis that *ey* is a master control gene that initiates the eye morphogenetic pathway and is shared between vertebrates and invertebrates. We are pursuing this hypothesis further by examining more primitive metazoa with primitive eyes. By polymerase chain reaction (PCR) amplification we have detected DNA sequences similar to those of *Pax-6* in flatworms (*Dugesia tigrina*) (*30*) and in nemerteans (*31*), which are among the most primitive metazoa with eyes (*28*). If the corresponding genes in flatworms and nemerteans are also involved in eye morphogenesis, the concept that the eyes of invertebrates have evolved completely independently from the vertebrate eye has to be reexamined. Also, the hypothesis that the eye of cephalopods has evolved by convergence with the vertebrate eye (*10*) is challenged by our recent finding (by means of PCR amplification) of *Pax-6*–related sequences in the squid *Loligo vulgaris* (*32*). These findings may throw some new light on the fascinating problems of eye evolution.

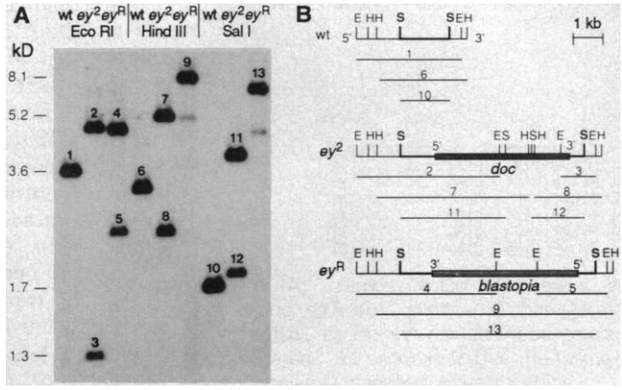

Fig. 4. Genomic Southern blots of ey^+, ey^2, and ey^R DNA. (**A**) DNA was isolated from mutant and wild-type (wt) strains, digested with the restriction enzymes indicated, run on a gel, blotted, and hybridized with the genomic 1.7-kb Sal I fragment (fragment 10 in Fig. 4B). Fragments are numbered for simplicity and are schematically shown in Fig. 4B. Some fragment sizes or marker lengths are indicated on the left. (**B**) Schematic drawings of wild-type and mutant DNA. Fragments detected in the genomic Southern blot (A) are numbered. The 1.7-kb Sal I fragment used as a probe is indicated in bold and the transposable elements with their orientation and insertion points are shown underneath.

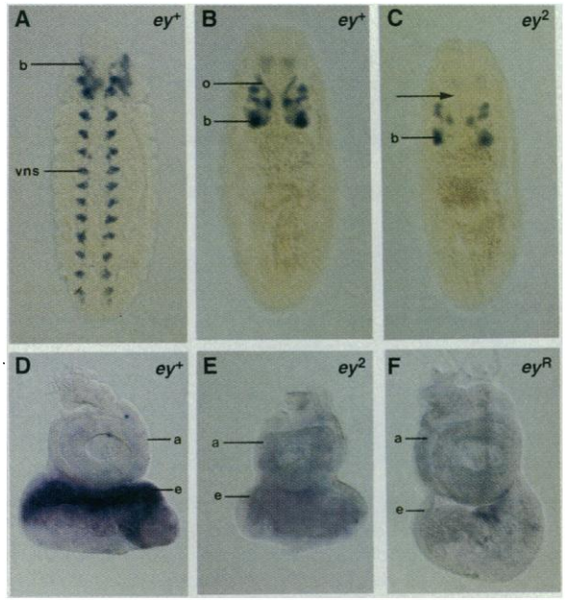

Fig. 5. Expression of *ey* transcripts in embryos and eye imaginal disks. In situ hybridization of ey^+ cDNA to RNA transcripts in embryos and larvae (*30*). (**A** and (**B**) Expression pattern in ey^+ embryos. (**C**) Expression in ey^2 embryo. Note the absence of labeling of the optic primordia (arrows) in ey^2. (**D**) Wild-type (ey^+) eye-antennal disk. The anterior portion of the eye disk is labeled. In the ey^2 mutant disk (**E**) and the ey^R mutant disk (**F**) hardly any labeling is detected. Abbreviations: b, brain; vns, ventral nervous system; o, optic primordia; e, eye imaginal disk; and a, antennal disk.

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13. For sequencing, the inserts were subcloned into the Bluescript KS (+) plasmid vector (Stratagene). Overlapping deletions were generated with Exo III and S1 nucleases (double-stranded nested deletion kit, Pharmacia) and sequenced on both strands with the dideoxynucleotide procedure of F. Sanger, S. Nicklen, and A. R. Coulson [*Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. Compressed DNA regions were sequenced using the fmol DNA sequencing system (Promega). DNA sequence analysis was done on a VAX with the GCG program [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)]. The homology searches through the EMBL and SWISSPROT data banks were carried out with the FASTA programs [W. R. Pearson and D. J. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 (1988)]. EMBL Data Library accession numbers: X79492 *D. melanogaster ey* mRNA (exon 1) and X79493 *D. melanogaster ey* mRNA (exons 2–9).
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30. DNA sequences with similarity to the *Pax-6* paired box were PCR-amplified, cloned, sequenced, and also detected by whole-genome Southern blotting in *D. tigrina* (P. Callaerts, E. Salo, W. J. Gehring, unpublished data).
31. A gene with significant homology to *Pax-6* in both the paired box and the homeobox was cloned from a genomic DNA library of the nemertean worm *Lineus sanguineus* (F. Loosli, M. T. Kmita, W. J. Gehring, unpublished data).
32. Sequences homologous to the paired box of *Pax-6* were PCR-amplified, cloned, and sequenced from DNA isolated from squid (*L. vulgaris*) sperm (P. Callaerts, J. Marthy, W. J. Gehring, unpublished data).
33. T. R. Bürglin, in *The Guidebook to the Homeobox Genes*, D. Duboule Ed. (Oxford Univ. Press, New York, pp. 25–72).
34. D. Tautz and C. Pfeifle, *Chromosoma* **98**, 81 (1989); M. Ashburner, *Drosophila, a Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989), protocol 27; M. Mlodzik, N. E. Baker, G. M. Rubin, *Genes Dev.* **4**, 1848 (1990). The probes were labeled with dig-dUTP and detected by digoxigenin antibodies (Boehringer).
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Blockage of NF-κB Signaling by Selective Ablation of an mRNA Target by 2-5A Antisense Chimeras

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Activation of 2-5A–dependent ribonuclease by 5′-phosphorylated, 2′,5′-linked oligoadenylates, known as 2-5A, is one pathway of interferon action. Unaided uptake into HeLa cells of 2-5A linked to an antisense oligonucleotide resulted in the selective ablation of messenger RNA for the double-stranded RNA (dsRNA)–dependent protein kinase PKR. Similarly, purified, recombinant human 2-5A–dependent ribonuclease was induced to selectively cleave PKR messenger RNA. Cells depleted of PKR activity were unresponsive to activation of nuclear factor–κB (NF-κB) by the dsRNA poly(I):poly(C), which provides direct evidence that PKR is a transducer for the dsRNA signaling of NF-κB.

Natural defense mechanisms can be allies in the quest for therapeutic approaches to disease. One such defense, the 2-5A system (*1*), mediates certain effects of interferons, such as the inhibition of encephalomyocarditis virus replication (*2*). Key components of this system include 2-5A, short oligoadenylates with 2′,5′-phosphodiester bonds; 2-5A synthetases that generate 2-5A from adenosine triphosphate (ATP) in response to dsRNA; and the effector of the system, the 2-5A–dependent ribonuclease (RNase) (*3*, *4*). This RNase, which is ubiquitous in the cells of mammalian, reptilian, and avian species (*5*), cleaves single-stranded RNA in response to 2-5A, with moderate specificity after UpNp sequences (*6*). Thus, this host defense mechanism is the basis for a strategy for the selective destruction of specific mRNA targets.

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