

dimers and tetramers occurs (the K_d value). Possibilities for this behavior involve a simple mechanism with compensating trailing of dimers and tetramers leading to increased peak widths in a symmetrical fashion to give a bell-shaped curve but not affecting peak position. Alternatively, a more complex dynamic mechanism involving the dissociation–association process itself could be involved. We are evaluating these possibilities for this behavior, which has not been observed by other methods for measuring protein dissociation but which can be analyzed accurately by the procedure described in this article.

Comments

Two aspects of determining subunit dissociation constants by FPLC on Superose-12 should be emphasized. First, the question of whether the gel-filtration procedure itself influences the equilibrium between tetramers and dimers has essentially been answered by Uversky²⁵ in a careful analysis of equilibrating native, molten globule and denatured states of proteins. He has shown in convincing fashion for many such protein systems that Superose-12 is inert and does not affect these equilibria. This conclusion is in agreement with ours regarding K_d values of hemoglobins calculated by this method compared with published values obtained by other methods as described in this article. With respect to proteins for which standard elution positions of equilibrating species are not known, curve fitting has been used as described in the section “Evaluation of K_d Values” to obtain accurate subunit dissociation constants.

²⁵ V. N. Uversky, *Biochemistry* **32**, 13288 (1993).

[7] Ectopic Expression Systems in *Drosophila*

By PIER PAOLO D’AVINO and CARL S. THUMMEL

Introduction

The development of higher organisms is dependent on the appropriate spatial and temporal patterns of gene expression. In the fruit fly, *Drosophila melanogaster*, misexpression of a single gene is often sufficient to disrupt normal development, generating dominant phenotypes that may provide important clues regarding gene function. In the past, the only means of ectopically expressing a gene in *Drosophila* was through the generation of

mutants, usually dominant, that caused misexpression and/or overexpression of a specific gene product. However, a variety of new tools for ectopic expression have been developed over the past few years, thanks to extensive efforts by the *Drosophila* research community. With these techniques in hand, it is now possible to manipulate gene expression both spatially and temporally, as well as to combine different systems in order to direct the expression of any gene in a predetermined stage- or tissue-specific manner. The use of these ectopic expression systems, combined with classical genetic studies in *Drosophila*, has led to a detailed understanding of a variety of key developmental pathways, including the establishment of cell fate, tissue patterning, and intercellular signaling mechanisms. These systems have also provided an invaluable means of obtaining markers for specific tissues, cells, or cell lineages, as well as a means of targeting cell ablation.^{1,2}

Drosophila ectopic expression systems utilize a promoter that drives either constitutive or regulated expression of the gene of interest. These constructs are prepared in a *P*-element vector and are introduced into the fly genome by germ line transformation.³ On establishing a transgenic line, ectopic expression of the gene of interest can be analyzed in either a wild-type or a mutant genetic background. This article provides a description of the different ectopic expression systems that have been developed in *Drosophila*, listing the strengths and limitations of each technique. Further information regarding the vectors described in this article can be accessed through FlyBase at <http://flybase.bio.indiana.edu:82/transposons/>.

Constitutive and Uniform Expression

Promoters from five *Drosophila* genes have been used in an effort to achieve constitutive and uniform expression during development: *tubulin*, *actin5C*, *armadillo*, *polyubiquitin*, and *EF-1 α* . Initially, the promoters from two cytoskeleton protein genes, *actin5C* and *tubulin*, were used for ectopic expression.^{4,5} Both promoters, however, have two important limitations: they direct relatively low levels of transcription and their expression is not uniform, especially during embryonic development.⁴⁻⁸ Nevertheless, the *actin5C* promoter has been widely used and has been shown to direct

¹ J. W. Sentry, M. M. Yang, and K. Kaiser, *BioEssays* **15**, 491 (1993).

² D. E. Buenzow and R. Holmgren, *Dev. Biol.* **170**, 338 (1995).

³ G. M. Rubin and A. C. Spradling, *Science* **218**, 348 (1982).

⁴ S. Bialojan, D. Falkenburg, and R. Renkawitz-Pohl, *EMBO J.* **3**, 2543 (1984).

⁵ E. A. Fyrberg, J. W. Mahaffey, B. J. Bond, and N. Davidson, *Cell* **33**, 115 (1983).

⁶ J. E. Natzle and B. J. McCarthy, *Dev. Biol.* **104**, 187 (1984).

⁷ T. C. Burn, J. O. Vigoreaux, and S. L. Tobin, *Dev. Biol.* **131**, 345 (1989).

⁸ J. O. Vigoreaux and S. L. Tobin, *Genes Dev.* **1**, 1161 (1987).

uniform expression in imaginal discs.⁹ Two versions of a *P*-element vector containing *actin5C* sequences, pCaSpeR-act, are available.¹⁰ This vector contains the *actin5C* proximal promoter and first exon as well as its 3' polyadenylation signals. The two versions of this vector differ in the unique cloning sites, *EcoRI* or *BamHI*, that are present between the promoter and 3' sequences.

The promoter of the segment polarity gene *armadillo* has been used in an effort to overcome the limitations of the *actin5C* and *tubulin* promoters. High levels of *armadillo* expression have been observed during embryogenesis and pupal development.¹¹ Similarly, transcription from the *armadillo* promoter is uniform during early embryogenesis and in imaginal discs.¹² However, its level is not constant throughout development. For example, when the *armadillo* promoter was used to drive expression of an *Escherichia coli lacZ* reporter gene, β -galactosidase staining was not detected in the larval midgut.¹² This observation suggests that the usefulness of the *armadillo* promoter for uniform ectopic expression may be restricted to embryonic development and imaginal discs.

Most recently, the *polyubiquitin* promoter has been employed for uniform ectopic expression.¹³ This promoter appears to direct higher levels of transcription than the other promoters just mentioned, in many tissues and throughout development. However, although a *polyubiquitin-lacZ* reporter gene is expressed uniformly in early embryos and imaginal discs, its expression pattern has not yet been characterized at other developmental stages.¹⁴ A useful *P*-element-*polyubiquitin* vector, Pwum2, has been described.¹⁵ This vector contains the *polyubiquitin* promoter fused to a small fragment encoding a *myc* epitope tag. Two restriction sites, *KpnI* and *NotI*, are located downstream from the *myc*-coding region and upstream from an *hsp70* 3'-untranslated region and polyadenylation signals (described later). The *myc* tag provides a useful means of detecting the encoded protein in transformed animals by immunolocalization of the *myc* epitope.

Finally, the peptide synthesis elongation factor *EF-1 α* promoter has been used for ectopic expression in *Drosophila*.¹⁶ This promoter was re-

⁹ G. Struhl and K. Basler, *Cell* **72**, 527 (1993).

¹⁰ C. S. Thummel, A. M. Boulet, and H. D. Lipshitz, *Gene* **74**, 445 (1988).

¹¹ B. Riggelman, E. Wieschaus, and P. Schedl, *Genes Dev.* **3**, 96 (1989).

¹² J.-P. Vincent, C. H. Girdham, and P. H. O'Farrell, *Dev. Biol.* **164**, 328 (1994).

¹³ H. S. Lee, J. A. Simon, and J. T. Lis, *Mol. Cell. Biol.* **8**, 4727 (1988).

¹⁴ J.-P. Vincent and C. Girdham, in "Methods in Molecular Biology" (R. Tuan, ed.), p. 385. Humana Press, Totowa, NJ, 1997.

¹⁵ M. M. S. Heck, A. Pereira, P. Pesavento, Y. Yannoni, A. C. Spradling, and L. S. B. Goldstein, *J. Cell Biol.* **123**, 665 (1993).

¹⁶ R. Ackermann and C. Brack, *Nucleic Acids Res.* **24**, 2452 (1996).

ported to direct high levels of uniform expression in embryos and adults, although no supporting data were presented.

In conclusion, no promoter provides completely uniform and constitutive expression throughout development. This is not surprising given that every promoter is subject to some level of spatial and temporal regulation. The choice of a promoter for widespread expression should, therefore, be based on the particular needs of each experiment. For example, the *armadillo*, *polyubiquitin*, or *EF-1 α* promoters should be useful for achieving uniform gene expression during embryogenesis or in imaginal discs.

Regulated Expression

One problem associated with constitutive ectopic gene expression is that this unregulated expression may lead to lethality, preventing the isolation of a transformed line. In addition, uniform expression may lead to undesirable phenotypes in tissues that do not normally express the gene of interest. These problems can be overcome by methods that allow stage- and/or tissue-regulated ectopic expression. Some of these methods are relatively simple whereas others are more sophisticated and require combining one or more different techniques.

The simplest method for achieving controlled expression of a foreign gene is to use regulatory sequences from a well-characterized promoter.¹⁷⁻²⁰ Such regulatory elements will drive gene expression in a well-defined tissue and/or stage-specific manner. Use of the *glass* multimer reporter (GMR) to drive ectopic gene expression in the developing *Drosophila* eye provides an ideal example of this system.²⁰ The GMR vector contains a pentamer of binding sites for the GLASS transcriptional activator, derived from the *Drosophila Rh1* promoter. GLASS expression is restricted to the developing eye, the larval photoreceptor organs, and a few cells in the larval brain, providing tight spatial regulation on ectopic gene expression.

The merit of using characterized regulatory sequences is that only one transgenic line has to be established in order to precisely drive gene expression in both time and space. This method is, however, limited by the availability of well-studied and cloned promoters. One means of overcoming this limitation is the "enhancer piracy" approach developed by Noll *et al.*²¹ This method is very similar to the "enhancer trap" technique in which the *lacZ* gene, fused to a minimal promoter, is inserted randomly at multiple

¹⁷ S. M. Parkhurst and D. Ish-Horowicz, *Development* **111**, 1121 (1991).

¹⁸ S. M. Parkhurst, D. Bopp, and D. Ish-Horowicz, *Cell* **63**, 1179 (1990).

¹⁹ C. S. Zucker, D. Mismar, R. Hardy, and G. M. Rubin, *Cell* **53**, 475 (1988).

²⁰ B. A. Hay, T. Wolff, and G. M. Rubin, *Development* **120**, 2121 (1994).

²¹ R. Noll, M. A. Sturtevant, R. R. Gollapudi, and E. Bier, *Development* **120**, 2329 (1994).

locations into the *Drosophila* genome. In over half of these transformed lines, the *lacZ* gene falls under the influence of one or more flanking genomic enhancers that activate its transcription in a spatially and temporally regulated pattern.²²⁻²⁴ In their strategy, Noll and colleagues substituted the *rhomboid* (*rho*) gene for the *lacZ* reporter gene present in enhancer trap vectors. Upon introducing this modified construct into multiple sites in the *Drosophila* genome, *rho* expression came under the influence of flanking genomic enhancer elements. This led to novel dominant phenotypes that could provide insights into *rho* developmental functions, which may also be useful for the development of genetic screens for suppressors or enhancers.²¹

Temporally Regulated Expression: Use of Heat Shock Promoters

Since its first applications, the use of heat shock promoters has been one of the most important and powerful tools for directing temporally regulated expression in *Drosophila*.²⁵⁻²⁷ The level of ectopic gene expression can also be modulated easily by altering the temperature and/or the duration of heat treatment. Indeed, this is one of the unique advantages of working with *Drosophila*, insofar as similar temporal regulation of ectopic gene expression is not currently available in other higher organisms.

Although heat shock ectopic expression experiments have been essential for elucidating many developmental mechanisms, there are at least three potential drawbacks in the use of this system. First, heat shock promoters have a low, but sometimes significant, basal level of transcription under non-heat shock conditions. This can be a problem when even small amounts of a gene product are toxic or lead to a premature mutant phenotype. Second, heat shock alone, at some developmental stages, can phenocopy certain mutations. This is because endogenous cellular gene expression is disrupted upon heat treatment, and the reduced levels of some key regulatory gene products lead to the generation of recognizable mutant phenotypes.²⁸ For example, a high temperature heat shock of 4-hr *Drosophila*

²² C. J. O'Kane and W. J. Gehring, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9123 (1987).

²³ E. Bier, H. Vaessin, S. Shepherd, K. Lee, K. McCall, S. Barbel, L. Ackerman, R. Caretto, T. Uemura, E. Grell, L. Y. Jan, and Y. N. Jan, *Genes Dev.* **3**, 1273 (1989).

²⁴ H. J. Bellen, C. O'Kane, C. Wilson, U. Grossniklaus, R. K. Pearson, and W. J. Gehring, *Genes Dev.* **3**, 1288 (1989).

²⁵ J. T. Lis, J. A. Simon, and C. A. Sutton, *Cell* **35**, 403 (1983).

²⁶ G. Struhl, *Nature* **318**, 677 (1985).

²⁷ S. Schneuwly, R. Klemenz, and W. J. Gehring, *Nature* **325**, 816 (1987).

²⁸ N. S. Petersen and H. K. Mitchell, in "Comprehensive Insect Physiology, Biochemistry and Pharmacology" (G. A. Kerkut and L. I. Gilbert, eds.), p. 347. Pergamon Press, New York, 1982.

embryos can reproduce the four-winged phenotype associated with *Bi-thorax Complex* mutations.²⁹ Finally, heat shock promoters are active in virtually all cell types and thus may not be useful for the analysis of tissue-specific functions.

There are a variety of ways in which the investigator can circumvent these problems. By isolating a number of transformed lines, it is often possible to select one or two transformants that provide a low level of background expression along with a relatively high level of heat-induced expression. Furthermore, it is possible to maintain transformed flies at low temperature (18–21°), reducing the background level of heat shock promoter activity. A non-heat-shocked control should also be included in each experiment. By maintaining one set of animals at 25°, while the second is treated at 35–37° for the desired period of time, the investigator can identify effects that are due solely to heat-induced expression.

Mutant phenocopies can be avoided by using relatively mild heat shocks ($\leq 37^\circ$) as well as avoiding developmental stages when the animal is most sensitive to heat shock.²⁸ Heat treatment of a nontransformed control stock, in parallel with the transformant of interest, can provide an effective control to identify phenotypes that are due solely to heat shock.

Techniques have also been developed that allow restricted heat treatment of specific target cells. Monsma *et al.*³⁰ reported the use of a heated needle to induce local heat shock induction at virtually any developmental stage. The temperature and diameter of the needle can be varied in order to determine the size of the affected region. More recently, Halfon *et al.*³¹ described the use of a finely regulated laser microbeam burst to induce heat shock treatments at the single cell level. These techniques, however, require that the cells of interest be easily accessible, and thus are primarily useful during embryogenesis in *Drosophila*.

In addition to ectopic expression experiments, heat-induced expression can be used to examine later phenotypes of some mutants that die early during development. For example, heat-induced expression of *spaghetti squash* (*sqh*), which encodes the nonmuscle myosin II regulatory light chain, can effectively rescue the early lethality of *sqh* mutants, facilitating the analysis of phenotypes later in development.³² This method requires that the gene of interest be widely expressed, reflecting the pattern of heat-

²⁹ H. Gloor, *Rev. Suisse Zool.* **54**, 637 (1947).

³⁰ S. A. Monsma, R. Ard, J. T. Lis, and M. F. Wolfner, *J. Exp. Zool.* **247**, 279 (1988).

³¹ M. S. Halfon, H. Kose, A. Chiba, and H. Keshishian, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6255 (1997).

³² K. A. Edwards and D. P. Kiehart, *Development* **122**, 1499 (1996).

induced transcription. In this way, the investigator can obtain the equivalent of a conditional lethal mutant when such mutants are not directly available.

Heat Shock Vectors and Protocols

Several *P*-element vectors are currently available for heat-inducible ectopic expression. These vectors have been constructed using *hsp26*, *hsp70*, and *hsp82* promoters and 5'-untranslated leaders, although the vectors that contain *hsp70* sequences are used most frequently. The presence of 5' leader sequences from *hsp* genes is crucial because these sequences are necessary for efficient translation at high temperatures.³³ Most of these vectors contain a polylinker for inserting the gene of interest, followed by a 3'-untranslated sequence derived from the *hsp70* or *Actin5C* *Drosophila* genes or SV40. The *hsp70* 3' trailer directs rapid mRNA degradation under non-heat shock conditions and is therefore recommended only when rapid turnover of the gene product is required. Two vectors that carry the *hsp70* promoter have been widely used, and their structures are presented in Fig. 1.

Many protocols have been published for heat-induced ectopic expression, and a good protocol for embryo treatment can be found in Brand *et al.*³⁴ This article describes an efficient protocol for heat treatment of late larvae and pupae. This protocol has been successful for the analysis of genetic regulatory hierarchies triggered by the steroid hormone ecdysone at the onset of metamorphosis.³⁵⁻³⁷

The inherent asynchrony of *Drosophila* larval development makes it difficult to stage larvae accurately. One simple and efficient method for staging third instar larvae is to grow them on food containing 0.05–0.1% bromphenol blue.³⁸ Late third instar larvae staged by this method can be used to select newly formed prepupae at 15-min intervals. These synchronized prepupae can then be allowed to develop for the appropriate period of time in order to obtain closely staged animals throughout pupal development.

³³ T. J. McGarry and S. Lindquist, *Cell* **42**, 903 (1985).

³⁴ A. H. Brand, A. S. Manoukian, and N. Perrimon, in "*Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology" (L. S. B. Goldstein and E. A. Fyrberg, eds.), p. 635. Academic Press, New York, 1994.

³⁵ G. T. Lam, C. Jiang, and C. S. Thummel, *Development* **124**, 1757 (1997).

³⁶ C. T. Woodard, E. H. Baehrecke, and C. S. Thummel, *Cell* **79**, 607 (1994).

³⁷ J. C. Fletcher, P. P. D'Avino, and C. S. Thummel, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4582 (1997).

³⁸ A. J. Andres and C. S. Thummel, in "*Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology" (L. Goldstein and E. Fyrberg, eds.), p. 565. Academic Press, New York, 1994.

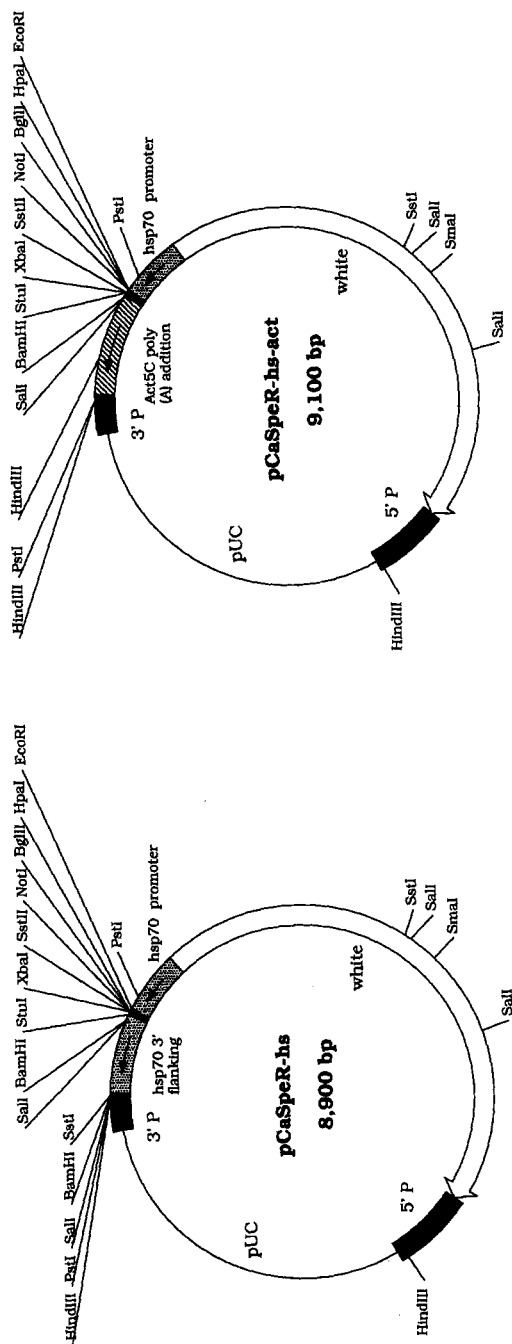


FIG. 1. Maps of *P*-element vectors for heat-inducible ectopic expression. pCaSpeR-hs has been described previously.⁵⁰ pCaSpeR-hs-act is a derivative that was constructed by C. Woodard and CST. An 800-bp *SalI*-*PstI* fragment from pCaSpeR-act, carrying the *actin5C* 3' trailer and polyadenylation signals, was used to replace the *hsp70* 3' region present in pCaSpeR-hs. This vector should increase the stability of ectopically expressed genes under non-heat shock conditions. The *white* gene provides a selectable marker to identify transformants. The following unique restriction sites are available for inserting foreign genes into either of these vectors: *EcoRI*, *HpaI*, *BglII*, *NotI*, *SstII*, *XbaI*, and *StuI*. GenBank accession numbers: pCaSpeR-hs, U59056; pCaSpeR-hs-act, U60735. These vectors are available on request.

1. Collect third instar larvae, prepupae or pupae, staged as described earlier, with a wet paintbrush and wash briefly in water at room temperature (no higher than 25°). As described earlier, it is best to use three stocks for each experiment: nontransformed control animals, transformants maintained at 25°, and transformants subjected to heat shock.
2. Transfer 10–20 animals to a 1.5-ml microcentrifuge tube. A second microcentrifuge tube, with small holes at the bottom, can be inserted into the first tube in order to prevent the larvae from escaping.
3. Transfer the tube to a 35–37° water bath for 30–60 min. The temperature and time may vary depending on the experiment, but ≤35° is recommended if incubations longer than 30 min are required.
4. Transfer the vials to room temperature and maintain at 25° for about 2 hr in order to allow the animals to recover from heat treatment. This period of time is usually sufficient to allow maximal accumulation of the protein of interest. It is best to try different heat shock temperatures, times of heat treatment, and recovery times in order to identify the appropriate conditions for optimal ectopic expression. This can be assessed easily by Northern or Western blot analysis.

Spatially Regulated Ectopic Expression: GAL4 System

The yeast transcriptional activator *GAL4* provides the most powerful method currently available for directing cell type or tissue-specific ectopic expression.³⁹ This method, including vectors, protocols, uses, strengths and limitations, has been reviewed previously.³⁴ We will, therefore, limit our description to the basic concepts of this technique.

GAL4 can activate transcription of any target gene by binding to a *GAL4* UAS (upstream activation sequence) positioned near a minimal promoter.⁴⁰ In this system, two different *P*-element transformant lines are established, one of which expresses *GAL4* in a particular spatial and temporal pattern and the other of which carries a target gene under the control of multiple UAS elements. Expression of *GAL4* has no detectable effects on *Drosophila* development, and the target gene is not expressed in the absence of *GAL4*. Only on crossing these two lines will the target gene be activated, in a temporal and spatial pattern that reflects that of *GAL4* (Fig. 2).

Two approaches have been used to generate lines that express the *GAL4* activator. First, *GAL4* can be expressed using either a defined *Drosophila* promoter or the *hsp70* promoter, as described earlier. Second,

³⁹ A. H. Brand and N. Perrimon, *Development* **118**, 401 (1993).

⁴⁰ J. A. Fischer, E. Giniger, T. Manaitis, and M. Ptashne, *Nature* **332**, 853 (1988).

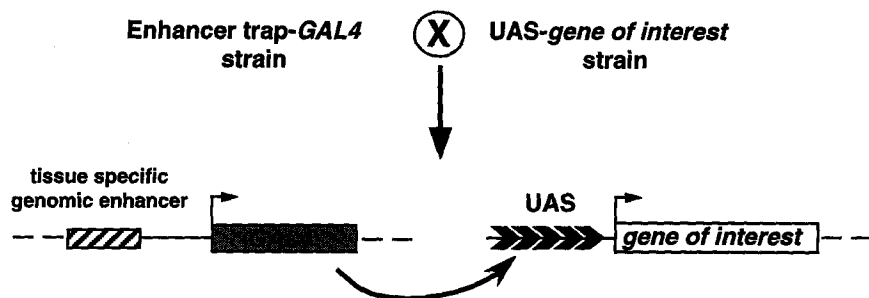


FIG. 2. Use of the GAL4 system to drive tissue-specific ectopic expression. A fly strain carrying *GAL4* under the control of a tissue-specific genomic enhancer is crossed to a strain containing the gene of interest under the control of UAS sequences. In the progeny, the gene of interest will be expressed in the same pattern as its activator, *GAL4*.³⁹

collections of *GAL4*-expressing lines have been established using the “enhancer trap” technique, in which the *GAL4* expression pattern is determined by flanking genomic enhancer elements. These transformant lines have been characterized by crossing them with flies that carry a *UAS-lacZ* reporter gene and analyzing the pattern of β -galactosidase expression at different stages of development. In this way, a large number of lines have been established that provide a wide range of different *GAL4* expression patterns.^{39,41,42} It should be noted that the level of ectopic expression can also be modulated by varying the number of UAS sequences. Finally, there are three possible drawbacks of the *GAL4* technique. First, *GAL4*-mediated expression cannot be detected in the female germ line and before gastrulation in embryos.³⁴ Second, the level of *GAL4*-mediated transcription is often variable from cell to cell within the same expression domain.³⁴ Finally, the effects of *GAL4*-mediated expression at later stages of development could be due to earlier expression of the *GAL4* activator. Some attention thus needs to be paid to the temporal and spatial patterns of *GAL4* expression up to the stage selected for phenotypic analysis.

The *GAL4* system has been developed into a method of screening for phenotypes caused by misexpression of endogenous genes.⁴³ In this method, a minimal promoter under the control of UAS elements was inserted into a *P*-element vector such that transcription from this promoter was directed into flanking genomic sequences. This element was introduced into multiple sites in the *Drosophila* genome and activated with tissue-specific *GAL4*

⁴¹ K. Gustafson and G. L. Boulianne, *Genome* **39**, 174 (1996).

⁴² L. Manseau, A. Baradaran, D. Brower, A. Budhu, F. Elefant, H. Phan, A. V. Philp, M. Yang, D. Glover, K. Kaiser, K. Palter, and S. Selleck, *Dev. Dyn.* **209**, 310 (1997).

⁴³ P. Rorth, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12418 (1996).

expression. Under these conditions, the UAS-regulated promoter is induced and a flanking endogenous gene will be expressed ectopically in a pattern that reflects that of the GAL4 activator. The resultant dominant phenotypes provide a novel means of screening for new regulatory genes.

Combined Techniques

Many combinations of one or more techniques described earlier have been established in order to overcome some of the limitations of each approach. Some of these techniques are very elegant and provide a means of ectopically expressing a given gene in clones of a single cell at a desired developmental stage.

For example, one method combines the temporal regulation provided by heat shock with the spatial regulation provided by an activator such as GAL4.^{20,39} In these methods, the gene of interest is under the control of responsive elements of a specific transcriptional activator (yeast GAL4 or *Drosophila* GLASS have been used), which, in turn, is under the control of an *hsp70* heat shock promoter. The transcriptional activator and the target gene are, however, separated in two different transgenic lines. In one line, the target gene remains inactive because of the absence of the activator protein, whereas in the other line the activator is present but there is no target gene to induce. The gene of interest will only be expressed after the two strains are crossed and the progeny are subjected to heat treatment (Fig. 3). This method is useful for expressing highly toxic genes in a spatially and temporally regulated manner.

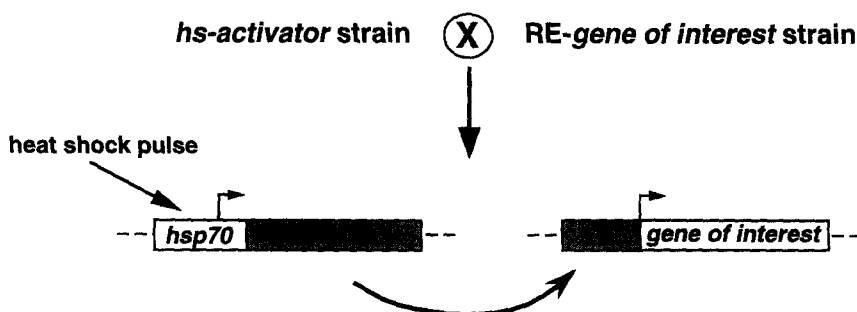


FIG. 3. Combining the heat shock technique with the use of characterized promoters. In this system, one fly strain carries a transgene in which a transcriptional activator is under the control of the *hsp70* heat shock promoter. Another transgenic fly strain carries the gene of interest under the control of response elements (RE) for the activator. After crossing these two lines, the progeny will carry both transgenes and thus the gene of interest can be expressed by heat induction of the transcriptional activator.

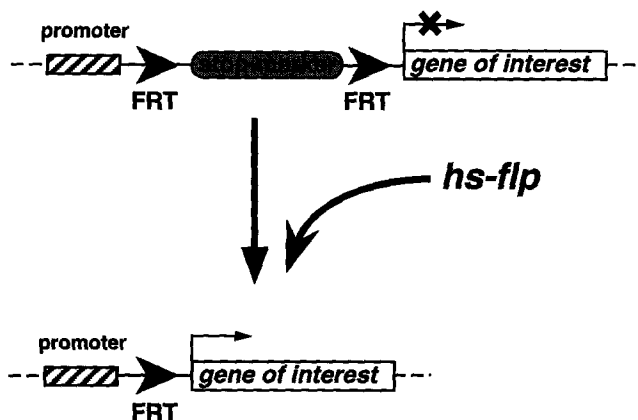


FIG. 4. The *FLP*-out technique. The gene of interest is separated from either a regulated or a constitutive promoter by a DNA fragment called a *FLP*-out cassette. This cassette contains one or more transcriptional stop signals (stop) and a genetic marker flanked by two directly repeated FRT sites. The *FLP*-out cassette thus prevents expression of the gene of interest. After heat-induced expression of FLP recombinase, the *FLP*-out cassette is excised randomly in the organism, juxtaposing the promoter and the gene of interest. In this way, clones of cells are generated that express the gene of interest under the control of a known promoter. These clones can be recognized easily by loss of the genetic marker.⁹

An elegant technique to direct patches of ectopic gene expression within a single tissue has been reported by Struhl and Basler.⁹ This method is based on the use of the FLP recombinase from the 2- μ m plasmid of *Saccharomyces cerevisiae*. FLP can efficiently catalyze site-specific recombination between two FLP recombination targets (FRTs) in flies.⁴⁴ When two FRTs are arranged as direct repeats, FLP-mediated recombination leads to the excision of the intervening DNA, leaving only one FRT and joining the sequences on either side.

Figure 4 shows how FLP-mediated recombination can be used in order to generate inducible ectopic expression. A promoter is separated from a given gene by a so-called "*FLP*-out cassette." This cassette contains a transcriptional stop signal along with a visible marker gene that is flanked by two direct FRT repeats. Transcription from the promoter is thus terminated upstream from the gene of interest, maintaining the target gene in a repressed state. The presence of the stop sequence can be followed by observing the phenotype of the marker gene (in this case, a *yellow* bristle marker). Upon ubiquitous activation of *FLP* expression by a heat pulse, the *FLP*-out cassette is excised randomly in the organism, establishing cell clones

⁴⁴ K. G. Golic and S. Lindquist, *Cell* **59**, 499 (1989).

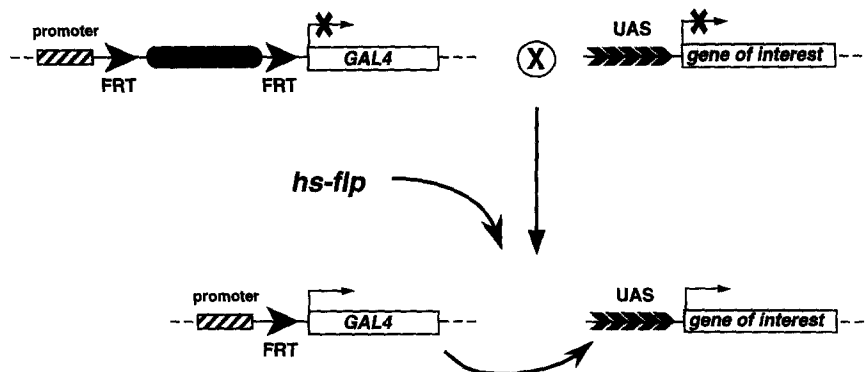


FIG. 5. Combining the GAL4 system with the *FLP*-out technique. In one transgenic line, a *FLP*-out cassette divides a promoter from the *GAL4* gene, whereas in another line the gene of interest is under the control of UAS sequences. The two lines are crossed and, in the progeny, heat-induced FLP recombinase expression generates cell clones that express *GAL4* under the control of the promoter. *GAL4*, in turn, activates the gene of interest through its binding to UAS sequences.^{46,47}

that express the gene of interest (see Fig. 4). Such clones can be recognized easily by the loss of the marker gene. Either a constitutive or a regulated promoter can be used with this technique, depending on the purpose of the experiment. Because this method directs patches of ectopic gene expression within a tissue, it is extremely helpful for analyzing the function of genes involved in establishing positional information during development.

A modification of the *FLP*-out method has been described that allows the analysis of gene expression at very early stages of development.⁴⁵ In this system, called IT for immediate and targeted, *FLP* expression is under the control of the β_2 -tubulin promoter. Because this promoter functions only in maturing spermatocytes, the target gene will only be active in progeny derived from sperm in which the *FLP*-out cassette has been excised. Furthermore, the use of an early promoter, such as that from the *hunchback* gene, restricts ectopic expression to early embryonic stages. The IT system can be used to direct the expression of a gene at earlier times than are possible with either the *hsp70* or *GAL4* systems.

The *FLP*-out method has also been combined with the *GAL4* system in order to facilitate the ectopic expression of target genes.^{46,47} In this method, outlined in Fig. 5, the *FLP*-out cassette divides a constitutive or regulated promoter from the *GAL4* gene, and different genes of interest

⁴⁵ E. A. Wimmer, S. M. Cohen, H. Jackle, and C. Desplan, *Development* **124**, 1509 (1997).

⁴⁶ F. Pignoni and S. L. Zipursky, *Development* **124**, 271 (1997).

⁴⁷ J. F. de Celis and S. Bray, *Development* **124**, 3241 (1997).

are under the control of UAS sequences. After FLP-mediated excision of the cassette, the *GAL4* gene is transcribed, which, in turn, activates expression of the gene of interest. Although this is formally identical to the *FLP*-out system depicted in Fig. 4, it provides more flexibility in that a variety of different UAS-target gene transgenes can be activated using different crosses. de Celis and Bray have devised a modification of this system in which they introduced a *GAL4-lacZ* dicistronic gene to mark cell clones not only by the loss of the marker, but also by the expression of *lacZ*.

Conclusions

The last few years have seen the development of elegant systems to ectopically express a gene of interest at virtually any time and place during development. These systems draw together the precise temporal regulation of the *hsp70* promoter with the spatial regulation provided by the *GAL4* system. In addition, judicious use of the FRT/FLP recombinase system provides an elegant means of further restricting the patterns of ectopic gene expression. It seems likely that future combinations of these systems, along with the CRE/loxP recombination system,⁴⁸ will provide additional ectopic expression systems over the next few years.

Despite the power of these techniques, however, it is important to bear in mind that ectopic expression provides only a partial understanding of gene function. These methods provide insights into what functions a particular gene can provide, not necessarily what the gene does in its normal developmental context. Furthermore, the high levels of ectopic expression associated with some systems may allow a factor to feed into a developmental pathway that it does not normally regulate. Despite these considerations, however, ectopic gene expression provides an essential and invaluable tool to study gene function. By combining gain-of-function studies with the analysis of loss-of-function mutant phenotypes, the investigator has an unparalleled opportunity to understand gene function during development.⁴⁹

Acknowledgments

P.P.D. is a Research Associate and C.S.T. an Associate Investigator with the Howard Hughes Medical Institute. We thank J. Broadus and S. Sakonju for critical comments on the manuscript.

⁴⁸ M. L. Siegal and D. L. Hartl, *Genetics* **144**, 715 (1996).

⁴⁹ Y. N. Jan and L. Y. Jan, *Cell* **75**, 827 (1993).

⁵⁰ C. S. Thummel and V. Pirrotta, *Dros. Inf. Serv.* **71**, 150 (1992).