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Ectopic Gene Expression in *Drosophila* Using GAL4 System

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Expressing a gene in cells in which it is not normally active is a powerful way of determining its function. The GAL4 system allows the selective expression of any cloned gene in a wide variety of cell- and tissue-specific patterns in *Drosophila.* A promoter (or enhancer) directs expression of the yeast transcriptional activator GAL4 in a particular pattern, and GAL4 in turn directs transcription of the GAL4-responsive (UAS) target gene in an identical pattern. The system's key feature is that the GAL4 gene and UAS–target gene are initially separated into two distinct transgenic lines. In the GAL4 line, the activator protein is present, but has no target gene to activate. In the UAS–target gene line, the target gene is silent because the activator is absent. It is only when the GAL4 line is crossed to the UAS–target gene line that the target gene is turned on in the progeny. In this article we describe, in detail, how to generate and characterize GAL4 lines and how to prepare UAS–target gene lines. Vector maps are provided for pGaTB, P[GawB], and pP[UAST]. In addition, we consider the range of UAS–reporters currently available and review several new modifications of the GAL4 system.
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The identity of a cell is determined, in large part, by its characteristic profile of gene expression. The ability to express a gene in cells in which it is not normally active is thus a powerful way of investigating its role in defining cellular identity. Traditionally, genes in *Drosophila* have been characterized on the basis of loss-of-function phenotypes, but ectopic expression phenotypes can be equally informative. For example, the loss-of-function phenotype of *eyeless,* a paired homeodomain transcription factor, is the partial or complete absence of the compound eyes (1), whereas ectopic expression of *eyeless* is sufficient to generate extra eye structures that are not only morphologically normal but also electrically active on illumination (2). Ectopic expression phenotypes can provide unique functional information in cases where there is no loss-of-function phenotype due to genetic redundancy.

If the gene of interest encodes an intercellular signal, the field of cells that are competent to respond can be defined by misexpressing the signal gene beyond its normal confines. Van Vactor *et al.* have used this approach to assay whether all the cells that express the *sevenless* receptor tyrosine kinase are competent to respond to the ligand *boss* (3). Different, or even opposing, modes of action by the same gene can be uncovered by misexpression in several different spatiotemporal domains. For example, at different stages of embryonic development, *even-skipped* acts as either a positive or a negative regulator of *fushi-tarazu* transcription (4). Quantitative aspects of gene function can be investigated by expressing a gene at higher than normal levels; for example, when the basic helix–loop–helix transcription factor *twist* is overexpressed within its wild-type mesodermal domain, ectopic muscle is formed at the expense of other mesodermal derivatives (5). Ectopic expression can be used to generate dominant phenotypes for a gene, facilitating its ordering within a genetic pathway by epistatic analysis (6).

Ectopic expression methods can also be used to produce loss-of-function phenotypes (rather than gain-of-function phenotypes) by driving the expres-

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sion of agents that will antagonize the endogenous gene product. This is desirable because it is currently impossible to perform targeted gene disruption in *Drosophila,* and despite the large repertoire of single-gene mutants available, there is still a need to obtain loss-of-function data for the thousands of genes being cloned without reference to phenotype. Antisense RNAs (7) and targeted ribozymes (8) have been ectopically expressed and used, albeit with varying success, to eliminate the mRNA of a particular gene. Likewise, agents that block endogenous protein function such as protein kinase inhibitors (9, 10) and pertussis toxin (which inactivates $G_o\alpha$ subunit) have also been ectopically expressed in *Drosophila* (11).

Methods of Ectopic Expression

There are now several methods for ectopic expression in *Drosophila,* and each has its own set of merits and limitations. The first technique is to drive expression of the target gene of interest by fusing it downstream of a characterized promoter. This method has proved invaluable in analyzing signal transduction and cell fate determination in the eye, using the *sevenless* promoter, for example (12). To change the spatiotemporal pattern of ectopic expression requires fusing the gene of interest to a different promoter, which in turn requires that such a promoter has been cloned and characterized. The level of expression can be varied, but only by exploiting position effects between lines or by changing copy number. Furthermore, if ectopic expression of the gene is toxic to the organism, it becomes impossible to establish stable transgenic lines.

A second method is to drive expression of a gene from a heat-shock promoter. The great advantage of this technique is that both the timing and level of expression can be conveniently varied by altering the timing and temperature, respectively, of the heat shock delivered (4). However, the spatial pattern of expression is not restricted to a subset of cells; it is always ubiquitous. Other disadvantages are that basal levels of expression are observed from heat-shock promoters and that the delivery of the heat shock can induce phenocopies (13–15).

The introduction of the FLP/FRT site-specific recombination system from *Saccharomyces cerevisiae* to *Drosophila* (16) has elegantly combined the heat-shock and characterized promoter methods of ectopic expression (17).

GAL4 System

In the defined promoter and heat-shock methods discussed above, the promoter driving ectopic expression is directly fused to the target gene of interest. In the GAL4 system (18), the promoter (or enhancer) drives expression of the yeast transcriptional activator GAL4 (19), which in turn activates the target gene (see Fig. 1).

The GAL4 protein activates transcription of only those genes bearing GAL4 binding sites [upstream activation sequence (UAS)]. The *GAL4* gene is placed near the promoter/enhancer driving ectopic expression, and an UAS is fused to the target gene. The promoter/enhancer directs expression of *GAL4* in a particular cell- or tissue-specific pattern, and GAL4 in turn directs transcription of the UAS–target gene in an identical cell- or tissue-specific pattern.

The key feature of the GAL4 system is that the *GAL4* gene and UAS–target gene are initially separated into two distinct transgenic lines. In the GAL4 line, the activator protein is present, but has no target gene to activate. In the UAS line, the target gene is silent in the absence of activator. When the GAL4 and UAS lines are crossed, the target gene is turned on only in the progeny of the cross. Thus a library of GAL4 lines can be built up, each line expressing GAL4 in a different spatiotemporal pattern. The UAS–target gene can then be ectopically expressed in a wide variety of patterns merely by crossing the UAS line to a library of GAL4 lines. In a complementary fashion, a library of UAS–target genes can also be constructed and a large number of different genes, or combinations thereof, can be misexpressed in precisely the same domain by crossing the appropriate GAL4 line to a UAS line library.

Another advantage of this system being bipartite is that since the UAS–target gene is silent in the absence of GAL4, the UAS line will be viable even if the UAS–target gene is lethal when ectopically expressed. This aspect of the GAL4 system has been exploited in performing targeted cell ablations (see Improving and Elaborating GAL4 System).

Two categories of regulatory element can be used to direct expression of *GAL4* in a particular cell- or tissue-specific pattern. The first is to drive *GAL4* transcription using a characterized promoter. The second is based on "enhancer detection" (20). The *GAL4* gene is fused to a minimal promoter which, depending on the particular genomic enhancer that acts on it, will direct GAL4 expression in a particular cell- or tissue-specific pattern. The advantage of this approach is that it eliminates the need to link numerous different promoters to the *GAL4* gene and allows expression in novel patterns from enhancers that have not yet been described. In addition, the enhancer detection–*GAL4* vector can be mobilized

to new genomic sites simply by P transposition. In this way a single transformant line can be used to generate a library of GAL4 lines.

METHOD

Generating a GAL4-Expressing Line

Promoter–GAL4 Vector

To construct a promoter–*GAL4* vector for P-element transformation, a three-way ligation is performed between (i) the desired promoter fragment, (ii) the *Bam*HI/*Not*I fragment of pGaTB (GAL4, Terminator, *Bam*HI), which carries the *GAL4* gene and the *Drosophila hsp70* terminator (see Fig. 2), and (iii) a P-element vector such as pP[CaSpeR 2, 3, or 4]. In selecting the length of the promoter fragment to drive GAL4 expression, as much sequence as possible should be used, up to, but not including, the ATG. Construction of pGaTB and the other two GAL4 system vectors, pP[GawB] and pP[UAST], is detailed in (18, 21).

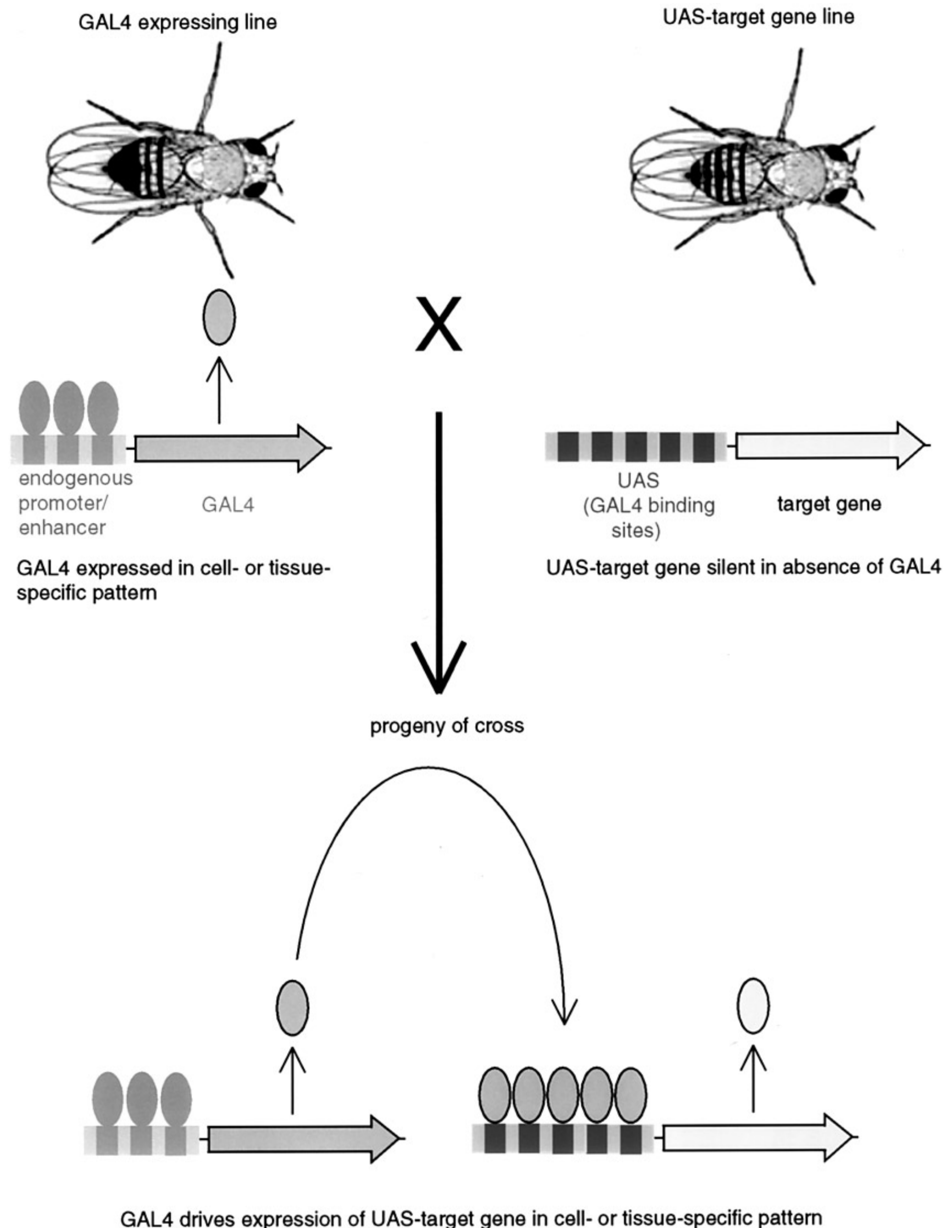

FIG. 1. GAL4 system.

Enhancer Detection–GAL4 Vector

Enhancer detection–*GAL4* lines are made using the vector pP[GawB] (GAL4, *white, Bam*HI), in which the *GAL4* coding sequence lies downstream of the P-transposase promoter (see Fig. 3).

P-Element Transformation

GAL4 lines are generated by the injection of pP[promoter–*GAL4*] or pP[GawB] (DNA prepared using the Qiagen Plasmid Midi Kit), at a concentration of 600 μg ml^{-1}, into embryos of the strain *yw; +/+;Sb, P*[*ry*$^+$, Δ*2-3*]/*TM6, Ubx* using standard procedures (22). This strain carries a defective P element on the third chromosome that expresses high levels of a constitutively active transposase, but cannot itself transpose. On average, we obtain three independent transformants per hundred embryos injected. pGaTB and pP[GawB] both carry the *white* selectable marker, which is dose dependent and thus allows one to distinguish between hetero- and homozygotes as well as between flies with different numbers of transgenes. Promoter–*GAL4* lines are then characterized, whereas enhancer detection–*GAL4* lines can be used to generate more lines by remobilizing P[GawB].

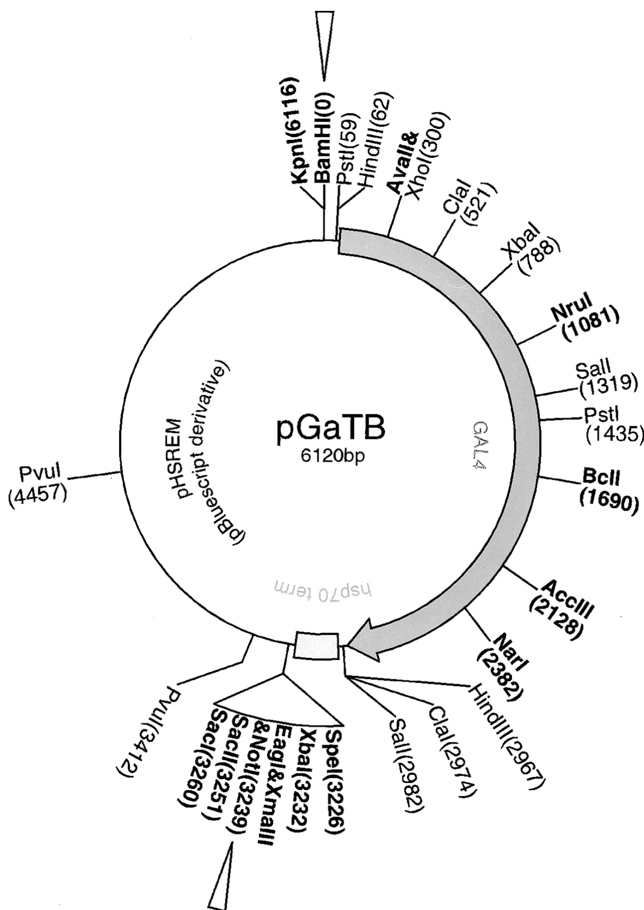

FIG. 2. *Bam*HI/*Not*I fragment of pGaTB is used to construct promoter–GAL4 fusions. Unique restriction sites are shown in boldface.

Mobilization of Enhancer Detection–GAL4 Vector to New Genomic Sites

P[GawB] is mobilized from its initial site of integration again using the strain *yw;+/+;Sb, P*[*ry*$^+$, Δ*2-3*]/*TM6, Ubx.* The frequency with which new P[GawB] lines are recovered is much lower than that reported for a similarly sized enhancer detection–*lacZ* vector. This could be due to alterations made in the sequence of the 5′ P end of P[GawB] that allows GAL4 to be expressed from its own ATG, rather than as a P-transposase–GAL4 fusion protein. Enhancer detection–*GAL4* lines are then screened.

Characterizing Ectopic Expression Pattern of GAL4 Line

It is essential to characterize the pattern in which a promoter–*GAL4* line will drive UAS–gene expression. It will not always recapitulate the pattern of the promoter's cognate gene "X". When discrepencies do arise, they may be due to position effects or GAL4-mediated expression. GAL4 can distort the temporal control of an expression pattern in two ways. First, there may be a delay before GAL4 reaches levels sufficient to activate the UAS–gene. The onset of UAS–target gene transcription will then lag behind the start of promoter–*GAL4* transcription. Second, since GAL4 may perdure, GAL4 protein will still be present and driving UAS–gene expression after the cessation of promoter–*GAL4* transcription. Thus, UAS–target gene expression is prolonged with respect to the promoter–*GAL4.*

Enhancer detection–*GAL4* lines must also be screened, first to eliminate lines that do not express GAL4 (i.e., those in which P[GawB] has failed to integrate near an enhancer), and second, to select lines that express GAL4 in a relevant cell- or tissue-specific pattern. When ectopic expression in the adult is required, it is often easier to cross uncharacterized GAL4 lines to the UAS–target gene line and to screen for phenotypes first, prior to characterizing the pattern of GAL4 expression. Alternatively, the UAS–*yellow* reporter gene can be used (see below).

GAL4 Antibodies

The most direct approach to determining the pattern of GAL4 expression in a particular line would be to perform immunohistochemistry using a GAL4 antibody. We and others have tried several GAL4 antibodies, but have yet to achieve satisfactory results.

UAS–Reporter Genes

Although the UAS–reporter gene strategy introduces a fly cross into the screening process and is conceptually less direct than GAL4 antibody staining, it provides information more relevant to the ectopic expression experiment since it reveals the pattern in which a particular GAL4 line will drive a UAS–gene rather than simply the raw GAL4 expression domain.

Requirements of UAS–Reporter Genes

UAS–reporter gene activity should be visualized by a convenient assay system. It is essential that the reporter gene product reveal the general sites of UAS expression within the embryo, larva or adult, but it should also facilitate high-resolution analysis of the pattern, in terms of how many and which kinds of cells express the reporter gene. To determine cell number it is useful for the reporter gene product to be specifically included or excluded from the nucleus, so that individual nuclei can be resolved and counted. Cell type can be inferred, in many cases, by cell morphology, so it is a distinct advantage if the body and processes of a cell are revealed by the reporter. However, cell identity is more definitively ascribed on the basis of which genes a particular cell does or does not express, as determined by *in situ* hybridization or immunohistochemistry. For this reason the reporter gene product should remain active after fixation so that UAS–gene activity can be directly compared with endogenous gene activity.

The function of a UAS–reporter gene is to reveal the pattern in which a particular GAL4 line will drive transcription of UAS–target genes. To provide an accurate temporal profile of this, two criteria must be met. First, the length of time taken for the reporter gene transcript to produce an active reporter gene product must be minimal; otherwise the onset of GAL4-mediated UAS expression will appear artificially late. Second, the reporter gene product should be rapidly turned over so that the end of UAS–reporter gene transcription is immediately followed by the loss of reporter gene product activity. Such distortions can be anticipated and corrected for if the duration of the "transcript to activity lag" and frequency of turnover are known. However, if the GAL4-mediated pattern is highly dynamic, and changes occur at a faster rate than the lag or turnover, fine details of the temporal expression pattern will be lost.

UAS–lacZ Reporters

The first generation of UAS–reporter genes used the *Escherichia coli* gene *lacZ,* which encodes the hydrolase β-galactosidase. The *lacZ* expression pattern in progeny of the GAL4 line crossed to a *UAS–*

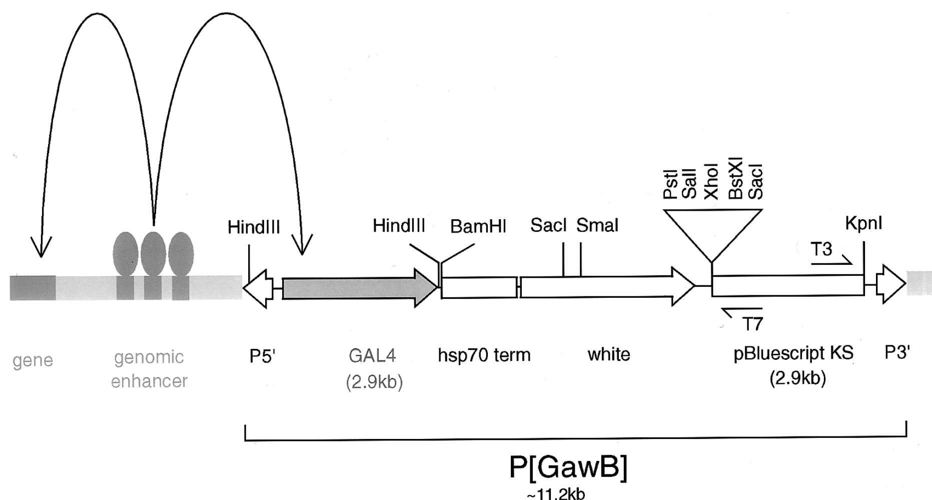

FIG. 3. P[GawB] "traps" neighboring genomic enhancers to drive GAL4 expression. The unique *Kpn*I restriction site is used in plasmid rescue of upstream genomic sequences and *Pst*I, *Sal*I, *Xho*I, *Bstx*I, or *Sac*I can be used to rescue downstream sequences.

lacZ line can be determined either by using X-Gal as a substrate for β-galactosidase or, more effectively, by using anti-β-galactosidase antibodies. β-Galactosidase and derived fusion proteins are extremely stable (for up to 6 h) and are not suitable for reporting dynamic expression patterns.

UAS–lacZ was first constructed by Fischer with four GAL4 binding sites (23). Higher levels of expression are obtained with the line *UAS–lacZ*$^{4\text{-}1\text{-}2}$ in which *lacZ* expression is driven from five GAL4 sites (18). β-Galactosidase is localized in the cytoplasm and reveals aspects of cell morphology, but is not effective at revealing cell numbers (see Fig. 4a).

UAS–nuclear lacZ (M. Muskavitch, unpublished) encodes a β-galactosidase with a nuclear localization signal (NLS), which targets it to the nucleus, facilitating accurate cell counting. Exclusion from the cytoplasm means that cell morphology is not revealed.

UAS–kinesin–lacZ (24). Kinesin is a plus end-directed microtubule motor protein. Kinesin-β-galactosidase is excluded from the nucleus and reveals cell shape, and in neurons it travels to and labels the growth cone of axons. However, the growth cone cannot be retraced to the cell from which it projects. *UAS–kinesin–lacZ* has been shown to cause phenotypes, including embryonic lethality, when expressed in neuronal cells. Swelling has been observed at the distal ends and branch points of axons (25). This may be due to the accumulation of the relatively large kinesin-β-galactosidase fusion protein at growth cones. No abnormalities have been observed in nonneuronal cell types such as glia, epidermis and muscle.

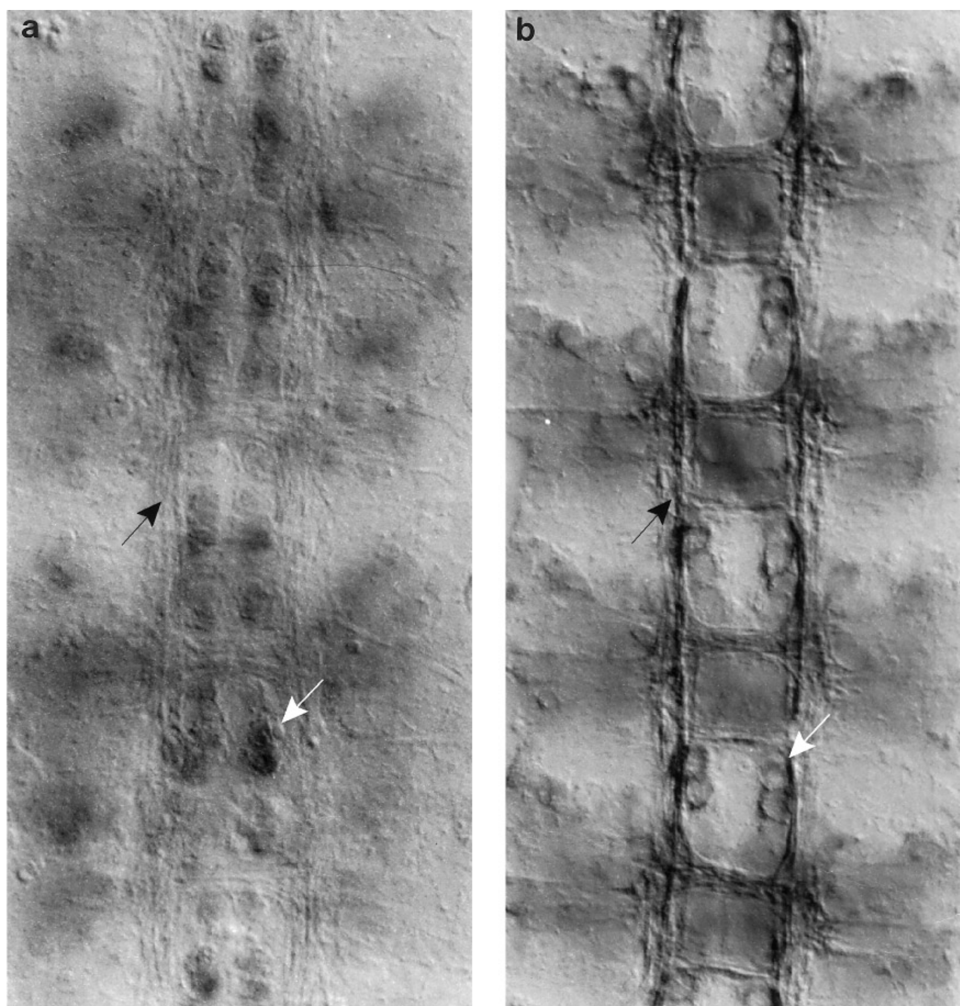

FIG. 4. (a) *fushi-tarazu–GAL4* driving *UAS–lacZ* expression in the ventral nerve cord. (b) *fushi-tarazu–GAL4* driving *UAS–tau–lacZ* expression in the ventral nerve cord. Tau–β-galactosidase reveals axon tracts more clearly than β-galactosidase (black arrow) and is excluded from the nuclei of cells (white arrow).

UAS–tau–lacZ (26) encodes a bovine Tau–β-galactosidase fusion protein (27). Tau is normally found in neuronal lineages where it binds and stabilizes microtubules; however, it can also bind microtubules in nonneuronal cell types. Tau–β-galactosidase fusion protein is excluded from the nucleus and labels the microtubule network of a cell which, in turn, reveals the gross morphology of that cell, facilitating its identification. Tau reporter fusions have proven to be extremely useful for defining GAL4 lines that direct UAS–gene expression in neuronal populations because, as well as the cell body, they highlight the whole length of axons (28) (see also Fig. 4b). This is particularly valuable when the axonal projection pattern of a neuron must be known to unambiguously assign it an identity. When expressed at high levels Tau–β-galactosidase can inhibit mitosis, causing phenotypes that are most readily seen in rapidly dividing tissues. For example, when *UAS–tau–lacZ* is driven in the imaginal disks by crossing to *engrailed*–GAL4, it leads to pupal lethality (A. H. Brand, unpublished).

Visualizing β-Galactosidase Pattern

With X-Gal. We no longer use X-Gal staining (21), primarily because the substrate can diffuse away from the site where β-galactosidase is active, and one loses the benefit of using *Tau–lacZ* to reveal subcellular architecture. Indeed, it can be hard to even achieve cellular resolution using X-Gal.

With anti-β-galactosidase antibodies. We use anti-β-galactosidase from Cappel and follow the standard methods of Patel (29) with a few variations (18).

UAS–GFP Reporters

The current generation of UAS–reporter genes are based on the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria.* GFP is an excellent reporter, the expression pattern of which can be visualized noninvasively in live specimens by virtue of its natural fluorescence (30). Thus, in stark contrast to X-Gal or anti-β-galactosidase staining, where it is necessary to analyze a large number of fixed embryos of different ages to construct a comprehensive time course, the GFP pattern can be monitored continuously through development in a single individual. Wild-type GFP suffers from a long "transcript to active gene product lag," which is due to an autoxidation step (31). Consistent with this, we observe wild-type GFP fluorescence approxi-

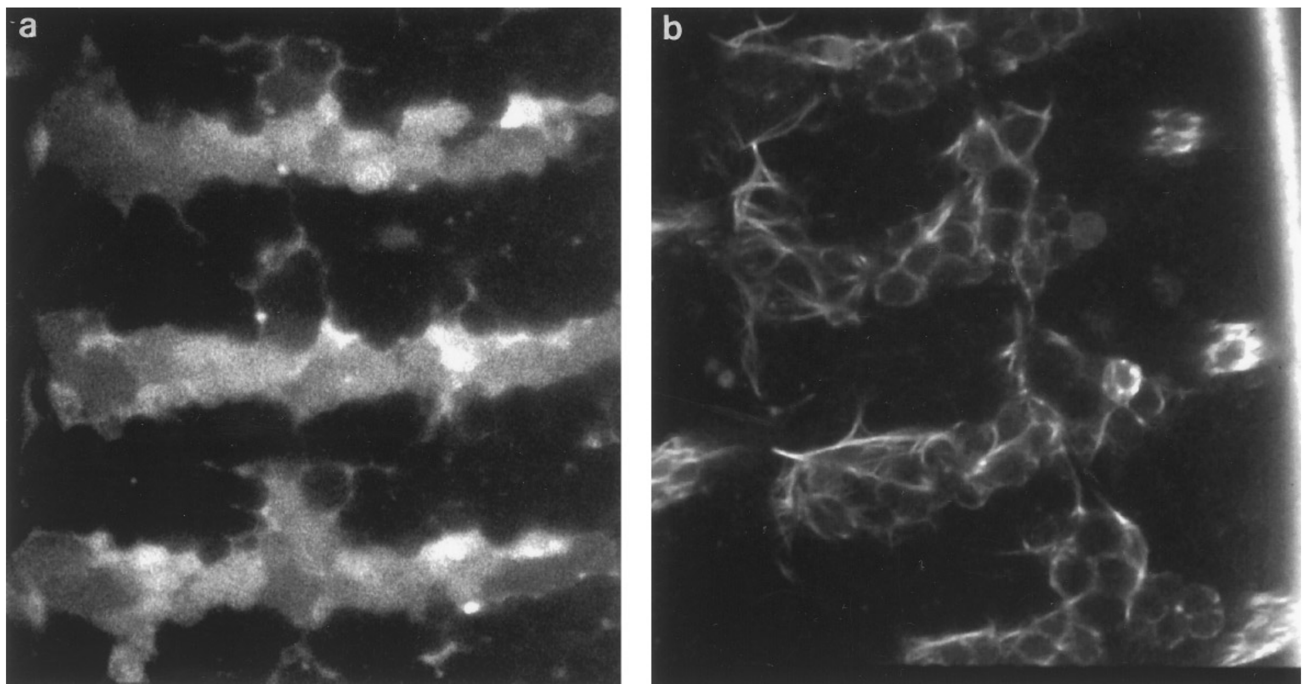

FIG. 5. (a) *engrailed–GAL4* driving *UAS–GFP* expression in epidermal stripes and a subset of neurons. (b) *engrailed–GAL4* driving *UAS–tau–GFP* expression in the same pattern. Tau–GFP fluorescence is excluded from nuclei and reveals axon tracts more clearly than GFP.

mately 3 h after β-galactosidase expression from a similar transgene (32). However, mutant GFPs have been selected that have a greatly reduced lag time of onset, which is almost comparable to that of β-galactosidase (C. M. Davidson and A. H. Brand, unpublished). GFP remains fluorescent after fixation, and although real-time analysis is sacrificed, *GFP* expression can be correlated with endogenous gene expression.

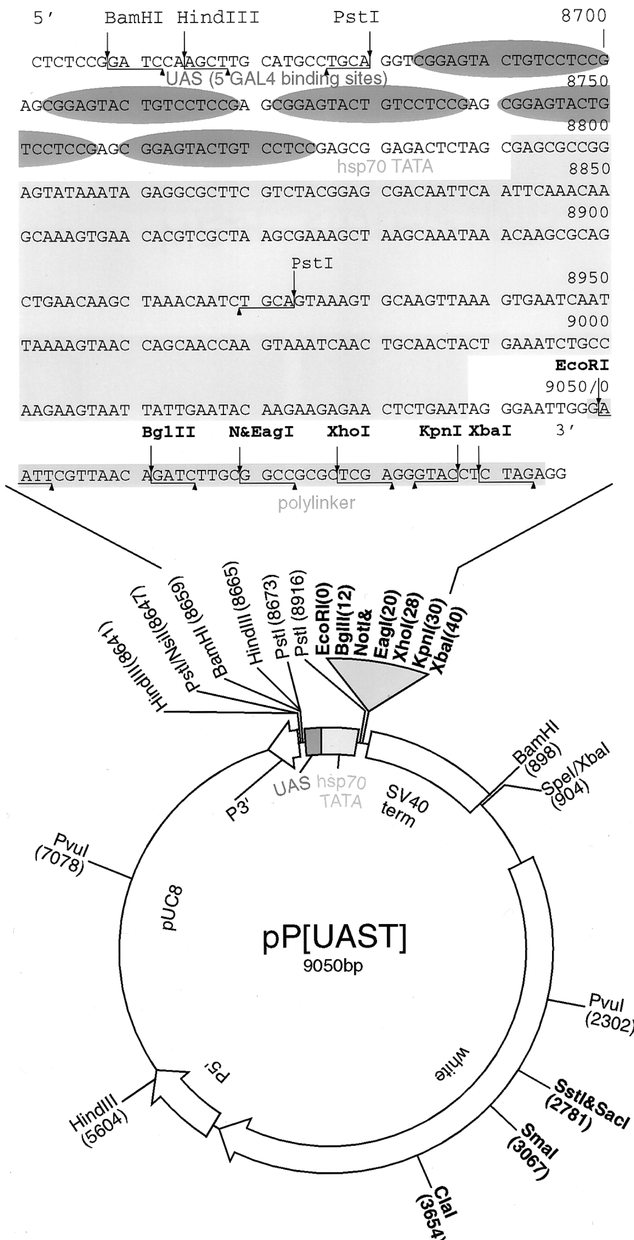

FIG. 6. Restriction map of pP[UAST] with unique sites shown in boldface. Sequence data are provided for the UAS, TATA box, and polylinker.

UAS–GFP encoding wild-type GFP has been generated by both Yeh (33) and Brand (32). GFP is a small protein (27 kDal), the fluorescence of which concentrates in nuclei, although it is excluded from nucleoli. GFP fluorescence is also detectable in the cytoplasm. A single GFP-positive cell can be resolved when surrounded by GFP-negative cells, but not when it is part of a GFP-positive cell cluster (see Fig. 5a).

With respect to *UAS–nuclear GFP–lacZ,* Shiga *et al.* (34) attempted to develop a strictly nuclear localized GFP by fusing it to a NLS, but fluorescence was detectable only in polytene larval tissue and not in diploid tissue. Further fusions to *lacZ* produced *nuclear GFP–lacZ,* which fluoresces brightly in the nuclei of several diploid larval and embryonic tissues.

UAS–tau–GFP (32) is currently our reporter gene of choice. Tau is a microtubule-binding protein that highlights the microtubular cytoskeleton within every cell. Fusing Tau to GFP produces a reporter the expression pattern of which can be followed in real time while also revealing the cytoskeletal architecture and, therefore, morphology of cells (see Fig. 5b). Tau–GFP is excluded from nuclei, facilitating cell counting. Tau–GFP causes much less severe phenotypes than Tau–β-galactosidase when expressed in rapidly dividing tissues. Expression driven by *engrailed–GAL4* yield viable adults with posterior wing defects. One explanation for why Tau–β-galactosidase causes more severe phenotypes than Tau–GFP is that β-gal can tetramerize, perhaps enhancing the stabilization and crosslinking of microtubules by Tau.

Visualizing GFP Expression

Embryos are dechorionated in 50% Clorox for 3 min, washed with demineralized water, and then mounted on an air-permeable Teflon membrane (designed by E. Wiechaus) in Halocarbon or Voltalef oil. GFP fluorescence can then be detected by epifluorescence or laser-scanning confocal microscopy (using most FITC sets or Chroma GFP set). We have observed GFP fluorescence through the embryonic vitelline membrane, the larval cuticle, and the early pupal case.

When fixing embryos, we have found that GFP fluorescence is preserved after fixation in 4% formaldehyde for 30 min, followed by removal of vitelline membrane in heptane, 30-s shake in methanol, 10-s wash with methanol, and then return to PBT.

UAS–yellow Reporter

Calleja *et al.* (35) have devised a *UAS–yellow* reporter gene system. The *yellow* (*y*) gene is responsible for the normal pigmentation of adult cuticle and bristles all over the body. y^- flies are a yellow color and are clearly distinguishable from wild type. Flies that express GAL4 drive expression of $UAS\text{–}y^+$. When expressed in a y^- fly, the domain in which the y^+ gene is turned on by GAL4 (during the period that y^+ directs pigmentation) will appear as a patch of y^+ territory on a y^- background.

Using the *UAS–yellow* reporter, Calleja *et al.* isolated 27 enhancer–GAL4 lines that drive region-specific expression in the adult. In addition to identifying several developmental genes known to play a role in patterning the adult, this approach revealed previously unsuspected genetic subdivisions of the thorax.

Generating UAS–Target Gene Line

UAS–Target Gene Vector

The gene of interest is cloned into the polylinker of the vector pP[UAST] (UAS, Terminator) (18, 21), downstream of the five optimized GAL4 binding sites (the "*Sca*I site") and a synthetic TATA box and upstream of the Simian virus 40 (SV40) small t intron and polyadenylation site (see Fig. 6 for restriction map and sequence data). In our experience, when subcloning the target gene into pP[UAST], we have found it best to minimize the amount of 5′ leader sequence included, although there are reports that in some cases 5′ untranslated regions (UTRs) may help translation.

P-Element Transformation

pP[UAS–target gene] is injected using the same protocol as for pP[promoter–GAL4] and pP[GawB]. We have found that UAS transgenes are subject to position effects, such that different insertions are expressed at different levels. We therefore test and maintain between five and six independent insertion lines. This allows the generation of a phenotypic series: each GAL4 line is crossed to several UAS lines, each of which expresses the same target gene at a different level.

GAL4-Mediated Ectopic Expression of UAS–Target Gene

To express the target gene ectopically, a line carrying the UAS–target gene is crossed to a GAL4 line which directs UAS–gene expression in the desired ectopic domain (as determined using a UAS–reporter). The phenotype of ectopic expression can then be examined in the progeny of the cross. The first step of the analysis should be to determine precisely where the target gene has been ectopically expressed. Ectopic expression of the UAS–target gene can be monitored directly by staining with antibodies against the target gene product or by *in situ* hybridization to the target gene mRNA. It is an advantage to be able to distinguish the UAS–target gene product from the endogenous gene product by fusing the coding region of the target gene to a molecular tag. Perhaps the most versatile tag is GFP, for the reasons discussed earlier. Having directly determined the spatiotemporal pattern of target gene ectopic expression, one can proceed with elucidating the biological consequences of the ectopic expression experiment.

FLYBASE GAL4 AND UAS DATABASES

A large number of GAL4 and UAS lines have been made, driving expression of a plethora of target genes in a diverse range of patterns in embryos, larvae, and adults. A database detailing this valuable resource is currently under construction. However, at present, there is a comprehensive database of promoter–GAL4 and UAS–target gene constructs, which can be used to track down the corresponding transgenic line. This GAL4/UAS database can be found at *http://www.embl-ebi.ac.uk:7081/transposons/.* Once made, details of your own promoter–GAL4 or UAS–target gene constructs can be added to the database at the above web site.

IMPROVING AND ELABORATING GAL4 SYSTEM

The GAL4 system has been extensively tailored to achieve a variety of experimental goals.

Overcoming the Temporal Block to GAL4-Mediated UAS Expression

Much of the power of the GAL4 system stems from the range of GAL4 lines available and the great diversity of ectopic expression patterns these lines afford. However, the temporal diversity of ectopic expression patterns has been curtailed by a block to

GAL4 mediated UAS expression occurring before 3–4 h of embryogenesis (21). D. St. Johnston (personal communication) has achieved GAL4-mediated UAS expression at earlier stages by modifying both GAL4 UTRs and the GAL4 protein. Both promoter– and enhancer trap–GAL4 constructs encode GAL4 transcripts that contain heterologous 5′ and 3′ UTRs from *Drosophila hsp70. hsp70* mRNA is selectively degraded at non-heat shock temperatures (36), probably through an element present in the 3′ UTR. In addition to mRNA degradation, a component of the temporal block may be a requirement for a coactivator that is absent at these early stages but present later. In yeast, GAL4 transactivation efficiency is reduced fivefold in the absence of GAL11, a coactivator that also facilitates transcriptional activation by a range of other transcription factors (37). No GAL4 or GAL11 homologues have yet been identified in *Drosophila.* To obviate the need for a GAL4 coactivator, the GAL4 activation domain was exchanged for the herpesvirus VP16 activation domain. By the combination of removing *hsp70* UTRs and using a GAL4 binding domain VP16 activation domain fusion, the onset of GAL4-mediated UAS expression has been pushed forward to stage 4.

The early temporal block has meant that previous enhancer detection–GAL4 screens have failed to recover early-expressing GAL4 lines. A screen with an enhancer detection–GAL4–VP16 vector without the *hsp70* UTRs might remedy this deficit.

Targeted Cell Ablation

The ablation of a cell is a definitive test of its requirement in a particular biological process. Noninvasive cell ablation in a defined spatiotemporal domain can be achieved by crossing a GAL4 driver line to a UAS line that carries a GAL4-responsive gene that will kill the cell. Viable UAS–"killer" lines can be established because all UAS genes are silent in the absence of GAL4.

Targeted cell death has been achieved using *UAS–ricin A* (26, 28). Ricin A is the catalytic subunit of a cytotoxin that kills cells by irreversibly blocking protein synthesis (38). Because one or two molecules of ricin A may be sufficient to kill a cell it is necessary to prevent transient expression of the toxin following injection by placing a transcriptional terminator flanked by FRTs between the *UAS* and *ricin A* gene. Following transformation, the translational terminator is excised by introducing FLP recombinase in a genetic cross. *UAS–ricin A* has been used to specifically ablate subsets of longitudinal glia and pioneer neurons in the embryonic central nervous system, to test their requirement in axon guidance.

Targeted apoptotic cell death has been achieved by coexpression of *UAS–reaper* and *UAS–head involution defective* (hid) (39, 40). *reaper* and *hid* are components of the *Drosophila* programmed cell death pathway that are reportedly unable to initiate the death program individually, but can when expressed together. We have found that *reaper* is no longer dependent on *hid* when it is expressed at higher levels (U. John and A. H. Brand, unpublished). Following injection, cells are more tolerant of reaper or hid than ricin A, and so an intervening FRT sequence is not required. Coexpression of *UAS–reaper* and *UAS–hid* under the control of GAL4 has been used to ablate subsets of midline cells in the embryonic nervous system to ascertain their contribution to axon guidance (39). McNabb *et al.* (40) have used GAL4-directed coexpression of *reaper* and *hid* to specifically ablate the two neurons that secrete the neuropeptide eclosion hormone (EH). In flies in which the EH neurons are ablated, larval and adult ecdyses are disrupted, yet one-third manage to emerge as adults, demonstrating that EH has a significant but nonessential role in ecdysis.

Specific Elimination of Synaptic Transmission

The targeted disruption of synaptic communication in a specific neuron is a powerful way of linking a neural circuit to a particular behavioural phenotype. To this end, Sweeney *et al.* (41) have developed a *UAS–tetanus toxin* line. Tetanus toxin cleaves synaptobrevin, a synaptic vesicle membrane protein required for neurotransmitter release. When tetanus toxin is expressed in embryonic neurons under the control of GAL4, evoked, but not spontaneous, synaptic vesicle release is blocked. Tetanus toxin expression in a particular subset of neurons reduces the olfactory escape response.

Screening for Ectopic Expression Phenotypes

Mutations derived from classical genetic screens are primarily loss-of-function alleles. Mis- or overexpression phenotypes can be equally informative, but are much less likely to arise by traditional mutagenesis. Rorth (42) has redressed this imbalance by developing a GAL4-based screen that exclusively generates ectopic expression phenotypes.

Rorth made a UAS–promoter construct without a target gene, which turns any endogenous gene it inserts upstream of into a GAL4-responsive gene. This construct was injected and then remobilized

to generate an extensive library of UAS–promoter lines, each carrying a single UAS–promoter construct inserted at a unique, random position in the genome. When the library of UAS–promoter lines is crossed to a particular GAL4 line, the endogenous gene directly downstream of the UAS–promoter element will be transcribed in cells expressing GAL4. This means, by selecting the appropriate GAL4 line, one can identify genes that generate ectopic expression phenotypes in a specified tissue.

Crossing the library of UAS–promoter lines to the *sevenless*–GAL4 line directed expression of endogenous genes to the eye imaginal disk. GAL4 expression in 5 of the 163 UAS–promoter lines resulted in a dominant rough eye phenotype, suggesting that in these lines the UAS–promoter construct had integrated upstream of genes that could influence eye development. Indeed, in one of these UAS–promoter lines, the construct had inserted 5′ of *gap1* which encodes the *Ras* GTPase-activating protein. This result fits very well with previous analyses that have implicated the *Ras* signaling pathway in eye development.

Restricting UAS–target Gene Expression Domain with FLP/FRT

Often the only GAL4 line available to drive expression in the cells of interest also drives expression in other cells. Smith *et al.* (43) have used FLP/FRT technology [see (19)] to restrict UAS expression to variable subsets of cells within the GAL4 expression domain. In this approach, the UAS and target gene are separated by a FRT–transcriptional terminator–FRT sequence, which terminates GAL4-dependent transcription before it reaches the target gene. Thus, expression of the target gene is conditional on two factors: (i) the presence of GAL4 and (ii) the excision of the intervening poly(A) sequence. The timing and frequency of FRT–poly(A)–FRT excision events can be controlled by modulating expression of FLP recombinase from a heat-shock promoter. GAL4 is continuously required to maintain target gene expression, but FLP recombinase is required only once in a cell. After the excision event has been catalyzed, target gene expression in that cell and all its descendents becomes solely dependent on GAL4.

Targeted Gene Expression without Tissue-Specific Regulatory Element

As stated above, a GAL4 line with the desired spatiotemporal pattern is not always available, especially when one is interested in a very limited number of cells or a very narrow time window. Discrete enhancer elements might not exist in such cases, and when they do, they are likely to be small parts of more complex promoter regions. Halfon *et al.* (44) and Cambridge *et al.* (45) have rendered tissue-specific elements unnecessary in their expression strategies by using laser or UV light beams to dictate the spatiotemporal pattern of ectopic expression and can consequently generate active GAL4 in any cell at any time point in embryogenesis.

Laser-Induced Heat Shock–GAL4

Heat shock–GAL4 lines have the capacity for exquisite temporal control but spatially, can only drive GAL4 expression ubiquitously. To introduce spatial restriction, Halfon *et al.* use a laser microbeam to specifically heat shock a single cell within the embryo. A 1 to 2-min laser treatment is sufficient to induce a heat shock but is not lethal to the heat shocked cells. Halfon *et al.* demonstrate induction of *UAS–lacZ* by laser heat shock in a variety of cell types including neurons and somatic muscle.

Caged GAL4

Cambridge *et al.* have done away with GAL4 driver lines altogether and instead deliver a “caged” form of GAL4–VP16 protein directly into the UAS–target gene embryo by microinjection such that, on cellularization and subsequent divisions, each cell inherits the modified GAL4 protein. The GAL4–VP16 fusion protein is “caged” by reacting it with 6-nitroveratrylchloroformate, which blocks the ability of GAL4–VP16 to bind the UAS target sequence. Thus, although caged GAL4–VP16 is present in every cell of the injected UAS–target gene embryo, it cannot activate target gene transcription. The ability of caged GAL4–VP16 to bind the UAS and activate transcription is only restored on illumination with 365-nm UV light. Thus, by aiming a fine beam of UV light at a particular cell, the caged GAL4–VP16 will be photoactivated in that cell alone and consequently drive UAS–target gene transcription in that cell alone.

Cambridge *et al.* used caged GAL4–VP16 in *UAS–lacZ* embryos to fate map single cells of adjacent mitotic domains and also successfully induced ectopic expression of Ubx in a *UAS–Ubx* line. This strategy cannot be used for manipulating later stages of development because the caged GAL4–VP16 fusion protein will have become too dilute.

GAL4 Systems in Other Organisms

GAL4 has been shown to transactivate UAS–target genes in a range of organisms. Thus it has been

feasible to establish the GAL4 system in organisms other than *Drosophila.* Ornitz *et al.* (46) have set up a mouse GAL4 system, that relies on characterized promoter–GAL4 lines to drive ectopic expression of UAS–target genes such as *int-2.* J. Haseloff (personal communication, see also *http://brindabella.mrc-lmb.cam.ac.uk*) has developed a GAL4 system in *Arabidopsis* that uses enhancer trap–GAL4–VP16 lines to drive ectopic expression.

Parallel Bipartite Ectopic Expression System

The ability to independently target the expression of two different genes to two different domains would add a new dimension to the analysis of gene function: the spatiotemporal basis of an interaction between two genes could be investigated. For example, is ectopic expression of genes X and Y required in the same cell or in different cells? Is ectopic expression of gene X required before the expression of gene Y for Y to exert its effects?

The GAL4 system can drive two different UAS–target genes simultaneously in the same pattern, but it cannot direct them in different spatiotemporal patterns. This can be achieved only by developing a parallel bipartite system. To this end U. John and A. H. Brand (unpublished) and B. Bello and W. Gehring (personal communication) are engineering into flies the tetracycline transactivator (tTA)/tetracycline operator (tetO) link, which has been pioneered in mice by Gossen and Bujard (47). Thus, within one fly, a promoter/enhancer–GAL4 construct would direct UAS–gene X in one pattern, while a promoter/enhancer–tTA would direct tetO–gene Y in a different pattern. The tTA/tetO system has the added advantage of inducibility.

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