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A nuclear GFP/β-galactosidase fusion protein as a marker for morphogenesis in living *Drosophila*

Yasuhiro Shiga,* Miho Tanaka-Matakatsu and Shigeo Hayashi†

Genetic Stock Research Center, National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan

A general, non-invasive method to trace morphogenesis in living *Drosophila* was developed. To label specific cells, green fluorescence protein (GFP) of jellyfish *Aequorea victoria* was expressed by the Gal4-UAS system. Green fluorescence from GFP fused to the nuclear localization signal was detectable in polytene larval tissue, but not in diploid tissue. Further fusion to bacterial β-galactosidase produced GFPN-lacZ, which fluoresced brightly in several diploid larval and embryonic tissues. GFPN-lacZ was used to trace dynamic cell movement during the formation of the embryonic tracheal system. These results indicate that GFPN-lacZ can be used to mark specific cells to study cell movement and gene expression in living animals.

Key words: *Drosophila*, fusion protein, green fluorescent protein, time lapse confocal microscopy, tracheal system.

Introduction

Pattern formation events during the development of a fruit fly, *Drosophila melanogaster*, have been extensively studied by genetic analyses. A large collection of mutations has facilitated molecular cloning of genes that are required for various developmental processes. Expression patterns of such genes have been studied by *in situ* hybridization, antibody staining and promoter analyses using P-element mediated germline transformation (Carroll & Scott 1985; Hiromi *et al.* 1985; Tautz & Pfeifle 1989). Furthermore, enhancer trap methods have made it possible to identify numerous tissue-specific enhancer elements throughout the genome (O'Kane & Gehring 1987). These markers have been used to reveal diverse cell types previously unrecognized by conventional histological methods. The dynamics of gene expression in normal development and its change in mutant animals were used to assess the function of genes and their regulation. Despite this progress, our knowledge is still limited. Part of the reason for this is that fixation of tissues is necessary to visualize most of the tissue specific markers, which inevitably kills the cell and destroys many intracellular structures. To overcome this problem and to trace marked cells in a living animal, cell marking techniques have been devised (Hiraoka *et al.* 1989; Bossing & Technau 1994), but they require technical excellence and make it difficult to process large numbers of samples.

Chalfie *et al.* (1994) described a non-invasive way to label cells in living nematode *Caenorhabditis elegance* by genetically marking with a reporter gene encoding green fluorescent protein (GFP) derived from a jellyfish *Aequorea victoria*. GFP is thought to autocatalytically form chromophores that absorb ultra-violet (UV) and blue light (excitation peaks at 395 nm and 478 nm) and emit green light (emission peak at 507 nm; reviewed in Heim *et al.* 1994; Prasher 1995). GFP has also been expressed in various organisms including *Escherichia coli*, *Dictyostelium*, *Drosophila* and in plant and mammalian cells (Chalfie *et al.* 1994; Heim *et al.* 1994; Inouye & Tsuji 1994; Brand 1995; Haseloff & Amos 1995; Hodgkinson 1995; Pines 1995; Yeh *et al.* 1995). Wang and Hazelrigg (1994) reported protein fusion between GFP and the Experentia (Exu) protein to study localization of Exu in the *Drosophila* ovary. Exu has been shown to be essential for the transport of *bicoid* mRNA, which encodes an anterior morphogen. The authors showed that the GFP-exu fusion protein co-localized with microtubules. Expanding the use of GFP to a more general purpose should allow tracing of various morphogenetic movements during development and help experimental manipulation of embryos. With these applications in mind, we made nuclear localized GFP fusion constructs and expressed them under the control of the Gal4-UAS system (Brand & Perrimon 1993). We show here that one such protein, GFPN-lacZ, can be detected in the living embryo and in dissected larval tissues.

†Author to whom all correspondence should be addressed.
*Present address: Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.
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Materials and Methods

Fly culture and strains

Flies were cultured in standard yeast-glucose-cornmeal-agar medium at 25°C. The salivary gland Gal4 enhancer trap strain (Gal4-lethal) was a gift from A. Brand and N. Perrimon (Harvard University, Boston, MA, USA). The *tsh*-Gal4 line is a second chromosome lethal enhancer trap line carrying the pGAW element (a gift from H. Nakagoshi and F. Matsuzaki, National Institute of Neuroscience, Tokyo, Japan). Its expression pattern, as revealed by the UAS-lacZ reporter (Brand & Perrimon 1993), is similar to the expression pattern of the teashirt gene (*tsh*; Fasano *et al.* 1991). The line also failed to complement tsh^8 and Df(2L)TW161. It is therefore likely that the line has an insertion near the *tsh* gene. The pros-Gal4 (Goto & Matsuzaki, unpubl. obs., 1994) was obtained from F. Matsuzaki. The Gal4 enhancer trap line G455.2 (Hinz *et al.* 1994) was obtained from U. Hintz (University of Köln, Köln, Germany). Other strains and plasmids related to the Gal4 system have been described in Brand and Perrimon (1993).

Vector construction

The plasmid pUAS-gfpn harbors a modified GFP open reading frame (ORF) fused to the SV40 nuclear localization signal (pTU62N: T. Ishihara, pers. comm., 1994) between the KpnI and XbaI site of pUAST downstream of UAS^G. To facilitate protein fusion, 3′ BamHI and HindIII sites were introduced to the modified GFP ORF by PCR. The 5′ primer was the universal primer complementary to the Bluescript vector (Stratagene, La Jolla, CA, USA) and the sequence of the 3′ primer was ACGGGATCCGAAGCTTTGTATAGTTCATCC. The amplified fragment was subcloned and then fused to the 5′ end of chloramphenicol acetyltransferase (CAT) ORF of pBS-CATDp or to the 5′ end of lacZ ORF of pUC-lacZ (Shiga *et al.* 1993). The plasmid pBS-CATDp was constructed by inserting the CAT ORF, flanked by the HindIII and SalI sites of pGR71 (Goldfarb *et al.* 1981), into HindIII–SalI digested Bluescript KS^+. Each fusion construct was introduced as a KpnI fragment to the KpnI site of pUAST to make pUAS-gfpn-CAT and pUAS-gfpn-lacZ. Bacteria colonies carrying the plasmids fluoresced under 365 nm UV light, verifying each construct. Fusions to bacterial proteins were further tested for resistance to chloramphenicol (pUAS-gfpn-CAT) or lacZ activity on X-gal plate (pUAS-gfpn-lacZ). To make *btl*-GAL4 construct, approximately 3 kb EcoRI–NdeI fragment carrying the *breathless* (*btl*) promoter (Ohshiro & Saigo, pers. comm., 1994) was placed upstream of the GAL4 coding sequence on pGATN (Brand & Perrimon 1993). The *btl*-GAL4 fusion was introduced into CaSpeR4 vector (Thummel & Pirrota 1991) to produce pCas-*btl*-GAL4. For P-element transformation, a y Df(1)w67c2 host embryo was injected under ethanol as previously described (Bartoszewski & Gibson 1994).

Fluorescent microscopy

Embryo and larval tissues were fixed as previously described (Hayashi *et al.* 1993). In brief, dechorionated embryos were fixed in 3.6% formaldehyde/PBS/heptane for 20 min, devitellinized with methanol, rehydrated in TBSTE (20 mmol/L Tris-Cl, 130 mmol/L NaCl, 0.1% Tween20, 0.2% BSA). Larval tissues were dissected in PBS, fixed in 3.6% formaldehyde/PBS and rinsed with TBSTE. Un-fixed dechorionated embryos were placed on glued coverslips and covered with Voltalef oil (10S; Atochem, Glenrock, NJ, USA). Unfixed larval tissues were mounted in PBS or 50% glycerol. Normally specimens were observed with a fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany) equipped with 40× planneofluoar lens (numerical aperture (N/A) = 1.3) and 63× planapochromat lens (N/A = 1.4). Blue excitation (Zeiss filter set #10) was used, whereas for confocal microscopy, Zeiss LSM410UV was used. Optimal results were obtained with a combination of a 40× planneofluoar lens (N/A = 1.3), an Ar 488 nm laser without attenuation, an FT488/543 dichroic mirror and a BP510-525 emission filter. Sets of optical sections were obtained using a combination of Z-sectioning and Time Series software (Carl Zeiss).

Histological methods

Immunostaining and X-gal staining of fixed larval tissues was performed according to Hayashi *et al.* (1993). Rabbit anti-β-galactosidase (Organon Technica-Cappel, Durham, NC, USA) and anti-CAT (Hayashi & Kondho, unpubl. obs., 1987) were used as the primary antibodies and were detected by the ABC elite kit (Vector Lab., Bourlingame, CA, USA).

Results

Construction of gfp fusion genes

We planned to express GFP in the cell nucleus because in the case of β-galactosidase, accumulation in the nucleus generally improves sensitivity of detection compared with the signal diffused in the cytoplasm. The gfp ORF fused to the nuclear localization signal (pTU62N; T. Ishihara, pers. comm., 1994) was used as a starting material to construct a series of vectors, as shown in Fig. 1. We chose the Gal4-UAS system developed by Brand and Perrimon (1993) to express the fusion proteins. In this system, an ORF of a protein

of interest is placed under the control of a promoter (UAS) that can be activated by the yeast transcription factor Gal4. Because a large collection of lines expressing Gal4 in various patterns are available, it is possible to express a protein of interest in targeted tissue simply by crossing a UAS and a selected Gal4 line. We first used a Gal4 enhancer trap line (Gal4-lethal) to express GFPN. In this line, Gal4 activity was first detectable in the salivary gland of stage 14 embryos and appears to be maintained until the end of the third instar (Fuse *et al.* 1994). The salivary gland cells endoreplicate to increase their ploidy, which reaches as high as 1000-fold larger than a haploid genome. This feature makes it possible to produce a large amount of GFPN in the salivary gland. In third instar larvae carrying both Gal4-lethal and UAS-gfpn, a strong fluorescence was detectable in the salivary gland which was visible through the cuticle of living larva (Fig. 2A). Fluorescence was localized to the nucleus, as expected (Fig. 2B).

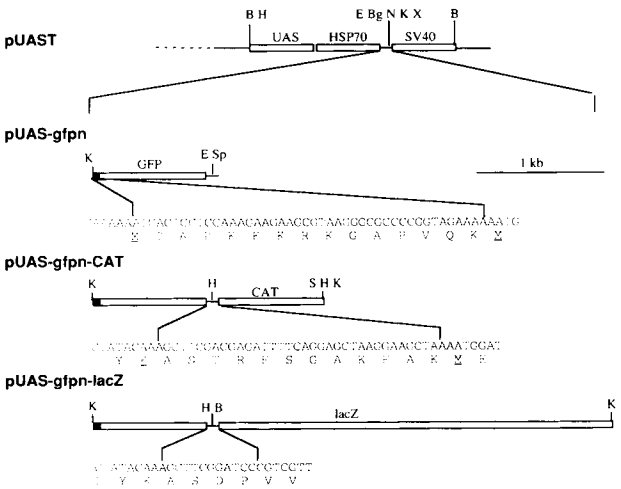

Fig. 1. Molecular maps of GFP expression vectors. All constructs are based on pUAST (Brand & Perrimon 1993), which carries six copies of the upstream activating sequence (UAS) of Gal4, the *hsp70* TATA box and leader sequence (HSP70) and the SV40 transcriptional terminator (SV40, this part of the map is not to the scale). pUAS-gfpn is predicted to encode a fusion protein carrying 15 amino acids of the SV40 nuclear localization signal placed N terminal to the authentic GFP starter methionine codon, which is underlined. pUAS-gfpn-CAT and pUAS-gfpn-lacZ are expected to encode GFPN fused to CAT and β-galactosidase. The last amino acid of GFP and the starter methionine coden of CAT are underlined. B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; N, NotI; S, SalI; Sp, SpeI; X, XbaI.

We next expressed GFPN in diploid cells in imaginal discs. A Gal4 enhancer trap line carrying an insertion near the *teashirt* gene (*tsh*-Gal4) was used. In the wing imaginal disc, *tsh*-Gal4 induced β-galactosidase expression from the UAS-lacZ construct in prospective notum and in the peripodial membrane. GFPN expressed in this manner gave a fluorescence barely detectable above background in a fixed wing imaginal disc (Fig. 3B).

We speculated that the low fluorescence intensity of GFPN may be due to protein instability or an inefficient post-translational modification to form the chromophore (Heim *et al.* 1994). We attempted to solve this problem by fusing GFPN to bacterial proteins CAT and β-galactosidase, known to be stable in *Drosophila* cells (Hiromi *et al.* 1985; Mitsialis *et al.* 1987). When expressed by

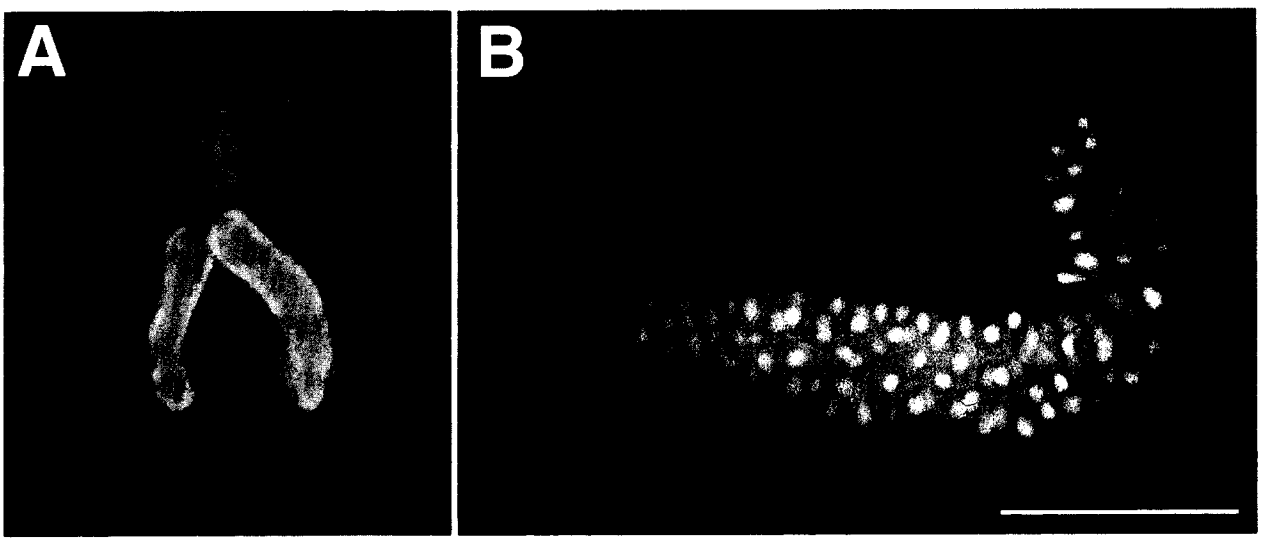

Fig. 2. GFPN expression in the larval salivary gland. (A) A living third instar larva carrying Gal4-lethal and UAS-gfpn was observed under fluorescence microscopy. (B) Fixed salivary glands from a negative control (top) and a Gal4-lethal; UAS-gfpn (bottom) larva. Bar, 800 μm (A); 200 μm (B).

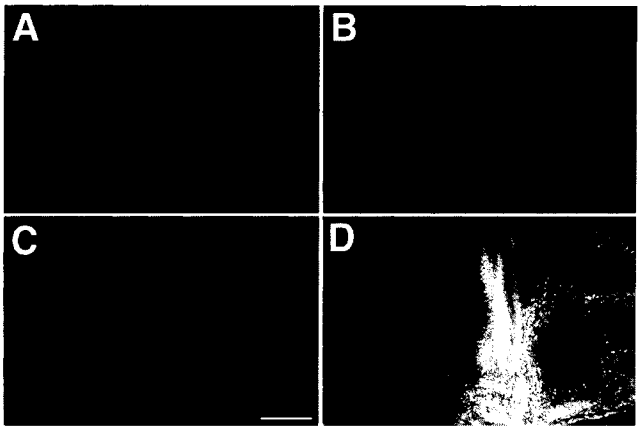

Fig. 3. Comparison of GFP fusion proteins. Wing discs from third instar larvae carrying *tsh*-Gal4 and one of the GFP fusion constructs were dissected, fixed and observed with a fluorescence microscope. The wing pouch is oriented to left, notum to the right and posterior up. Conditions for photographic exposure and subsequent processing were identical for all samples so that fluorescence intensity can be directly compared. (A) A negative control (y w; *tsh*-Gal4/+). (B) y w; *tsh*-Gal4/UAS-gfpn wing disc. Fluorescence is barely visible. (C) y w; *tsh*-Gal4/uas-gfpn-CAT wing disc. Fluorescence is weaker than the sample in B, but is above background. (D) y w; tsh-Gal4/UAS-gfpn-lacZ wing disc. Bright fluorescence is visible in prospective wing hinge (center), notum (right) and in peripodial membrane covering wing pouch (left). Bar, 80 μm.

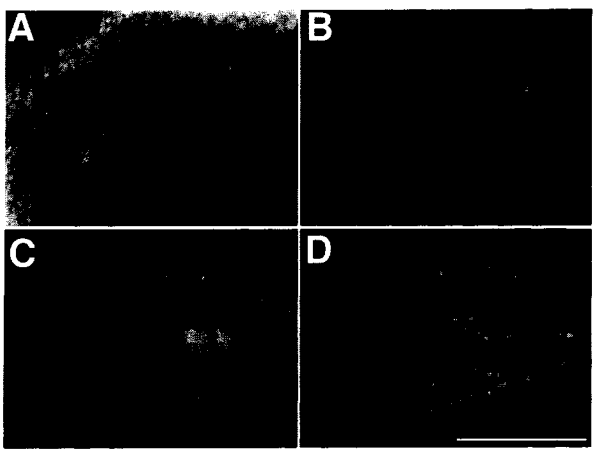

Fig. 4. GFPN-lacZ expression in larval tissues. (A) y w; *tsh*-Gal4/UAS-gfpn-lacZ wing disc. A part of the peripodial membrane is shown; green fluorescence is localized to the nucleus, but is excluded from the nucleolus. (B) y w; UAS-gfpn-lacZ/+; G455.2/+ wing disc. G455.2 induced fluorescence in prospective scutellum. (C) Central nervous systems of a control (y w; left) and y w; UAS-gfpn-lacZ/+; *pros*-Gal4/+ (right) third instar larvae. The bright spots associated with the CNS on the right is the ring gland. (D) A ventral nerve cord of y w; UAS-gfpn-lacZ/+; *pros*-Gal4/+ larvae. Bar, (A) 63 μm; (B) 200 μm; (C) 400 μm; (D) 100 μm.

the *tsh*-Gal4 driver, GFPN-CAT still fluoresced very weakly (Fig. 3C). To confirm that GFPN-CAT accumulates in the imaginal disc cells and is stable, the discs were stained with anti-CAT antibody. Staining revealed a pattern of expression expected for the *tsh* promoter (data not shown), suggesting that GFPN-CAT is present in cells but does not fluoresce effectively. When GFPN-lacZ was expressed, however, very bright fluorescence was detected (Fig. 3D). Fluorescence was mainly localized to the nucleus (Fig. 4A) and was stable for more than two weeks when the samples were stored at 4°C. An equal intensity of fluorescence was obtained from un-fixed preparations. An identical staining pattern was obtained with X-gal staining (data not shown). GFPN-lacZ was also expressed in the wing disc by the Gal4 driver G455.2, which is specifically expressed in prospective scutellum (Fig. 4B). When the *prospero* (*pros*, Doe *et al.* 1991; Vaessin *et al.* 1991; Matsuzaki *et al.* 1992) promoter in *pros*-Gal4 construct (Goto & Matsuzaki, unpubl. obs., 1994) was used, green fluorescence was detected in the ventral cord and the optic lobe of the central nervous system and in the ring gland (Figs 4C,D). These results demonstrate that GFPN-lacZ can be detected in various diploid tissues in the larva.

GFPN-lacZ expression in the embryo

We next expressed GFPN-lacZ in embryos in order to study development of the tracheal system. We chose the *breathless* (*btl*; Glazer & Shilo 1991; Klämbt *et al.* 1992), also known as DFR2 (Shishido *et al.* 1993), promoter to express GFPN-lacZ. When expressed under the control of the *btl*-Gal4 driver, green fluorescence from GFPN-lacZ was detectable in tracheal cells in living embryo (Fig. 5A). However, autofluorescence from yolk masked GFP fluorescence in the central part of the embryos. The green fluorescence was unstable under these conditions. When observed with a high magnification objective (×63, NA1.4), the fluorescence decayed within 1 min. In order to solve this problem, a confocal microscope was used. By choosing an Ar 488 nm laser and an appropriate filter combination, it was possible to eliminate a significant portion of the background fluorescence. Thus, the entire tracheal system was traced by serial confocal optical sectioning (Fig. 5B). The GFP fluorescence was also stable under these conditions; the embryos could be scanned for more than 5 min without significant loss of fluorescence. We noted that pre-exposure to the Ar 488 nm laser increased fluorescence intensity. Expression of *btl* was studied with enhancer trap strains and by *in situ* hybridization (Glazer & Shilo 1991; Klämbt *et al.* 1992; Shishido *et al.* 1993). Its RNA expression started at stage 10,

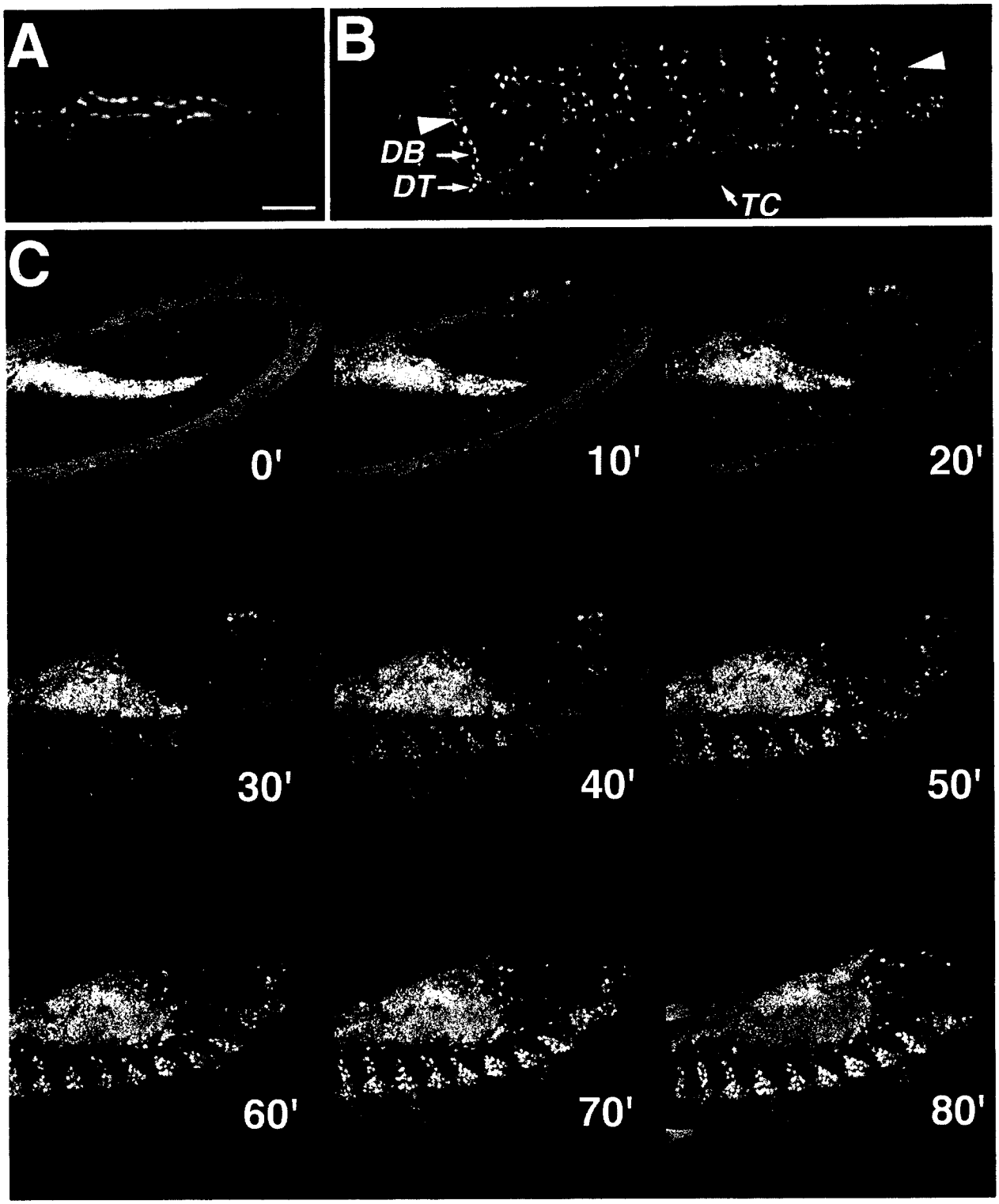

Fig. 5. GFPN-lacZ expression in the tracheal system of living embryos. Embryos are oriented anterior to left. (A) Lateral view of a stage 17 embryo observed with conventional fluorescence microscopy. Fluorescing nuclei of tracheal cells surrounding the tracheal cuticle are visible. (B) A dorso-lateral view of a stage 16 embryo observed with confocal microscopy. This image was reconstituted from 15 serial sections (2 μm). DT, dorsal trunk; DB, dorsal branch; TC, transverse connective. The dorsal midline is marked with arrowheads. (C) Confocal pictures of a single living embryo taken every 10 min. Each image was reconstituted from 18 serial optical sections (1.5 μm). The first image was taken in early stage 12 (time 0'). The last image was taken in early stage 13 (time 80'). The signal surrounding the periphery of the embryo is due to autofluorescence of the vitellin membrane. The signal in the middle is yolk autofluorescence. Bar, (A,B) 25 μm.

when tracheal precursors invaginated to form tracheal pits, and persisted throughout the later larval and adult stages. In the strain carrying both the UAS-gfpn-lacZ and *btl*-Gal4, GFP fluorescence was first detected in late stage 11. Therefore, the onset of GFP fluorescence was delayed approximately 2 h relative to *btl* RNA expression. A similar level of a delay of the appearance of GFP fluorescence was observed in mid-line cells in the central nervous system (data not shown).

Development of the tracheal system was observed by time lapse confocal microscopy. Serial optical sections were taken every 10 min over an 80 min period starting from early stage 12 (Fig. 5C). Embryos developed over a normal time course and the fluorescence became stronger in later stages of development. This result demonstrates that it is possible to visualize the formation of the tracheal network in living embryos.

Discussion

In the present study, we have shown that GFP fused to the nuclear localization signal and to β-galactosidase fluoresced brightly in embryos and in larvae. In contrast, fluorescence from GFPN and GFPN-CAT was weak in diploid imaginal disc cells. The precise reason for such different fluorescent properties is not yet known. It was recently shown that fluorescence from unfused GFP is detectable in *Drosophila* diploid cells (Yeh *et al.* 1995). Although direct comparisons have not been made, this may suggest that the fusion of the nuclear localization signal reduced fluorescence. As GFPN-CAT was detectable with antibodies against CAT and GFP was proven to be a very stable protein (Shimomura & Shimomura 1981; Ward & Bokman 1982), instability of GFPN-CAT is unlikely to be the reason for the low fluorescence. Alternatively, it is possible that the fusion affected the efficiency of the post-transcriptional modification of GFP. It has been suggested that the formation of the GFP chromophore occurs in two steps. The first step involves cyclization of Ser–Tyr–Gly at position 65–67 and the second step involves its oxidation (Heim *et al.* 1994). This modification occurs autocatalytically in many different cellular environments. The first step is thought to occur very rapidly, but the second step requires oxygen and is the rate limiting step. In *E. coli*, it takes approximately 4 h to complete the modification of the wild type GFP. The fluorescence of GFPN and GFPN-CAT in larvae did not increase after prolonged culture. We propose that most of the GFPN and GFPN-CAT are rapidly transported to the nucleus where they take on an alternative conformation in which chromophores do not form, as in the case of native GFP in an inclusion body in *E. coli* (Heim *et al.* 1994). Fusion to β-galactosidase may have improved the efficiency of the modification. Indeed, in embryos carrying GFPN-lacZ and *btl*-Gal4, fluorescence was first detectable in late stage 11, approximately 2 h after the initiation of *btl* RNA expression. As the production of GFPN-lacZ requires the accumulation of Gal4 expressed from the *btl* promoter, the actual time lag between the synthesis of GFPN-lacZ and the formation of the chromophore must be less than 2 h, which is significantly shorter than the time required for the chromophore formation of wild-type GFP in *E. coli*. Consistent with our observations with GFPN and GFPN-CAT, Davis *et al.* (1995) recently reported expression of a nuclear localized GFP fusion protein (PUbnlsGFP) driven by the strong polyubiquitin promoter in embryos. Their data suggest that the appearance of fluorescence of zygotically produced PUbnlsGFP is delayed relative to the accumulation of the protein and that its intensity is weak.

We have shown that GFPN-lacZ can be used to observe the sequence of tracheal cell migration and fusion in developing embryos. The tracheal system is a network of tubular epithelium (Campos-Ortega & Hartenstein 1985; Manning & Krasnow 1993). It is derived from 10 pairs of precursors that originate from invaginations of the dorsal ectoderm (tracheal pits) of each thoracic and abdominal segment. While keeping epithelial and tubular morphologies and without mitosis, the tracheal precursors form branches that migrate in several directions. Some of the branches migrate to target tissues. Dorsal trunk (DT) branches migrate anteriorly (DTa) and posteriorly (DTp) to fuse to homologous branches derived from adjacent segments to form a continuous tube of the dorsal trunk. Dorsal branches (DB) migrate dorsally to make connections at the dorsal mid-line with branches derived from the other side of the segment. Therefore, the formation of the entire tracheal network is achieved by mutual rearrangements and shape changes of tracheal cells to drive the migration of branches and also by recognition and adhesion between cells at fusion points. These features make the embryonic trachea a good model system for the study of dynamic cell behavior during tissue development. Several mutations that affect various stages of tracheal development have been identified (Klämbt *et al.* 1992; Klämbt 1993; Affolter *et al.* 1994a,b; Anderson *et al.* 1995). It should be possible to use GFPN-lacZ to analyze changes in cell behavior in these mutant backgrounds. Insertion of GFPN-lacZ into balancers should allow for the identification of mutant embryos for analyses such as dye filling of neurons and electrophysiology. Another possible application is to label cell nuclei for an ablasion experiment using laser beams. Mutations that change the spectrum

of absorbence and emission and the intensity of fluorescence (Heim *et al.* 1994, 1995) have been described. These mutations should expand the use of GFP in the future.

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