

Molecular Genetic Analysis of Ethanol Intoxication in *Drosophila melanogaster*¹

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SYNOPSIS. Recently, the fruit fly *Drosophila melanogaster* has been introduced as a model system to study the molecular bases of a variety of ethanol-induced behaviors. It became immediately apparent that the behavioral changes elicited by acute ethanol exposure are remarkably similar in flies and mammals. Flies show signs of acute intoxication, which range from locomotor stimulation at low doses to complete sedation at higher doses and they develop tolerance upon intermittent ethanol exposure. Genetic screens for mutants with altered responsiveness to ethanol have been carried out and a few of the disrupted genes have been identified. This analysis, while still in its early stages, has already revealed some surprising molecular parallels with mammals. The availability of powerful tools for genetic manipulation in *Drosophila*, together with the high degree of conservation at the genomic level, make *Drosophila* a promising model organism to study the mechanism by which ethanol regulates behavior and the mechanisms underlying the organism's adaptation to long-term ethanol exposure.

INTRODUCTION

Alcohol is among the most widely used and abused drugs in the world, yet our understanding of the mechanisms by which it regulates brain function and behavior is rudimentary. Some of the difficulties in understanding ethanol's mechanism of action derive from the fact that, unlike other abused drugs (such as nicotine, cocaine, and heroin), ethanol appears to have a broad spectrum of molecular targets in the nervous system. Ethanol readily crosses the blood-brain barrier and intercalates into cell membranes, changing membrane fluidity. It has been argued that ethanol's effects in the nervous system are caused primarily by non-specific alterations in membrane properties (Wood *et al.*, 1991). However, increasing evidence implicates certain proteins—mostly membrane proteins—as direct targets of ethanol in the nervous system (Peoples *et al.*, 1996). How ethanol acts on these proteins and how these effects relate to ethanol-induced behaviors and the complex process of alcohol addiction is poorly understood.

When ingesting low doses of ethanol, most humans exhibit responses such as disinhibition and euphoria. Higher doses cause incoordination and confusion, and in extreme cases, coma and death. The degree of response to ethanol is at least in part due to genetic predispositions. For example, young men with a family history of alcoholism are less sensitive to the motor, perceptual, and biochemical changes induced by intoxicating levels of ethanol than those from families without alcoholism (Schuckit and Gold, 1988; Schuckit *et al.*, 1996). In addition, when reexamined a decade later, a significantly higher proportion of subjects with reduced ethanol sensitivity had developed alcoholism

(Schuckit, 1994; Schuckit and Smith, 1996). These studies show that the initial level of response to ethanol is influenced genetically and may be a good predictor of risk for alcoholism. Recently, several chromosomal regions that harbor genes that may relate to this low responsiveness to ethanol have been identified (Wilhelmsen *et al.*, 2003). A causal relationship between ethanol sensitivity and risk for alcoholism has however not been demonstrated and the biological bases for this correlation remain unknown. Yet, these studies imply that an understanding of fairly simple behaviors induced by acute ethanol exposure may help gain insights into the more complex process of alcohol addiction.

Drosophila melanogaster is one of the most thoroughly studied organisms in biology and has provided essential insights into developmental and cellular processes that are conserved in mammals, including humans. Flies have a relatively sophisticated nervous system (approximately 300,000 neurons) and are capable of many complex behaviors (DeZazzo and Tully, 1995; Hall, 1994; Hall, 1998; Sokolowski, 2001). They are easy and inexpensive to rear in the laboratory and their life cycle is only 10 days at 25°C. The major advantage of flies is the simplicity and scale with which they can be manipulated genetically and molecularly. An analysis of the recently completed *Drosophila* euchromatin sequence revealed a high degree of molecular similarity between flies and mammals (Adams *et al.*, 2000; Rubin *et al.*, 2000). For example, *Drosophila* has most—if not all—major neurotransmitters, molecules involved in synaptic vesicle release and recycling, receptors and channels for neurotransmission, and signal transduction mechanisms involved in neural function in mammals (Littleton and Ganetzky, 2000; Lloyd *et al.*, 2000). Genes implicated in the actions of ethanol are, for the most part, conserved. These include N-methyl-D-aspartate receptors, γ -aminobutyric acid type A, 5-hydroxytryptamine, adenosine, and nicotinic acetylcholine receptors, multiple

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potassium and calcium channels, and signaling molecules involved in various second messenger cascades. Here we review some recent work from our laboratory that aims to develop and validate *Drosophila melanogaster* as a model system to study the molecular and neural bases of acute ethanol intoxication. A review of ethanol tolerance can be found elsewhere (Guarnieri and Heberlein, 2003; Wolf and Heberlein, 2003).

RESULTS

Behaviors induced by acute ethanol exposure

As has been described for most organisms in which it has been studied, *Drosophila* respond to acute ethanol exposure with a complex and biphasic behavioral response: an initial increase in locomotion is followed by incoordination, loss of postural control, and eventually sedation and immobility. Because current assays rely on continuous exposure to ethanol vapor under non-steady state conditions, the level of ethanol absorbed by the flies is directly proportional to exposure time. Thus, low levels of accumulated ethanol stimulate locomotion and high levels depress it.

In flies, ethanol-induced changes in walking activity and pattern were first quantified using simple line-crossing assays (Bainton *et al.*, 2000; Singh and Heberlein, 2000). When exposed to ethanol vapor in a chamber, flies show a transient increase in walking velocity; when exposed in narrow tubes, they show a temporary but dramatic increase in turning that is not explained simply by increased walking velocity (Singh and Heberlein, 2000). More recently, the development of automated assays has greatly enhanced the analysis efficiency and resolution of locomotor behaviors. The Ruden laboratory developed the inebri-actometer, an assay that measures simultaneously the activity of 128 flies (Parr *et al.*, 2001). Flies are placed individually in narrow tubes and as they move across the middle of each tube they break an infrared beam, which is recorded as motion. The kinetics and magnitude of locomotor activation and sedation measured with the inebri-actometer are essentially identical to those seen in the line-crossing assays. Finally, recent advances in computer and video technology have made feasible the automated and simultaneous tracking of several groups of freely walking flies (Fig. 1A) (Wolf *et al.*, 2002). In this system, two-dimensional traces of the movement of individual flies are established by following their position over time at 0.1 sec intervals. Information about the velocity of movement, degree of turning, and position in the box can be extracted by specialized software. The high temporal and spatial resolution of this system led to the discovery of aspects of ethanol-induced locomotor behaviors that had been previously missed. For example, upon ethanol exposure, flies show an immediate and short-lived increase in locomotion; this is a startle response to the smell of ethanol. After a brief quiescent period, flies enter a sustained hyperactive phase that dissipates gradually as flies become sedated (Fig. 1B). The kinetics of onset

and dissipation of this hyperactivity as well as the maximal velocities achieved are highly concentration-dependent.

The inebriometer, originally developed for selective breeding purposes (Cohan and Hoffman, 1986; Weber, 1988; Weber and Diggins, 1990), is an apparatus that measures the effect of ethanol (or any gas) on fly postural control (Fig. 2). It consists of a vertical cylinder fitted with a series of oblique mesh baffles (on which flies can stand and walk) that is perfused with ethanol vapor of defined concentrations. Groups of flies are introduced into the top of the cylinder, where they remain temporarily due to their natural propensity for negative geotaxis. As flies absorb and accumulate ethanol, they lose the ability to stand or walk properly and fall from one baffle to the next, eventually eluting from the bottom of the inebriometer. Because the level of ethanol absorbed by the flies is relatively linear over the time period of the assay, the mean elution time (MET) of the population is proportional to the ethanol dose needed to cause loss of postural control, and thus is a measure of fly sensitivity to the acute intoxicating effects of ethanol. The inebriometer has been used successfully to isolate mutants with altered sensitivity to volatile anesthetics (Krishnan and Nash, 1990) and to ethanol (see below).

In summary, ethanol exposure causes clear and measurable effects on *Drosophila* locomotion and postural control. In general, lower concentrations stimulate walking speed, while higher concentrations cause reduced movement, loss of postural control, and immobility. Interestingly, the ethanol concentrations that stimulate locomotion in flies are very similar to those causing the same effect in rodents and those generating disinhibition and euphoria in humans; the concentrations that cause incoordination and sedation are also comparable (Scholz *et al.*, 2000; Singh and Heberlein, 2000).

Genetic screens for mutants with altered ethanol-induced behaviors

Alkylating agents, such as ethylmethane sulfonate (EMS), and transposable elements, such as P-elements, have been used to generate and isolate mutants with altered ethanol sensitivity in the inebriometer (Moore *et al.*, 1998; Singh and Heberlein, 2000). Because flies are only exposed as long as they can maintain postural control, the inebriometer can be used to select for mutagenized flies with altered ethanol sensitivity in a manner that is similar to previous selective breeding experiments. In a genetic screen for X-linked mutations that cause altered ethanol sensitivity, approximately 30,000 progeny from EMS-mutagenized flies were subjected to several rounds of selection in the inebriometer. This resulted in the isolation of about twenty mutants with altered ethanol sensitivity; five of these mutants display altered ethanol pharmacokinetics (Singh and Heberlein, 2000). Two mutants with normal pharmacokinetics, *barfly* and *tipsy*, show strongly reduced and increased ethanol sensitivity in the inebriometer.

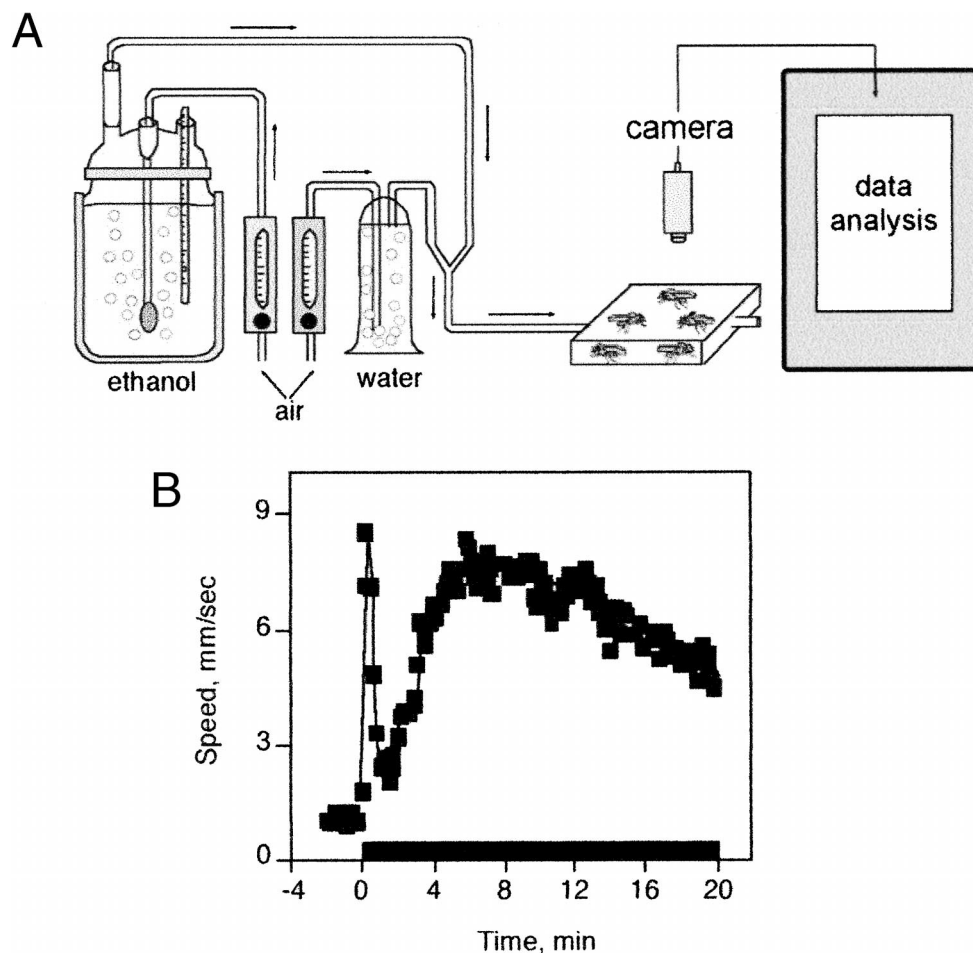


FIG. 1. A. Diagram of the apparatus used to measure ethanol-induced changes in locomotion in *Drosophila*. Air at controlled flow rates is bubbled through 95% ethanol maintained at 20°C, and separately through water. Humidified air and ethanol vapor are mixed and delivered to an exposure chamber made of clear plastic. Flies are introduced into this chamber and filmed with a digital video camera. Video is captured directly onto a computer and data is analyzed to extract information about speed of movement. B. Data from a typical experiment conducted at an intermediate ethanol vapor concentration. An initial transient peak of activity is a response to the smell of ethanol, while the second period of hyperactivity is caused by ethanol accumulation in the fly (see Wolf *et al.*, 2002 for details). The horizontal black bar represent the time of ethanol vapor exposure.

briometer, respectively. The altered inebriometer phenotype appears to be caused by an effect of the mutations on ethanol's sedative effect. While both mutants display normal ethanol-induced locomotor stimulation, *barfly* shows delayed sedation, while *tipsy* sedates prematurely. A genetic screen using P-element-mediated mutagenesis targeting all chromosomes has been carried out for ethanol sensitivity mutants in the inebriometer (Moore *et al.*, 1998). Two mutants with increased ethanol sensitivity, *cheapdate* and *lightweight*, have been analyzed in detail (Moore *et al.*, 1998; K. Berger and M. S. Moore, personal communication).

cheapdate was the first P-element-induced mutant isolated based on its increased sensitivity to ethanol-induced loss of postural control (Moore *et al.*, 1998); mutant flies elute from the inebriometer faster than wild-type flies. Because ethanol absorption and metabolism are normal in *cheapdate*, the mutant loses postural control at an ethanol dose that is lower than that

causing the same behavioral effect in wild-type flies. The P-element responsible for the *cheapdate* mutant phenotype was found to disrupt the *amnesiac* gene (Moore *et al.*, 1998). The *amnesiac* mutant was originally isolated due to its inability to perform normally in an olfactory classical conditioning paradigm (Quinn *et al.*, 1979), and was later shown to disrupt a gene predicted to encode a PACAP-like neuropeptide (Feany and Quinn, 1995). In vertebrates, PACAPs signal through G-protein-coupled receptors that activate adenylyl cyclase and cAMP-dependent protein kinase (PKA) (Vaudry *et al.*, 2000). Consistent with a similar role for *amnesiac* are findings that mutations in the calcium/calmodulin-sensitive adenylyl cyclase *rutabaga* and the catalytic subunit of PKA (*pka-C1*) also cause increased ethanol sensitivity in the inebriometer (for a simplified diagram of the cAMP pathway see Fig. 3). Moreover, the ethanol sensitivity defect of *amnesiac* can be reversed by pharmacological activation of adenylyl cyclase or PKA in adult flies. This obser-

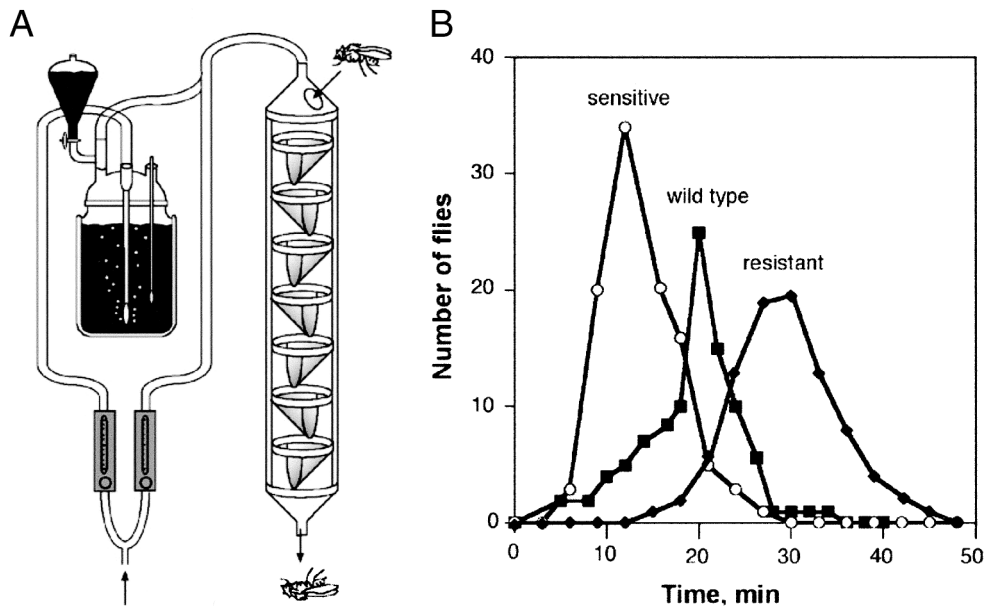


FIG. 2. A. Diagram of the inebriometer, an apparatus that measures ethanol-induced loss of postural control. Ethanol vapor is generated as described in Figure 1A. Flies are introduced into the top of a 4-foot glass column through which ethanol vapor of controlled concentration circulates. As they become intoxicated, flies progressively lose postural control and tumble downwards; their fall is retarded by their ability to cling to oblique mesh baffles distributed along the length of the column. As flies fall out of the bottom of the inebriometer, they are counted at 3-minute intervals. B. Representative inebriometer elution profiles of wild-type flies, a mutant with increased sensitivity (sensitive), and one with reduced sensitivity (resistant) to ethanol.

vation, together with the finding that expression of a transgene-encoded *amnesiac* in adult flies is sufficient to rescue the ethanol sensitivity phenotype of cheap-date, suggests that *amnesiac* function is required acutely during ethanol exposure to regulate the flies' behavior.

Screens for brain regions involved in the regulation of ethanol-induced behaviors

The results described above implicate cAMP signaling in the regulation of ethanol-induced behaviors in *Drosophila*. To define brain regions and, eventually, neural circuits where cAMP signaling may regulate ethanol-induced behaviors, we took an unbiased approach (Rodan *et al.*, 2002). Specifically, we used the GAL4/UAS gene expression system (Brand and Perrimon, 1993) (Fig. 4), to target the expression of a PKA inhibitor to different brain regions using a collection of GAL4 lines with diverse expression patterns in the CNS (see <http://www.fly-trap.org/>). The PKA inhibitor is a *Drosophila* PKA regulatory subunit with mutated cAMP-binding sites (Li *et al.*, 1995); it remains bound to the endogenous PKA catalytic subunit inhibiting its activation in a dominant fashion. Of the nearly 70 GAL4 lines tested, only 3 showed a specific alteration of ethanol sensitivity in the inebriometer; two of these three lines also showed a delay in sedation when assayed in the locomotor tracking system. We concluded that different behavioral effects of ethanol—loss of postural control and locomotor sedation—seem to be regulated by separable (yet overlapping) brain regions. Another important conclusion

from this study is that disruption of PKA function in only a few brain regions alters the fly's sensitivity to ethanol.

Although clearly not the only cells involved in regulating ethanol-induced behaviors, a group of neurosecretory cells located in the pars intercerebralis are the most promising candidates defined by this study. These cells are known to express different neuropeptides in *Drosophila* and in other flies, and to project axons to the ring gland—an endocrine gland of flies (Nassel, 1993). Interestingly, an enhancer trap insertion in the *amnesiac* gene, which presumably reports the activity of the *amnesiac* gene, is strongly expressed in the ring gland (our unpublished data). It is therefore tempting to speculate that there is a functional connection between the pars intercerebralis neurosecretory cells and *amnesiac* in the regulation of ethanol sensitivity.

In one of the GAL4 lines that showed an inhibitor-dependent change in ethanol-induced loss of postural control and sedation, the PKA inhibitor was strongly expressed in the mushroom bodies—prominent brain structures that are involved in olfactory conditioning (Roman and Davis, 2001). This observation, together with the fact that several olfactory learning and memory mutants such as *amnesiac*, *rutabaga* and *fasciclin II* alter ethanol sensitivity (Cheng *et al.*, 2001; Moore *et al.*, 1998), motivated an analysis of the role of mushroom bodies in ethanol sensitivity. Chemical ablation of the mushroom bodies, a procedure that completely abolishes olfactory conditioning (de Belle and Heisenberg, 1994), did not affect ethanol sensitivity in

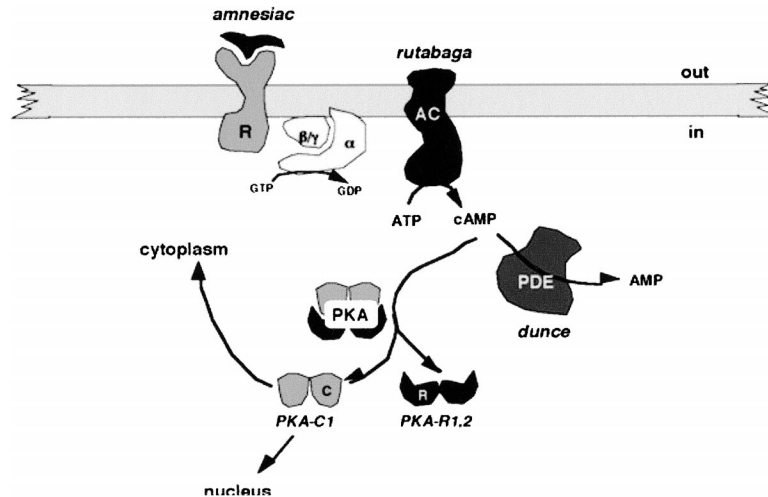


FIG. 3. Cartoon of the cAMP pathway, which is presumably activated upon binding of the amnesiac neuropeptide to its receptor (R) (embedded in the cell membrane). Known components of this pathway include the calcium/calmodulin-activated adenylyl cyclase (AC) encoded by the *rutabaga* gene, the phosphodiesterase (PDE) encoded by the *dunce* gene, and cAMP-dependent protein kinase (PKA) with its catalytic (C) and regulatory (R) subunits. Activation of PKA will lead to phosphorylation of cytoplasmic and nuclear targets. For a detailed review of the role of these genes, many of which were originally identified as mutants with abnormal learning and memory, see Davis *et al.* (1995).

the inebriometer (Rodan *et al.*, 2002). Thus, despite the overlap among the genes involved in regulating ethanol sensitivity and olfactory conditioning, the brain regions and neural circuitry regulating these behaviors is distinct.

CONCLUSIONS

In its natural environment, rich in fermenting plant materials, the fruit fly *Drosophila melanogaster* encounters relatively high levels of ethanol. Fruit flies are well equipped to deal with the toxic effects of ethanol; they use it as an energy source and as a precursor for lipid biosynthesis. The effects of ethanol and its metabolites on *Drosophila* have been studied for decades, as a model for adaptive evolution. More recently, *Drosophila* has been introduced as a model to

study the molecular bases of ethanol-related behaviors. While these fields of study have different primary goals, the information gained can be nicely complementary. For example, a definition of the genes (by mutagenesis) that regulate various ethanol-induced behaviors in the laboratory may provide candidate genes for evolutionary biologists and population geneticists. Conversely, knowledge of the role of ethanol in the fly's ecology has important implications for the design of mutagenesis-driven approaches that aim to understand behavior. It has been postulated that the evolutionary origins of alcohol consumption in humans may be related to primate frugivory, which led to consumption of substantial amounts of ethanol (Dudley, 2000). Humans and *Drosophila melanogaster* share a common history of ethanol exposure.

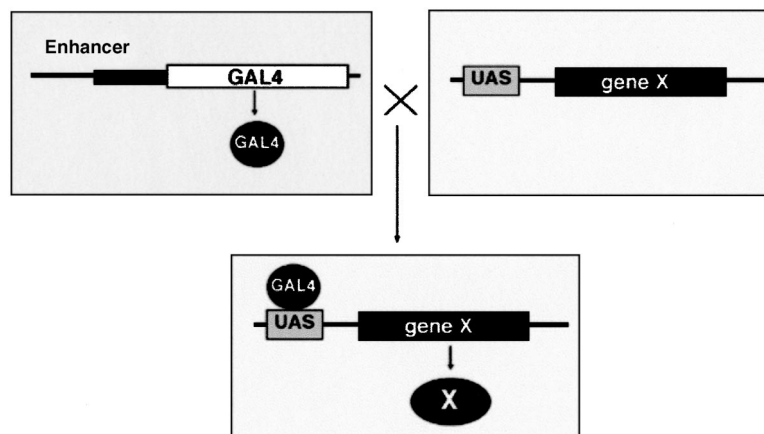


FIG. 4. Cartoon of the GAL4/UAS binary expression system. Flies expressing the yeast transcription factor GAL4 in specific areas of the fly brain (upper left box) are crossed to flies in which the gene of interest (gene X) is under the control of a UAS element (upper right box). In the progeny of this cross (lower box), GAL4 binds to the UAS sequence leading to the transcriptional activation of gene X. For a detailed description of this system see (Brand *et al.*, 1994; Kaiser, 1993).

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