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Targeted gene expression without a tissue-specific promoter: Creating mosaic embryos using laser-induced single-cell heat shock

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ABSTRACT **We have developed a method to target gene expression in the *Drosophila* embryo to a specific cell without having a promoter that directs expression in that particular cell. Using a digitally enhanced imaging system to identify single cells within the living embryo, we apply a heat shock to each cell individually by using a laser microbeam. A 1- to 2-min laser treatment is sufficient to induce a heat-shock response but is not lethal to the heat-shocked cells. Induction of heat shock was measured in a variety of cell types, including neurons and somatic muscles, by the expression of β-galactosidase from an *hsp26-lacZ* reporter construct or by expression of a UAS target gene after induction of *hsGAL4*. We discuss the applicability of this technique to ectopic gene expression studies, lineage tracing, gene inactivation studies, and studies of cells *in vitro*. Laser heat shock is a versatile technique that can be adapted for use in a variety of research organisms and is useful for any studies in which it is desirable to express a given gene in only a distinct cell or clone of cells, either transiently or constitutively, at a time point of choice.**

Transgenic organisms have become essential tools in the study of both animal and plant development. One powerful use of transgene expression has been to study the functions of a given gene by causing it to be expressed in a cell or tissue in which it is normally not expressed, or by altering the timing of its expression in a given cell or tissue. Such ectopic expression experiments are limited, however, by the availability of methods to direct transgene expression to the tissue of choice at the time point of choice. In *Drosophila*, where it is relatively simple to create stably transformed fly lines via *P*-element-mediated transformation (1), there are several common methods for ectopic gene expression (2, 3). These methods can be grouped into two basic classes: methods that use a defined promoter sequence to drive transgene expression in a tissue-specific fashion, and those that use a heat-shock promoter to induce transgene expression upon heat shock. While each class has certain advantages, each also has significant limitations.

Ectopic expression by means of a defined promoter or enhancer sequence has the distinct advantage of allowing expression to be regulated in a known spatial and temporal fashion. However, use of this method requires that appropriate promoter or enhancer sequences be cloned and available. Many *Drosophila* promoters have been characterized, and the introduction of the GAL4 system of Brand and Perrimon (4) has made it possible to take advantage of enhancer-trapping techniques to generate large numbers of ectopic expression drivers with many different expression patterns. Nevertheless, a promoter, enhancer element, or GAL4 driver with the desired expression pattern is not always available, especially when one is interested in causing expression in a very limited number of cells or during a very narrow time window. Discrete enhancer elements might not exist in many such cases, and even when they do, they are likely to be small parts of more complex promoter regions and thus labor-intensive to isolate.

Use of the heat-shock promoter allows expression of the transgene to be induced at any time during development. Moreover, by varying the temperature or duration of heat shock, the level of transgene expression can be regulated (5). However, heat-shock induction causes transgene expression throughout the organism; specific cells or tissues cannot be targeted. Drawbacks to the heat-shock method include the problem that prolonged heat shock alone can induce various defects ("phenocopies") and lead to increased lethality, particularly before gastrulation (6, 7), and that the heat-shock response is not present in embryos before cellularization (8). Also, there is often a basal level of transcription from the heat-shock promoter (9). A further consideration with regard to heat shock is that heat-shock-induced expression is transient.

Constitutive ectopic expression can be induced by making use of the yeast flp recombinase (10, 11). In this system, the heat-shock promoter is used to induce recombination in a clone of cells to activate the expression of a given gene. This system has been widely exploited in *Drosophila* both for ectopic expression studies and for lineage tracing (10–14). However, as with other heat-shock-induced expression methods, the flp/FRT system does not allow for targeting of specific cells. While this problem conceivably could be circumvented by using a tissue-specific rather than a constitutive promoter, many of the problems discussed above with relation to both heat shock and to specific promoters still would pertain.

We present here a method to target gene expression to a specific embryonic cell or cell lineage in an inducible, either transient or constitutive fashion, without having a promoter or GAL4-expressing line that directs expression in that particular cell or clone. A digitally enhanced imaging system is used to identify single cells within the living *Drosophila* embryo. We then activate a heat-shock-responsive transgene by applying a heat shock to each cell individually by means of a laser microbeam, similar to a technique previously described for *Caenorhabditis elegans* (15, 16). We show that this method can be used to induce expression of a reporter gene in individual cells of various cell types within a single living embryo. Laser heat shock combines the advantages of inducible expression via heat shock with the targeted tissue-specific expression previously obtainable only by virtue of having a specific promoter. At the same time, it eliminates many of the drawbacks associated with these other methods. Laser heat shock thus provides a powerful new technique for inducing gene expression under strict spatial and temporal control in circumstances where such specificity cannot otherwise be achieved.

MATERIALS AND METHODS

***Drosophila* Lines.** *hsp26-lacZ* flies were strain −351.94h and are described by Glaser *et al.* (17). *hsGAL4* was a third-

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chromosome insertion of the *hsp70* promoter fused to the GAL4 coding region, gift of S. Hayashi (National Institute of Genetics, Mishima, Japan; line N630). *UAS-lacZ* and *UAS-GFP*[*S65T*] lines are available from the Bloomington Stock center.

Laser Setup. The laser setup was essentially as described previously for laser ablation (18, 19). A VSL337 nitrogen pulsed-dye laser driving a mirror-to-mirror dye module (Laser Science, Cambridge, MA) with Coumarin 440 as the dye was focused with a 75-mm planoconvex lens and directed through the epifluoresence lamp port of a Zeiss IV FL fluorescence microscope. A 510-nm dichroic mirror was used to direct the light through a 63× planapochromatic objective to give a focused spot of approximately 1×2 μm. Neutral density filters to a total of neutral density = 1.9 were placed in the beam path to attenuate the beam energy. With the filters in place, the beam energy at the back aperture of the 63× objective was 0.05 μJ/pulse as measured by a pyroelectric detector (Molectron, Sunnyvale, CA; model J4–05). A second laser setup also was used, consisting of the same model laser directed through a Laser Sciences laser adapter into a Zeiss Axioskop microscope. With this system, a neutral density = 1 neutral density filter was used to give a measured energy output of about 0.02 μJ/pulse. Both systems performed equally well for purposes of laser heat shock.

Standard Heat Shock. For standard (i.e., not laser-induced) heat shocks, embryos on an apple juice/agar egg-lay plate were placed in a 35°C incubator for 15–20 min. After heat shock, the plate was transferred to either 18°C or 25°C for 1 to 2 hr until fixation and staining as described below.

Laser Heat Shock. Embryos were reared at 18°C until they reached the desired stage, were manually dechorionated, and were oriented on a slide on a small piece of double-stick tape. They were then covered with Halocarbon 700 oil and a coverslip. To avoid damage to the embryos, the corners of the coverslip were raised with small pieces of plasticine or with vacuum grease. The slide was placed on the microscope stage, and the embryo visualized with digitally enhanced microscopy as described by Halpern *et al.* (20). Individual cells were heat-shocked by applying a 75- to 120-s laser burst of 3-ns pulses at a frequency of 4–10 Hz. After heat shock, the coverslip was removed and the embryo allowed to recover, under oil, for at least 1 hr at either 18°C or 25°C.

Fixation and Staining. Embryos were individually transferred to a small dish and fixed in heptane saturated with 25% glutaraldehyde for 12 min (21). Embryos then were transferred and manually devitellinized as described by Vincent and O'Farrell (22). After a brief wash in PBS, embryos were transferred to 5-bromo-4-chloro-3-indolyl β-D-galactoside for 1–4 hr at 37°C to detect β-galactosidase activity.

RESULTS

To heat-shock single cells, we used a nitrogen pulsed-dye laser setup similar to that used for cell ablation (see *Materials and Methods*). A series of neutral density filters was placed in the path of the laser beam to attenuate its energy below levels that would be lethal to the targeted cells. Our intention was to find conditions that would allow rapid heating to a temperature high enough to activate the heat-shock response but not so high that we would inhibit it. Previous experiments have shown that the *Drosophila* embryonic heat-shock response is inhibited at at least 49°C (23). Because we could not measure the actual change in temperature caused by the laser treatment, we selected a level of attenuation well below that at which we could see the least visible signs of cell damage (e.g., coagulation in the nucleus).

Live, dechorionated embryos were mounted on slides under a small drop of Halocarbon oil and covered with a coverslip. Embryos mounted this way can survive for at least 20 min without any noticeable effect, including heat-shock induction (data not shown). An individual cell then was targeted with the aid of digitally enhanced differential interferences contrast microscopy optics (Fig. 1*A*). As our microscope objective has a working distance of 90 μm, we can visualize cells as far as halfway through the embryo; by mounting an embryo in the proper orientation we are thus able to visualize virtually any embryonic cell. Once a cell was targeted, a series of laser pulses was applied at a frequency of 4–10 Hz. The process was repeated until the desired number of cells were treated. Recovered embryos were allowed to continue to develop at either 18°C or 25°C for 1 or more hr, followed by fixation and histochemical processing.

Our initial experiments were performed on embryos bearing an *hsp26-lacZ* transgene (gift of J. Lis, Cornell University, Ithaca, NY). In the absence of heat shock, these embryos have a discrete but limited pattern of β-galactosidase expression (Fig. 2*A*). When treated to moderate heat shock (15–20 min at 35°C; see *Materials and Methods*), however, β-galactosidase is expressed strongly in most embryonic tissues (Fig. 2*B*). Using the protocol described above, we induced β-galactosidase expression in individual cells in both the somatic musculature (Fig. 2*C*) and the central nervous system (Fig. 2*D*) of stage 15–16 embryos. Because embryonic development in the *Drosophila* embryo is rapid (some cells have a cell cycle as short as 20 min; ref. 24), we optimized conditions for the shortest possible duration of laser treatment. Monsma *et al.* (23) have shown that the heat-shock response can be induced after as little as 30 s of exposure to a heat stimulus. However, we were able to see laser-driven heat-shock induction in only 25% of the embryos ($n = 3/12$) at treatment durations of less than 75 s. In embryos in which we were successful in inducing heat shock, induction occurred in close to 50% of the targeted cells at a

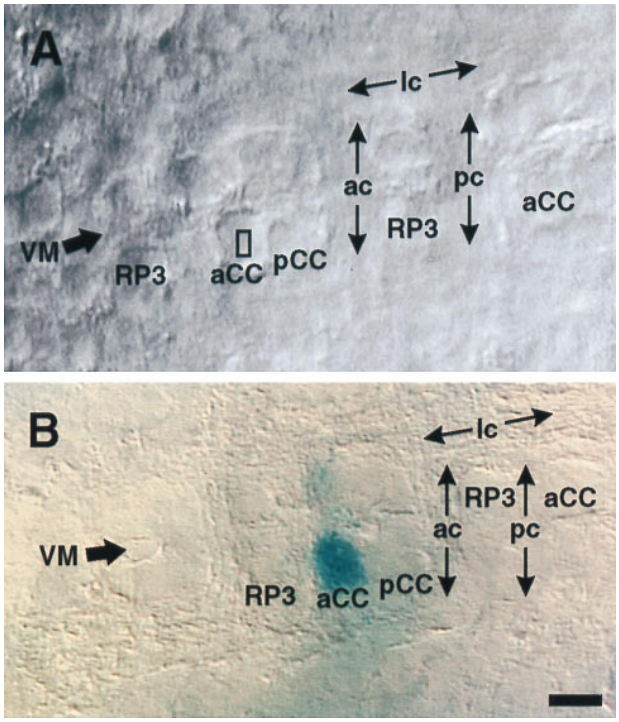

FIG. 1. Specific cells can be identified and accurately targeted for laser heat shock. (*A*) Motoneuron aCC, which lies just posterior to the posterior commissure and anterior to interneuron pCC, is targeted for heat shock in a living stage 16 *hsp26-lacZ* embryo. The box represents the 1×2 μm target area of the laser beam. (*B*) Expression of β-galactosidase in an embryo in which aCC was targeted as shown in *A*. Although several aCCs were targeted, not all were successfully induced (see text). VM, ventral midline; ac, anterior commissure, pc, posterior commissure; lc, longitudinal connective. (Bar = 200 μm.)

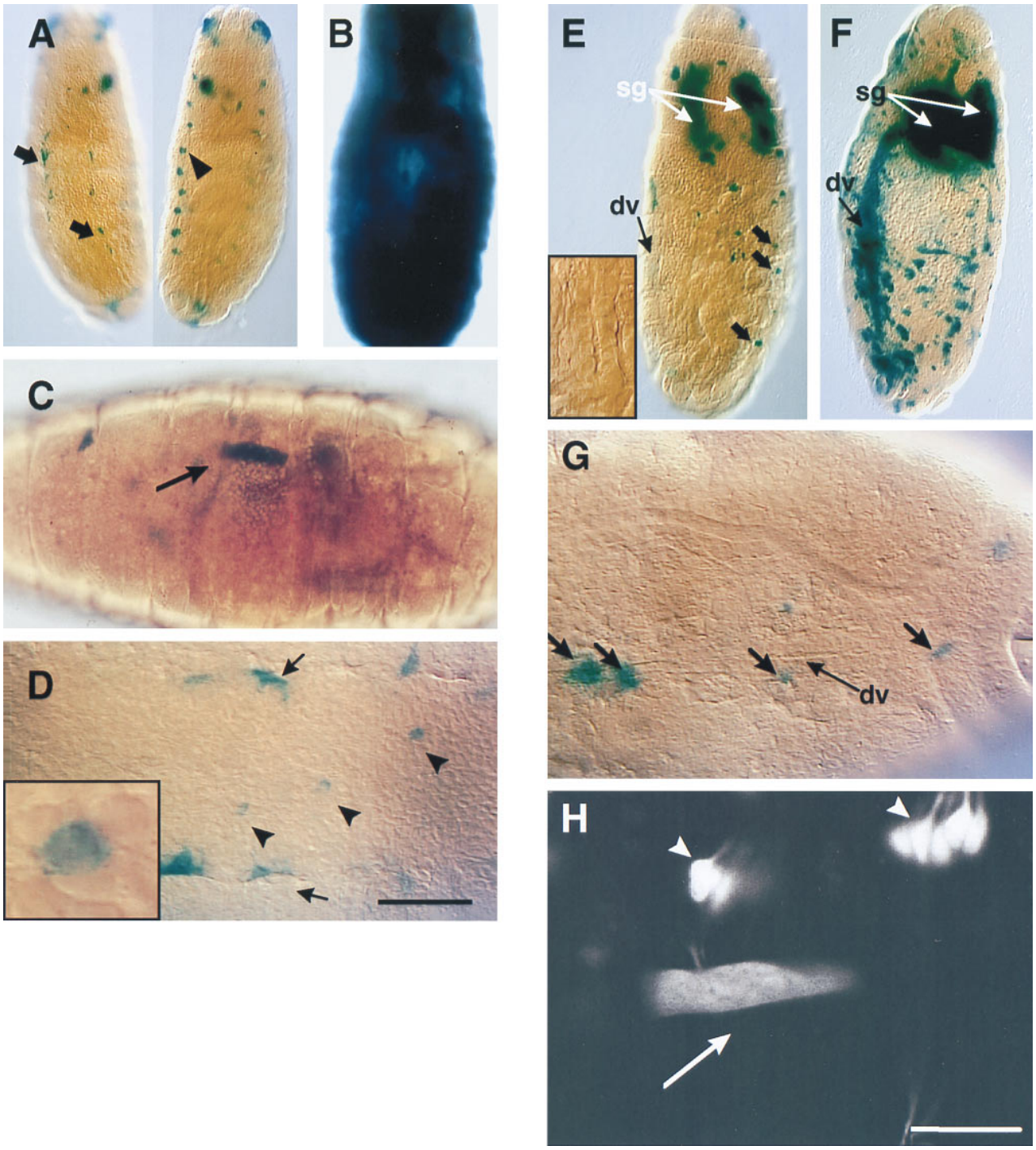

FIG. 2. Induction of heat shock in either *hsp26-lacZ* embryos (*A–D*) or with the GAL4-UAS system (*E–H*). (*A*) Control embryos, maintained at 18°C without heat shock until fixation at stage 16. In the dorsal view (*Right*), non-heat-shock-dependent β-galactosidase expression can be seen in cells along the tracheal trunks (arrowhead) and in several dorsal anterior cells. The ventral view (*Left*) shows expression along the edges of the central nervous system (arrows), but not within it. (*B*) Late-stage embryo subjected to 20 min of standard heat shock. Cells throughout the embryo express β-galactosidase. (*C*) Induction of β-galactosidase expression in a single ventral somatic muscle fiber (arrow) by laser heat shock. Note that no other muscle fibers are stained. (*D*) Induction of β-galactosidase expression in a row of single cells within the central nervous system (arrowheads). The expression along the borders of the central nervous system (arrows) is non-heat-shock dependent. (*Inset*) A closeup of the center stained cell. The single-cell nature of the laser heat-shock induction can be seen clearly. (*E*) In the absence of heat shock, the GAL4 driver *hsGAL4 N630* drives expression in a subset of sensory neurons (wide arrows) and in the salivary glands (sg, white arrows), measured here with a *UAS-lacZ* reporter gene. Note that there is no expression in the dorsal vessel and surrounding tissues (dv, black arrow; *Inset*). (*F*) When a 20-min heat shock is given, expression is induced most consistently in cells in the dorsal portion of the embryo, especially in and around the dorsal vessel (dv, black arrow), and more sporadically elsewhere throughout the embryo. White arrows indicate the salivary glands (sg). (*G*) Laser heat shock can induce expression of the *UAS-lacZ* target gene in specific single cells; here cells in the dorsal vessel (dv) were targeted (thick arrows). (*H*) Use of a *UAS-GFP*[*S65T*] target gene allows *in vivo* imaging of expression induced by laser heat shock. In this confocal micrograph, expression can be seen in a single ventral muscle fiber (muscle fiber 13, arrow). The non-heat-shock-dependent expression of the *N630* driver is evident in the lateral chordotonal neurons and other sensory axons (arrowheads). (*A–G*) 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining. (Bars: *A*, *B*, *E*, *F* = 100 μm; *C* = 60 μm; *D* = 30 μm; *G* = 35 μm; *H* = 25 μm.)

duration of laser treatment between 75 and 120 s ($n = 133/297$ cells in 61 embryos, average = 48% cells per embryo). Longer periods of heat shock (3–5 min) did not appear to significantly increase the frequency of induction. In roughly 35% of the treated embryos, no heat-shock induction was observed ($n = 34/95$). In all cases of successful induction, β-galactosidase expression was confined to single cells (Fig. 2*D*, *Inset*); at no time did we observe expression in clusters or pairs of adjacent cells.

By targeting cells of known identity, we were able to demonstrate that our targeting is accurate. Fig. 1*A* shows a targeted motoneuron aCC as it appears in a live stage 16 embryo; Fig. 1*B* shows β-galactosidase expression in a similarly targeted cell. aCC lies several cell layers deep from the ventral surface of the embryo, indicating that our heat-shock method can induce expression in internal cells. Similarly, when specific ventral muscle fibers that also lie several layers internal to the embryonic surface were targeted, expression was induced only in the targeted fibers (e.g., Fig. 2*C*). This is consistent with our experiences with other laser-based techniques, such as cell ablation, in which an internal cell can be ablated without damage to the overlying and underlying cells (18, 25).

Due to factors such as chromosomal position effects and a given transgene's 3′ untranslated sequence, individual transgenic lines carrying heat-shock-dependent transgenes have different sensitivities to heat shock and different amounts and patterns of non-heat-shock-dependent transcription (9, 26). As a result, the required duration and frequency of laser treatment may differ from line to line; a similar problem has been reported for laser heat-shock protocols in *C. elegans* (15). To avoid having to determine empirically the proper conditions for each new transgenic line, we began using the GAL4 system (4) for our laser heat-shock experiments. By having one or two *hsGAL4* drivers with precisely defined heat-shock conditions, any UAS target gene can easily be ectopically expressed using laser heat shock.

Like the *hsp26-lacZ* line, the *hsGAL4* line we used (N630; gift of S. Hayashi) has a limited degree of non-heat-shock-induced expression (Fig. 2*E*). Upon standard heat shock (20 min at 35°C), expression is strongly induced in cells of the most dorsal embryonic tissues, with additional expression spread sporadically throughout the embryo (Fig. 2*F*). Using the same conditions described for the *hsp26-lacZ* flies (above) we were able to induce the expression of UAS target genes in specific embryonic cell types, such as the embryonic heart (Fig. 2*G*) and somatic musculature (Fig. 2*H*) with efficiencies similar to those reported above (average = 50% of targeted cells, 80% of embryos showing activation; $n = 20$ embryos). However, in tissues that responded poorly to standard heat shock (e.g., late-stage central nervous system, Fig. 2*F*) the efficiency of induction was lower (<15% of embryos, $n = 5/35$). It therefore may be necessary to find *hsGAL4* lines with a more evenly distributed heat-shock response. Nevertheless, using the GAL4 system provides a convenient and standardized way of inducing the expression of multiple different target genes.

DISCUSSION

We have developed a method to target gene expression to a specific embryonic cell or cell lineage without having a promoter or GAL4-expressing line that directs expression in that particular cell or clone. By using a focused laser beam to cause a localized heat shock, we are able to induce expression of a gene in a single cell of choice, at the time point of choice. This method presents a powerful new way of generating mosaic animals and for targeting gene expression.

Properties of the Heat-Shock Response. The heat-shock treatment we provided was sufficient to induce heat-shock-dependent transcription in an average of about 50% of the targeted cells. It is not clear why 50% of the cells failed to respond. Previous studies of the temperature-response profile of the heat-shock promoter have shown that the heat-shock response occurs mainly at temperatures between 33°C and 38°C (27). Although we could not make direct measurements of the temperatures generated by laser heat shock, longer heat shocks did not significantly increase the frequency of response, whereas shorter durations of treatment gave a lower response frequency. Therefore, the laser treatment appears to be providing heating in the appropriate range.

Interestingly, Stringham and Candido (15) report a similar 50% success rate in laser heat-shock treatments of *C. elegans*. One possibility is that the level of heating achieved is highly dependent on how the animal is mounted and on the exact plane of focus of the laser beam. Excess Halocarbon oil or a focus just above the surface of the cell may be sufficient to reduce the amount of heat being generated. However, it is also possible that there is a degree of randomness inherent in the heat-shock response such that any given cell has only a certain probability of responding to increased temperature. Alternatively, there may be a non-cell-autonomous component to the heat-shock response through which cells reinforce the response in neighbors that also receive the heat stimulus. However, our laser heat shocks always induced reporter gene expression in single cells only, consistent with previous suggestions that the heat-shock response is at least in part cell autonomous (15, 23).

Using the GAL4 System to Overcome Disadvantages Associated with the Heat-Shock Promoter. Laser heat shock combines the advantages, incompatible under traditional ectopic expression methods, of inducibility and tissue specificity. At the same time, it eliminates many of the drawbacks associated with other methods. Because the laser heat-shock method relies on the heat-shock promoter, however, certain of the difficulties associated with heat-shock-driven expression remain. One such concern when using heat-shock-promoter-driven transgenes is that in many transgenic lines they are expressed in certain tissues even in the absence of heat shock. Conversely, certain transgenic lines have tissues that appear to be at least partially refractory to heat shock. This may be due in part to chromosomal position effects, but also may reflect an inherent tissue-specific difference in the heat-shock response (28, 29). In addition, the overall strength of the heat-shock response can vary considerably from line to line.

This problem of variable response to short-duration heat shock can be obviated by making use of the GAL4/UAS system to standardize conditions so that the same heat-shock regimen can be used to express any gene placed downstream of a UAS control region. While none of the *hsGAL4* lines we have tested to date completely lack uninduced expression, we are continuing to test lines for minimal uninduced expression and maximal sensitivity to heat shock in all tissues. As long as the uninduced expression is in tissues that will not cause serious interference with the desired experimental outcome, however, the currently available *hsGAL4* lines are acceptable for many applications.

The main unavoidable limitation to heat-shock methods is the absence of the heat-shock response in early embryos (8). However, a method conceptually similar to laser heat shock, yet not reliant on the heat-shock promoter, has recently been described by Cambridge and Minden (J. Minden, personal communication), who used focused UV light to release a caged GAL4 compound and activate a UAS target gene in a cell-specific manner. While not effective after embryonic stage 11, this method can be used at early embryonic stages. Between the two methods, therefore, and with an appropriate selection of UAS target transgenes, virtually all stages of *Drosophila* development are accessible to single-cell inducible gene activation.

Induction of Multiple Target Genes. Laser heat shock combined with the GAL4 system also can be used to express multiple different genes in a single cell with a single heat shock. Having several different genes each under the control of an individual heat-shock promoter would raise the concern that not all of the promoters responded strongly to the heat shock, requiring that activation of each gene be independently confirmed. However, because under the GAL4 system expression of multiple genes would be dependent only on the induction of GAL4 expression, a single successful heat-shock event will cause all of the dependent UAS-controlled genes to be expressed (Alan Michelson, personal communication; M.S.H., unpublished observations). Confirming the expression of only one of the genes therefore would be sufficient. The availability of a GAL4-responsive vital label, *UAS-GFP* (30) makes this an especially attractive option, as the effects of a specific ectopic induction could be monitored *in vivo* (see Fig. 2*H*).

Additional Applications of the Laser Heat-Shock Technique. Several additional applications of the laser heat-shock technique can be brought about by combining laser heat shock with the flp recombinase system to induce constitutive reporter gene expression (10, 11). These include tracing cell lineages, expressing a desired gene throughout a cell lineage, and inactivating a gene throughout a lineage, either through antisense RNA production (31), expression of a ribozyme (32, 33), or through "flp-out" of a rescue construct (34). By allowing a specific embryonic or larval cell to be labeled, laser heat shock in combination with the flp system also would allow for the following of cell fates through metamorphosis. This is extremely difficult to do with current lineage tracing methods; as these methods induce clones at random, one is unable to determine which embryonic or larval cell may have been the lineage founder of an observed clone in the adult fly. In preliminary experiments, we have successfully used *hs-flp* to induce β-galactosidase expression in neuronal lineages (data not shown).

In addition to its use in whole-embryo studies, the laser heat-shock technique has application to cell culture systems. It may be especially useful in exploring questions of the autocrine vs. paracrine signaling properties of known signaling molecules. By inducing expression in just a single cell in a culture it should be easy to measure the responses of surrounding cells without being concerned that they, too, are producing the signaling protein.

The fact that we observed only single-cell induction even when the cells we targeted were small (3 μm diameter) indicates that the temperature increase caused by the laser is confined to a small area. This raises the possibility that the laser heat-shock technique could be used in larger cells to study possible subcellular differences (e.g., nuclear vs. cytoplasmic; axon vs. soma) in heat-shock response. The ability to use a focused laser beam to affect subcellular processes previously has been demonstrated by the use of microscale chromophore-assisted laser inactivation to inactive proteins in a subset of filopodia in a single chicken neuronal growth cone (35).

Conclusion. Inducible transgene expression from a heat-shock promoter has been demonstrated in a wide variety of organisms in addition to *Drosophila* and *C. elegans*. These include arthropods such as mosquitoes, mites, and crustacea (36–38); mice (39); mammalian cell culture systems (40); and several plant species (41–44). Inducible activation of constitutive gene expression by means of *hs-flp* has been reported in transgenic plants (45, 46). Laser heat shock thus could be adaptable for use in many organisms. The laser heat-shock method is a versatile technique that should prove useful for testing gene function in ectopic tissues, expressing mutant genes in specific cells, removing gene function from specific cells, lineage analysis, and any other purposes in which it is desirable to control gene expression in an inducible fashion at the single-cell level.

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