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Prophenoloxidase as a Reporter of Gene Expression in *Drosophila*

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ABSTRACT

We have developed a novel reporter for the analysis of gene expression using a *Drosophila* prophenoloxidase (*proPO*) gene. We have expressed *proPO* in transgenic flies using the *GAL4-UAS* system and characterized ectopic expression in imaginal discs. *proPO* staining is fast and robust, requiring only inexpensive reagents, and performs similarly to β -galactosidase (β -gal) staining. Double staining using *UAS-proPO* and β -gal reporters is straightforward and provides a convenient method for examining the expression of two genes simultaneously.

INTRODUCTION

Reporter gene assays are a critical tool for the study of developmental gene regulation. By allowing the analysis of gene expression by proxy, reporter genes often obviate the need for laborious immunochemical and RNA hybridization procedures. Moreover, "enhancer trapping" by reporter constructs can be used to study the expression of genes that have not been cloned (3). Functional dissection of *cis*-regulatory regions of genes that are expressed in complex spatial and temporal patterns by fusing genomic fragments to reporter genes has also been an indispensable technique in elucidating the mechanisms of gene regulation.

In *Drosophila*, the two widely used reporters are the bacterial β -galactosidase (β -gal) (12) and the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (5). Depending on the application, their expression can be driven either directly from a minimal constitutive or native promoter or indirectly by the yeast *GAL4/UAS* system (4). The latter approach has an added level of flexibility in that a single *UAS- β -gal* or *UAS-GFP* reporter can be combined with any *GAL4* enhancer trap or expression construct.

We have further enhanced the utility of the *GAL4/UAS* system in *Drosophila* by generating a new *UAS*-driven *in situ* reporter based on an endogenous prophenoloxidase (*proPO*). We show that *proPO* satisfies the two main requirements placed on reporter genes: ease of use and compatibility with other staining procedures. Histochemical staining for *proPO* is quick and robust, requiring only inexpensive reagents, and can be readily combined with staining for β -gal to analyze the expression of two genes simultaneously.

MATERIALS AND METHODS

Construction of *UAS-proPO*

An incomplete 2.1-kbp *proPO* cDNA was received from K. Fujimoto (Meikai University, Japan) as an *EcoRI* clone in the pBluescript® II SK vector (Stratagene, La Jolla, CA, USA). This cDNA lacked 135 bp at the 5' end, as compared with the published complete cDNA sequence (GenBank® accession no. D45835). To synthesize a full-length *proPO* cDNA, we amplified and sequenced a 882-nucleotide fragment containing the 5' end of the cDNA from wild type *D. melanogaster* Oregon-R genomic DNA, using the forward primer 5'-CCTCGAGCCATGACTAACA CGGATC-3' and the reverse primer 5'-ATTGTAGCGCGGAGTATCTGG-3'. The ATG in the forward primer is the presumptive translation initiation codon. This genomic DNA fragment matched the published cDNA sequence exactly, except for the presence of two small introns: a 56-bp intron starting at position 356 (numbering from the initiation codon starting at nucleotide position 1) and a 69-bp intron starting at position 549. This fragment was cloned into the original pBluescript plasmid containing the incomplete *proPO* cDNA, using the *XhoI* site in the forward primer and the *BssHII* site in the reverse primer, thus replacing the incomplete 5' end of the original clone. The presumptive full-length *proPO* cDNA was then cloned into the pUAST expression vector (4) as an *XhoI-XbaI* fragment (the presence of introns in pUAST constructs does not affect *in vivo* expression).

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Transgenic *Drosophila* lines were created using standard P-element transformation methods (14). Native polyacrylamide gel electrophoresis of adult and larval protein extracts confirmed that PO activity was overexpressed in hsp70-Gal4/UAS-proPO flies compared to wild-type strains and strains carrying either the GAL4 or the UAS-proPO constructs alone (data not shown).

Transformant lines with insertions of P{UAS-proPO} on each of the three major chromosomes with the stock names P{UAS-proPO}X, P{UAS-proPO}2, and P{UAS-proPO}3 have been deposited in the Bloomington *Drosophila* Stock Center and can be ordered through FlyBase (<http://flybase.bio.indiana.edu/>).

PO Staining

Third instar imaginal discs were dissected in potassium phosphate buffer

(100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{PO}_4$, 150 mM NaCl, pH 6.3). PO staining was performed by incubating the discs in 0.4 mg/mL dopamine in 40% potassium phosphate buffer/60% isopropanol for 20 min to 2 h at room temperature (solution should be made fresh before use). Combining isopropanol activation with dopamine staining in a single reaction saves time without sacrificing sensitivity and produces a brighter, orange-brown product. The alternative substrate L-Dopa can be used at the same concentration as dopamine but produces somewhat weaker staining. The colorimetric reaction was stopped by washing discs twice for 10 min each in potassium phosphate buffer, followed by dehydration in an ethanol series.

For proPO/ β -gal double stainings, the proPO-stained discs were fixed in 1% glutaraldehyde in sodium phosphate buffer (100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.0, 150 mM NaCl, 1

mM MgCl_2 , 0.1% Triton[®] X-100) for 10 min at room temperature, followed by a 10-min wash in the same buffer. β -gal staining was developed by incubating the discs in 0.2% 5-Bromo-4-Chloro-3-indolyl- β -D-galactoside (X-Gal) in staining buffer (10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.0, 150 mM NaCl, 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, 5 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$, 0.2% Triton X-100) for 1 h to overnight at room temperature. Staining solution was prepared immediately before use by adding 1/25 volume of 5% X-Gal in dimethyl formamide to staining buffer prewarmed to 70°C and allowing it to cool down to room temperature [as suggested by Struhl et al. (15)]. Stained discs were washed in standard PBS and dehydrated in an ethanol series to remove X-Gal crystals. Stained material can be stored in 100% ethanol at -20°C indefinitely. Stained discs were rehydrated in PBS, mounted in 80% glycerol/20% PBS,

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and examined under Nomarski optics.

Endogenous PO activity occurs in hemocytes and in the epidermis during cuticle secretion (2), and the latter can be detected by incubation with dopa or dopamine (not shown). During the third instar larval and early pupal stages, in which the proPO reporter is most useful, detectable endogenous PO activity occurs only in the body wall and is absent from the imaginal discs. In mid to late pupal stages, when extensive cuticle synthesis takes place, endogenous PO activity appears throughout the imaginal epidermis, thus limiting the usefulness of our reporter to earlier stages.

RESULTS AND DISCUSSION

Insect phenoloxidases catalyze the oxidation of L-3,4-dihydroxyphenylalanine (L-dopa) and 3,4-dihydroxyphenethylamine (dopamine) to their reactive quinone derivatives and are involved in melanin pigmentation, cuticle sclerotization, and wound healing (11,13,16). PO activity in *Drosophila* is thought to be composed of at least three structural proteins and an unknown number of regulatory cofactors (6,16). Only one of the structural enzymes has been cloned in *Drosophila*, and this protein is highly homologous to other insect phenol ox-

dases (8). The corresponding gene, called variously *Mox*, *Phox*, and *Dox-A1*, may be allelic to the dominant neomorphic mutant *Black cells*, which causes melanization of the hemocytes.

Drosophila phenoloxidase and its *Manduca* and mosquito homologs are produced as inactive prophenoloxidases (proPOs), requiring cleavage by a protease at a conserved arginine-phenylalanine site to be activated (10). This proteolytic activation step can be bypassed in native gels by incubation with isopropanol or other alcohols, which is thought to cause a conformational change that converts the protein to its active state (1,9). We have developed an in situ staining procedure that makes use of this property of *Drosophila* proPO. We find that gene expression can be analyzed easily and effectively by incubating the tissue expressing a UAS-proPO reporter in a solution containing isopropanol and melanin precursors.

Single PO Staining

Figure 1A shows a dopamine-stained wing imaginal disc of *Dll-GAL4/+*; UAS-proPO/+ larvae. The pattern of PO activity closely reflects the pattern of Dll protein expression (7), confirming the accuracy of the reporter.

Depending on the GAL4 driver used, the full pattern of PO expression develops within 20 min to 2 h at room temperature, with no noticeable background or “bleeding” of the melanin product. Longer incubations or higher temperatures do not appear to increase the signal but can produce high background. Substitution of L-dopa for dopamine changes the color of the product from orange-brown (Figure 1A) to a darker brown (Figure 1B).

We find that for a given GAL4 driver, the UAS-proPO reporter usually works more quickly than UAS- β -gal and is equally sensitive and robust. The single-step proPO staining protocol is faster and less laborious than β -gal staining. Additionally, the proPO substrates dopamine and L-dopa are two orders of magnitude less expensive than X-Gal (\$0.01–\$0.02 vs. \$2.40 per hundred 250- μ L reactions), can be stored at room temperature, and used in aqueous solutions, whereas X-Gal must be stored frozen and used with haz-

Figure 1. UAS-proPO staining using dopamine (A, C, and D) or L-dopa (B) in third instar imaginal discs of *Drosophila*. Dorsal is up, and anterior is to the left in all panels. (A) In the wing discs of *Dll-GAL4/+*; UAS-proPO/+ larvae, PO activity is present in the *Dll* pattern along the dorsoventral compartment boundary; note that expression diminishes gradually in both directions. (B) In the *ptc-GAL4/+*; UAS-proPO wing discs, PO is expressed along the anteroposterior compartment boundary. The L-dopa substrate gives a slightly darker, grayish brown color, especially around the edges of the pattern. (C and D) proPO/ β -gal double staining in *ptc-GAL4/en-lacZ*; UAS-proPO/+ wing and leg discs (C) and *Dll-GAL4/+*; UAS-proPO/*bab-lacZ* leg disc (D). In C, *en* expression in the posterior compartment and *ptc* expression in the anterior compartment abut but do not overlap. In D, *Dll* and *bab* expression overlap in the tarsal region of the leg, producing a dark blue or black color.

ardous organic solvents such as formamide. All of these features make proPO staining more convenient than β -gal for high-throughput and classroom applications.

PO/ β -Gal Double Staining

Another advantage of proPO staining is that it can be readily combined with β -gal staining to study the expression of two genes simultaneously. Figure 1 shows imaginal discs of *ptc-GAL4/en-lacZ*; UAS-proPO/+ (Figure 1C) and *Dll-GAL4/+*; UAS-proPO/*bab-lacZ* (Figure 1D) larvae double-stained for PO (brown) and β -gal (blue). The two colors are easily distinguishable, and both the sharp, abutting boundaries of gene expression (Figure 1C) and overlapping expression patterns (Figure 1D) can be identified.

The double-staining protocol is rapid and straightforward compared to double antibody staining and thus may be preferable for the analysis of large numbers of genotypes or individuals. This method may prove particularly useful for gene products for which no antibodies are available and for the analysis of GAL4 gene constructs.

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