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Rapid Tissue-Specific Expression Assay in Living Embryos

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The GAL4 technique (Brand and Perrimon, 1993) has initiated a major change in the way gene function can be studied in *Drosophila* (reviewed by Phelps and Brand, 1998), vertebrates (e.g., Hartley *et al.*, 2002; Long *et al.*, 2001; Scheer and Camnos-Ortega, 1999) and plants (e.g., Boisnard-Lorig *et al.*, 2001). The major advantage of GAL4-targeted expression is that it allows ectopic expression to be restricted to specific tissues at discrete developmental times. In contrast, the widely used methods of DNA or RNA injection into eggs or embryos result in widespread ectopic expression with little control over the onset of expression. Although targeted expression by GAL4 overcomes these drawbacks, the GAL4 technique lacks the speed of simple DNA or RNA injection. In particular, the generation of transgenic *Drosophila* lines can take 4–6 weeks. In this report, we show that injection of plasmids carrying UAS-transgenes into GAL4-expressing embryos combines the best of both techniques. The timing and site of expression of the transgene is controlled by GAL4 and the subcellular localisation and possible function of the gene can be assayed in the tissue of interest in hours instead of weeks.

We first tested if the expression of UAS plasmids depends on the presence of GAL4. To ensure the maximum uptake of DNA into cells, embryos were injected at the syncytial blastoderm stage (2 h after egg lay), when there are no membrane barriers to DNA diffusion or nuclear uptake. Injection of UAS-mGFP5 plasmids (Brand, 1998) into Oregon P flies never results in fluorescent cells (n = 15 embryos). In contrast, injection of the same plasmid into the maternal $GAL4^{V2b}$ line (Hacker *et al.*, 1997) yields fluorescently labelled cells in the ectoderm, CNS, midgut, and muscles in 87.5% of embryos (n = 16; Fig. 1b). In crosses of stable transformants, $GAL4^{V2b}$ drives ubiquitous expression of GFP from gastrulation onwards (data not shown). After injection, fluorescence is first detectable 2 h after gastrulation. The majority of labelled cells are in the ectoderm. Labelled cells are distributed over up to four segments, with the highest number of cells at the site of injection. The distribution of GFP-expressing cells indicates that the injected plasmid diffuses over only a short distance.

We also tested if injection of two plasmids carrying different genes would allow co-expression of these genes in the same cells. Co-expression of the gene of interest together with a GFP tagged gene, which encodes a protein that outlines the cell or part of the cytoskeleton, would greatly facilitate phenotypic analysis. 90% (n = 10) of $GAL4^{V2b}$ embryos injected with an equal mixture of UAS-GFP and UAS-lacZ plasmids express both proteins (Fig. 1). Co-expression varies between 50–100%, with two embryos showing a complete overlap.

To test if the site and timing of expression is defined by GAL4 expression, we injected UAS-GFP plasmids into the line $GAL4^{MZ1580}$ (Hidalgo *et al.*, 1995), which expresses GAL4 in longitudinal glial cells and a subset of central and peripheral neurons from Stage 10 onwards. We also used line $GAL4^{43}$, in which expression in longitudinal glial cells and a subset of peripheral neurons starts at Stage 12. GFP-expressing cells are found in 92% (n = 26) of injected $GAL4^{MZ1580}$ embryos. Expression is restricted to neurons and longitudinal glial cells. We do not detect any expression before Stage 12, about 2 h after the onset of expression in stable transformants. After injection into $GAL4^{43}$ embryos, GFP expression is restricted to longitudinal glial cells and is first detectable at Stage 15. In contrast to $GAL4^{V2h}$ and $GAL4^{MZ1580}$, in which up to 30 cells over four segments express GFP, we only find one to two GFP-expressing cells per $GAL4^{43}$ embryo (in 14% of embryos; n = 14). A reduction in the frequency of expression in GAL4 lines with a late onset of expression may not be unexpected. In *Drosophila* a small fraction of injected DNA enters the nucleus, where it is replicated only once (Steller and Pirrotta, 1985). The lack of replication coupled with degradation of the cytoplasmic fraction (Steller and Pirrotta, 1985) could reduce the concentration of UAS plasmid present later in embryogenesis.

GFP expression generated by plasmid injection is significantly stronger than expression in stable transformants, possibly due to the higher copy number of the injected plasmid. High levels of expression could interfere with the localisation of endogenous proteins or reduce the viability of expressing cells. We investigated this possibility by generating and injecting constructs encoding cytoskeletal proteins, UASp-CFP-actin and

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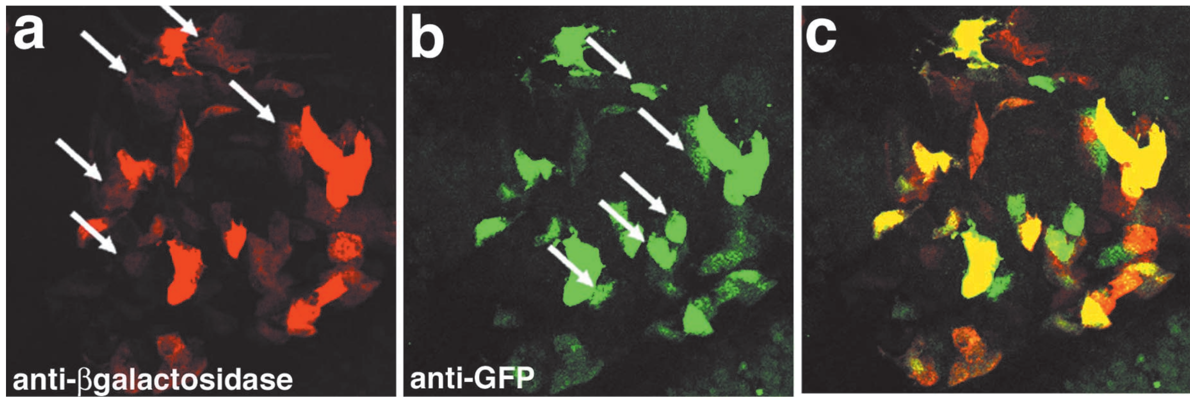

FIG. 1. Co-injection of two UAS plasmids into GAL4 transformants allows co-expression of two different proteins in the same cell. An equal mixture of UAS-GFP and UAS-β-galactosidase plasmids were injected into the maternal driver line *GAL4*^{V2h}. **a–c:** Expression of the two proteins does not completely overlap. A few cells express only β-galactosidase (**a**, arrows) or GFP (**b**, arrows). The majority of cells express both proteins (**c**, merged image). The cells are located in the midgut. A lateral view of a Stage 17 embryos is shown. Ventral is down; anterior is left.

UASp-GFP-tubulin, and a transcription factor, UAS-engrailed-GFP (Heemskerk *et al.*, 1991; Kornberg *et al.*, 1985). We injected into GAL4 driver lines that express the transgene in the same domain as the endogenous genes. This enables us to evaluate any cell damage caused solely by the strength of expression. Injection of CFP-actin plasmids into *GAL4*^{V2b} embryos only leads to fluorescent cell fragments engulfed by macrophages (three out of nine embryos). This suggests that strong expression of CFP-actin driven by the maternal GAL4 driver can cause cell death. We also injected CFP-actin plasmids into GAL4^{227}, a line in which expression in the epidermis and the CNS starts at Stage 10. 40% of the injected embryos (n = 15) express CFP-actin in epidermal cells and CFP-actin accumulates at the base of the forming hairs, a site of actin bundling (Fig. 2a,b; Turner and Adler, 1998). No macrophages are labelled.

GFP-tubulin was injected into the maternal driver *GAL4*^{V37} (Hacker *et al.*, 1997) and *sca-GAL4* (Klaes *et al.*, 1994), a neuronal driver. One out of 16 *GAL4*^{V37} embryos showed cell fragments engulfed in macrophages; the remaining embryos were nonfluorescent. 58% of injected *sca-GAL4* embryos (n = 12) have labelled neurons or glial cells (Fig. 2c,d). Despite the strong accumulation of GFP-tubulin in axons, we did not observe any errors in axonal pathfinding.

UAS-engrailed-GFP was injected into *engrailed-GAL4* embryos (AHB, K. Yoffe and N. Perrimon, unpublished; Fietz *et al.*, 1995). In 71% of the injected embryos (n = 7), ectodermal cells exhibit nuclear fluorescence (Fig. 2e). We conclude that overly strong expression generated by plasmid injection can result in cell death, a problem that can be avoided by injection into a less potent GAL4 driver. The subcellular localisation of proteins we have assayed so far is normal.

In this report we show that injection of UAS plasmids into GAL4 lines is a rapid means of assaying protein localisation and function in tissues of interest. The timing and expression of the injected plasmids depends on the GAL4 expression pattern. Compared to crosses of stable transformants, which take several weeks, the expression of fluorescently labelled proteins shows only a 2–3 h delay. Only a small number of embryos need to be injected because of the high frequency of expressing cells. GAL4 lines with an early onset of expression are more suitable for injections, as they give a higher frequency of expressing cells. We cannot rule out the possibility that frequency of expression might also depend on the UAS-transgene. Injection of UAS plasmids into GAL4-expressing embryos is a quick and easy tool to study the function of gene constructs, to analyse the subcellular localisation of unknown proteins or engineered genes, and to assess possible phenotypes (Bossing and Brand, 2002).

Immunhistochemistry

Immunohistochemistry was performed as previously described (Bossing *et al.*, 1996). The following primary antibodies were used: anti-β-galactosidase, 1:10 (Promega, Madison, WI) and anti-GFP, 1:1000 (Abcam, Cambridge Science Park, UK). Secondary antibodies conjugated to Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR) were used at a dilution of 1:500. All antibodies were diluted in PBTS (PBS, 0.3% Triton, 20% newborn calf serum).

Injected embryos were prepared for antibody staining at Stage 17. The coverslip was covered with PBT (PBS, 0.3% Triton) and the halocarbon oil removed by a stream of PBT (from a drawn-out Pasteur pipette). The embryos were fixed in 8% formaldehyde in PBT for 20 min on a shaker. During the fixation, five washes of fixative were squirted along the row of embryos from a drawn-out Pasteur pipette. Fixation was followed by three rinses in PBS, a 10-min incubation in PBS, and manual removal of the vitelline membrane with an injection capillary. After

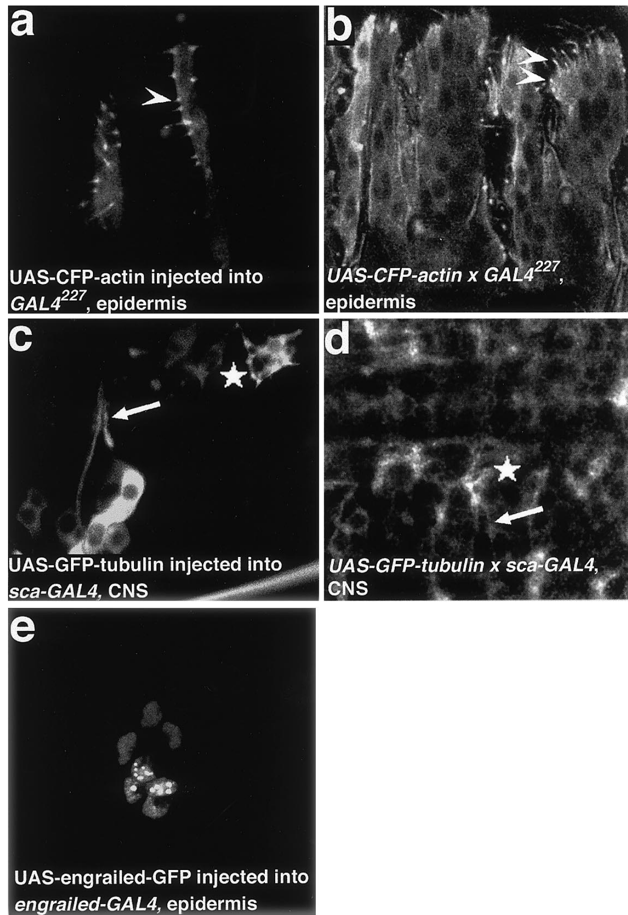

FIG. 2. Injection of UAS plasmids into Gal4-expressing embryos results in the correct localisation of the expressed proteins. **a,b:** Actin-CFP can be expressed either by injection of UASp-actin-CFP plasmids (**a**) or by crossing stable transformant lines (**b**, *UASp-actinCFP* × *GAL4*^{227}). In both cases Actin-CFP localises to the cytoplasm and is enriched at the base of the forming hairs (arrowhead) in epidermal cells. **c,d:** Tubulin-GFP is localised in the cytoplasm of cells expressing the protein after plasmid injection (**c**) and in stable transformants (**d**, *UAS-tubulin-GFP* × *sca-GAL4*). In neurons, Tubulin-GFP can be seen in axons (arrow). In glial cells (asterisk), Tubulin-GFP is found in filopodia. **e:** In cells expressing Engrailed-GFP after plasmid injection the protein is localised to the nucleus. The cause for the punctate staining in the nuclei of Engrailed-GFP expressing cells is unclear. One possibility might be the accumulation of the homeoprotein at its chromatin binding sites. All images were recorded from live embryos. Horizontal views of the ventral (**c,d**) or the dorsal side (**a,b,e**) of Stage 17 embryos are shown. Anterior is left.

three washes in methanol, the embryos were rehydrated in PBT and incubated in primary antibody.

Injection of DNA

Plasmids were gel-purified (Qiagen, Chatsworth, CA) and dissolved in deionised water. Embryos were manually dechorionated about 2 h after fertilisation, glued to a coverslip, desiccated at room temperature (23°C) for 4–6 min, and covered with halocarbon oil (Voltalef 10 s). The DNA (100–500 μg/ml) was injected laterally into GAL4-expressing embryos at the syncytial blastoderm stage. We used a plastic syringe attached to a hand-pulled polypropylene tube to control the pressure in the capillary. Capillaries were bevelled using a Bachofer microtip grinder. After injection embryos were either incubated at 25°C to define the start of expression or kept overnight at 18°C. Before examination the embryos were shifted for 1 h to 25°C to enhance GAL4-mediated expression.

Images of live embryos were collected with a MRC 1024 confocal scanhead (BioRad, Cambridge, MA) mounted on a Nikon E800 microscope. Images were assembled in Adobe PhotoShop v. 6.

Molecular Biology

To generate the UAS-Engrailed-GFP5 plasmid we used a UAS-Engrailed plasmid as template (Yoffe *et al.*, 1995). We added by PCR amplification an EcoRI site in front of the start codon and replaced the stop codon with three glycine codons followed by a BamHI site. The PCR fragment was cloned into the pCR2.1-TOPO vector, sequenced, and joined with mGFP5 by three-way ligation into the pUASt vector using the EcoRI and XbaI sites.

The UASp-CFP-actin plasmid was created using BglII/BamHI-cut DNA encoding human cytoplasmic β-actin (pEGFP-human-β-actin plasmid; ClonTech, Palo Alto, CA). CFP was PCR amplified from pECFP plasmid (ClonTech). The 5′ primer introduces a BglII site upstream of the start codon. The 3′ primer deletes the stop codon and introduces a BamHI site, ensuring in-frame fusion to the N-terminus of actin. The two products were joined by a three-way ligation into the pUASp (Rorth, 1998) vector using the BamHI site. The fusion was checked for correct orientation of insertion of the products and the PCR-amplified region was sequenced.

The UASp-mGFP5-tubulin plasmid was generated using a XhoI/BamHI-cut DNA encoding human α-tubulin (pEYFP-human α-tubulin plasmid, ClonTech). mGFP5 (Siemering *et al.*, 1996) was PCR-amplified. The 5′ primer adds a KpnI site immediately upstream of the start codon. The 3′ primer deletes the stop codon and introduces an XhoI, ensuring in-frame fusion to the N-terminus of tubulin. The PCR-amplified region was sequenced. The two products were ligated into the pUASp (Rorth, 1998) vector using the KpnI and BamHI sites.

Transgenic flies were generated by DNA injection into *yw; P(ry, Δ2-3), Sb/TM6, Ubx* embryos (Robertson *et al.*, 1988) as described previously (Brand and Perrimon, 1993).

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