THE DETERMINATION of REPORTER GENE EXPRESSION in AN ENHANCER TRAP LINE, P[GAL4]c682

Bir İnhansır Trap Olan P[GAL4]c682'de Raportör Gen Ekspresyonunun Saptanması

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SUMMARY

In this study, the tissue localisation of the enhancer trap line P[GAL4]c682 was determined in D. melanogaster, fruitfly. The expression pattern for the reporter gene, β-galactosidase, was visualised at the adult-embryonic nervous system and male-female genitalia using chromogenic substrate X-gal or polyclonal β-galactosidase antibody.

P[GAL4]c682 produced an expression in the cell bodies and axons of the giant fibre system *(GFS). Reporter gene expression was also observed in the longitudinal glia and some other cell bodies on the ventral nerve cord of the fly. The enhancer-trap line which had been thought as homozygous male sterile was examined using cytochemistry, and the reporter gene expression was found in the secondary cells of the paragonial gland. These expression patterns for the enhancer trap P[GAL4]c682 may be produced by the same candidate gene or genes. The data related to the expression in the male genitalia may also suggest that either there is link between the enhancer-trap expression in the paragonial gland and the sterility phenotype or the enhancer-trap does not represent the complete expression pattern.

Key Words: Enhancer Trap Technique, P[GAL4]c682, β-galactosidase, Paragonial gland, Secondary cells.

ÖZET

Bu çalışmada sirke sineğindeki (D. melanogaster) bir inhansır trap olan P[GAL4]c682'nin doku lokalizasyonları tespit edilmiştir. Raportör genin (beta-galaktozidaz) yetişkin ve embriyonik sinir sistemi ile erkek-dişi üreme organlarında lokalize olduğu bölgeler renkli bir substrat olan X-gal ve poliklonal beta-galaktozidaz antikorunun histokimyasal olarak ortaya konulmasıyla tespit edildi.

İnhansır trap olarak bilinen P[GAL4]c682'nin merkezi sinir sistemi sinir hücreleri ile aksonlarında lokalize olduğu, ayrıca embriyonik dönemde raportör genin ventral nöral kordda bulunan bazı hücreler ile birlikte longitudinal glia hücrelerinde de bulunduğu belirlendi. Homozigot kısır olduğu düşünülen inhansır trap P[GAL4]c682'de, raportör genin erkek üreme sisteminde paragonial bezdeki ikincil hücrelere lokalize olduğu belirlendi.

Raportör genin farklı dokularda değişik lokalizasyonları bir veya birden fazla genin etkisi altında gerçekleşebilir. Bu durum beta-galaktozidazın paragonial bezdeki lokalizasyonu ile kısırlık fenotipi arasındaki bir ilişkinin bulunabileceğini ifade eder. Ancak raportör genin paragonial bezde gösterildiği bölgeler kesin olarak P[GAL4]c682'nin tüm lokalize olduğu bölgeleri temsil etmez.

Anahtar Sözcükler: İnhansır Trap Tekniği, P[GAL4]c682, β–galaktosidaz, Paragonal bez, İkincil hücreler.

INTRODUCTION

The standard approach to studying development of the fruit fly, *Drosophila*, has been to identify mutations on the genes that disturb the process of interest and to characterize the gene or genes. Extensive classic genetical screenings have been used to identify many genes involved in early developmental processes in *Drosophila* (1-3). Other approaches have been devised to isolate developmental genes in *Drosophila*. In recent years, a family of transposable elements,

P-elements, have been used extensively. In the last two decades, particular attention has been given to the P-elements family (4), Which has been the subject of intensive research as a molecular tool.

Complete P-elements are 2907bp in length and feature a perfect 31bp short inverted terminal repeat and an 11bp subterminal inverted repeat (5). The elements encode a well characterized, germline limited transposase and

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regulate their own copy number and activity in wild type strains. In addition to the study of their interesting properties, P-elements have been developed as an important tool of genetics and molecular biology in *Drosophila*. They have been used in numerous applications such as insertional mutagenesis (6-8), transposon tagging of genes for cloning (9), P-element-mediated transformation (10,11), site-specific recombination (12-14) and generating flaking deletions (15,16). P-element based vectors also have been applied to search for cis-acting sequences which confer tissue-specific expression of a *lac* Z gene (17).

Enhancer trapping was performed by constructing an in vitro-engineered *Drosophila* P-element. This modified P-transposable element carrying a reporter gene, the *E. coli lac* Z dominant eye colour gene (e.g. white+) to score flies that contain it and plasmid sequences that facilitate the cloning of the flanking genomic DNA from around the point of insertion. The P-element insertion can be also used to identify the cytological location of the gene. There are now several examples of the genes that have been identified and cloned using enhancer-traps [e.g., *teashirt, musashi, degenerative spermatocyte, lingerer*] (18-21). The principle of enhancer trap technique is schematized in figure 1.

Fischer and his co-workers (22) showed that the yeast transcriptional activator, GAL4, can activate transcription in *Drosophila*. When

GAL4 is expressed from the *Drosophila* alcohol dehydrogenase promoter (Adh), it activated a GAL4 responsive transgene in which five GAL4 binding sites, known as the upstream activating sites (UAS), were linked to *Lac* Z. The reporter gene was expressed in the tissues where *Adh* was normally expressed.

To exploit the basic enhancer-trap principle even further, "second generation" enhancer-trap systems have been developed (23). The GAL4 system allows the rapid generation of individual strains in which ectopic expression of a target gene can be directed to different tissues and cell types. The method also separates the target gene from its transcriptional activator in two distinct transgenic lines. The target gene is only activated when it is crossed to its transcriptional activator and turned on in the progeny.

The enhancer-trap GAL4 system can potentially be used to identify and analyse important developmental genes in other organisms (24-27). One such line is P[GAL4]c682 subjected to this study and was firstly identified in a screen for enhancer-trap lines expressing in specific cell bodies of the adult nervous system by Dr. David Shepherd, (Biological Sciences, University of Southampton), who noted expression in the giant fibre system. A project student, Jamie Honeychurch at the University of Warwick, also reported the line as homozygous

Figure 1. The principles of Enhancer Trapping Technique
Resim 1. Enhansır Trap Tekniğinin Prensibi

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male sterile. This article aims to characterize the enhancer-trap line P[GAL4]c682 in the nervous system of the embryo and the adult fly, and concentrates on expression pattern analysis of the mutation causing male sterility.

MATERIALS and METHODS

Materials

Flies were generally maintained on standard cornmeal, agar molasses and yeast medium. Fly stocks were standard laboratory stocks (except where stated) and were cultured at either 18 °C or 25 °C in plastic vials (Regina Industries Ltd.) or glass bottles of food. White Canton S was used as a wild type in all the genetical and molecular analysis and is shown with apostrophe, 'wild type'. Mutant and balancer chromosomes are listed in Lindsley and Zimm (28). Some other flay strains were provided from Bloomington Stock center.

Examination of fertility with mating and dissection

One male was crossed with three females which were confirmed as virgin by keeping at 25 °C for 3 days. The cross was kept at 25 °C for 3 days and in the third day it was searched for larvae under microscope in the vial. The vial was kept five days more at 25 °C to see pupae, unless we could see larvae.

Antibody Labelling of Embryos

Antibody labelling were standardly performed firstly by incubating with a rabbit primary antibody and then with a biotin labelled anti-rabbit secondary antibody. An ABC complex consisting of streptavidin bound to horse radish peroxidase (HRP) was then bound to the secondary antibody. The peroxidase activity was detected using the substrate, diamino benzoate (DAB), which gives a brown-black precipitate when cleaved by the peroxidase. DAB is carcinogenic and all operations after adding DAB, were performed on a tray and everything was washed with sodium hypochloride before disposal. The secondary antibody and streptavidin/peroxidase were supplied by the ABC elite kit from Vector laboratories. β-galactosidase anti-rabbit antibody (Cappel) was used to label the enhancer trap line P[GAL4]c682 embryos. Antibody labelling of embryos with β-galactosidase antibody was performed as previously described (29).

X-Gal Staining to adult brains and reproductive system of the male and female flies materials;

1X PBS: NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1 litre, pH adjusted to 7.2. Fix Solution: 4% paraformaldehyde in PBS. Staining Solution: The stock solution was made 500 ml as depicted as below. 5 ml NaP (150 ml of 1M Na_2PO_4 and 80 ml NaH_2PO_4 were mixed until pH was 6.8), 75 ml 1M NaCl, 0.5ml 1M MgCl2, 10ml $K_4(Fe_3(CN)_6)$, 10 ml $K_3(Fe_3(CN)_6)$, 1.5ml 100% Triton X-100, 398 ml distilled water. $20_\mu l$ 10% X-gal in Dimethylsulfoxide (w/v) was added to 1ml of staining solution at 37 to prevent crystals of X-gal forming. Histoclear : RA LAMB, Mounting Solution : Depex (BDH).

Method

The enhancer-trap pattern in the adult brain and male-female genitalia is visualised by using X-gal chromogenic substrate which gives a permanent blue colour. The brains and genitalia dissected in 1X PBS were fixed in fixing solution for 5 minutes and then transferred into 1X PBS solution for 2 minutes. The prewarmed staining solution (37 °C for 5 minutes) applied to the tissues until the permanent blue colour develops. The colour reaction was stopped with three, 1X PBS washes. The brains and genitalia were then dehydrated in ethanol series (from 30% to 100%) and then cleared in histoclear and mounted in Depex.

Microscopy and Photography

Low power microscopy for fly sorting and dissections were performed on a Meiji stereo microscope and Zeiss Stemi SV6. For high power microscopy, Nikon Optiphot and Zeiss Axioskop were used with the aid of nomarski and phase optics.

Photographies were taken by using either a Pentax K1000 or an Olympus OM4 camera. Films used were 35mm 100 ASA Kodacolor Gold II print film (Kodak) and 160T tungsten slide film (Kodak)

RESULTS

The expression pattern of the enhancer-trap line, P[GAL4]c682 in the adult nervous system

In order to confirm the expression pattern of the line P[GAL4]c682 in the adult nervous system, the line was first crossed with flies containing UAS-lacZ. The adult brain and thoracic ganglia from the progeny of this cross were dissected and stained with the chromogenic substrate X-gal. The expression pattern of P[GAL4]c682 is shown in Figure 2. β-galactosidase expression of P[GAL4]c682 showed a striking similarity to the expression of the line P[GAL4]307 line (30). A pair of large, descending neurons (red arrowhead) were stained with X-gal as well as some other cell body stainings in the brain (green arrowhead). The β-galactosidase protein was located in both nucleus and cytoplasm. The cell body of the giant fibres is located just below the dorsal protocerebral surface. The projections of these cells towards the thoracic ganglia of the central nervous system (CNS) were also stained. The projections can be clearly seen to descend through the cervical connective along the dorsal surface of the prothoracic neuromere (T1). Some cell bodies were also stained in T1. The projections extend further posteriorly to the mesothoracic neuromere (T2) and here the characteristic bend can be seen to connect to the tergotrochanteral motorneuroun (TTMn). Some other cell body stainings were also visualised in the T1, T2 and T3 neuromeres. The staining in the T2 neuromere might be candidate cell bodies for TTMn, but we could not identify those with X-gal preparations because no axons can be seen connecting to the cell bodies.

Embryonic expression pattern of the enhancer-trap line, P[GAL4]c682

Expression pattern of the enhancer-trap P[GAL4]c682 was also analysed embryonically as well as in the adult. The embryos were collected from the enhancer-trap line P[GAL4]c682 containing UAS-lacZ and labelled with polyclonal β-galactosidase antibody. The expression was detected on the longitudinal tract in the longitudunal glial cells (LGs, black arrowhead) at a very late stage embryogenesis, stage 16 (Figure 3). It also detected some other

stainings on the midline of the ventral nerve cord, VNC. Enhancer trap lines containing GAL4 are also nonspecifically expressed in the salivary glands (SG) of the fly as visualised in figure 3.

Glial cells fulfill different tasks in supporting neuronal cells including insulation, homeostasis and providing nutrition (31,32). Studies have also showed that they play roles in the compartmentalisation and the complex axonal folding formation within the central nervous system (33). Recently, a number of genes have been identified that are expressed in different glial cells and may play role in differentiation of these cells such as the spitz group (34-41). Expression of the enhancer-trap P[GAL4]c682 in the ventral nerve cord may mark a new candidate gene required for the developmental processes of the embryonic nervous system.

The enhancer-trap pattern analysis of P[GAL4]c682 males

The enhancer-trap line P[GAL4]c682 had already shown an expression in the nervous system of adult and embryo (Figure 2 and 3). A similar approach was also chosen again to try and detect an expression pattern in the male genitalia. The male heterozygous flies for P[GAL4]c682 containing UAS-lacZ were dissected and stained with X-gal (Figure 4). The expression pattern was localised to the secondary cells of the paragonial gland and ejaculatory bulb [black arrowhead shows the expression in the paragonial gland (PG) and ejaculatory bulb (EB)]. There was no expression in the testes, only background staining (unfilled arrowhead). Both the paragoinal gland and ejaculatory bulb are known to produce secretory products.

The paragonial gland, the so-called accessory gland, plays an important role for successful reproduction by providing multiple components of the seminal fluid, including short peptides, longer prohormone-like molecules with the potential to be cleaved into bioactive peptides (42-46). The secretions of the accessory gland are transferred to the female fly during copulation (44,45) and elicit stereotyped behavioral and physiological changes in the female. Such behavioral changes include a decrease in female receptivity to courtship by males, and increase

in agg laying (44,47-49).

Anatomically and structurally, the paragonial glands are two lobes, and each lobe is composed of a single layer of two morphologically distinct cell types (50). The major portion of the accessory gland epithelium is hexagonally shaped main cells (96%) (51). These cell are flat and binucleate cells that secrete their products into the lumen of each lobe. Embedded between the main cells at the tip of each lobe are the secretory 'secondary' cells which are large, spherical and binucleate cells with large vesicular vacuoles (5mm) (50). The muscle layer of the glands squeeze the glands content into the papillar region of the ejaculatory duct. Although the two secretory cell types are morphologically distinct, little is known about functional differences between each other. The morphological distinction between the cell types is reflected in biosynthetic and regulatory distinctions.

Enhancer-trap lines provide markers for each unique cell types in the gland. The effects of mating and ageing on the expression of ß-galactosidase in these enhancer-trap markers has been observed in a cell-type specific manner. Each cell type in the gland respond to cues of ageing and mating in a manner characteristic to that cell type (51).

A molecular analysis of genes specifically expressed in the paragonia was started a decade ago (42-44,52). At least 85 spots are seen upon two-dimensional gel electrophoretic analysis of the gland secretion (53). Several genes expressed in the male accessory gland have been identified by hybridisation techniques, such as main cell-specific 316-mst316 (42), msP355a and msP355b are expressed at both cell types (45), mst57D (52).

The examination of the P[GAL4]c682 female flies

During preliminary analysis, homozygous flies for the insert also showed reduced viability in female as well as male (29). Thus, the possibility existed of the homozygous mutant also affected female fertility. Twenty homozygous female flies for the insert were aged 3 days at 25 °C, and single crosses were set up with 'wild type' male flies. As a control similar crosses were performed between 'wild type' flies. Twelve out of 20 gave viable embryos of whiche 8 produced fertile eggs, their numbers were reduced (data not shown). Therefore, the homozygous females for the insert are classified as semi-sterile. The ovaries and eggs of the homozygous female flies for the insert were examined for morphological changes and they were found to be as the 'wild type' (data not shown).

The enhancer-trap was also analysed for the expression in the female genitalia. Female flies were aged 3 days at 25 °C and the genitalia was dissected. X-gal staining was performed to the dissected genitalia (Figure 5). The expression pattern was localised to the terminal filaments (red arrow) at each ovariole which are 20 tube-like structures forming a ovary (54). Each ovariole has 8-9 disc-shaped terminal filament cells in the anterior region and are required for ovary morphogenessis (55).

DISCUSSION

Initial characterization of P[GAL4]c682 reporter gene expression

The line had been reported to express in the giant fibre system (GFS) of the adult nervous system. It was therefore important, to choose an enhancer-trap line whose reporter gene expression pattern suggested that the enhancer trap might have inserted near to gene of interest. P[GAL4]c682 produced an expression in the cell bodies and axons of the GFS as reported (Figure 2) the same as other enhancer-trap lines in the group (30,56,57). Reporter gene expression was also found in the longitudinal glia and some other cell bodies on the midline in the ventral nerve cord of the embryo (Figure 3).

The enhancer-trap line had also been reported as homozygous male sterile (29), reporter gene expression was therefore investigated in the male genitalia. The expression was localised to the secondary cells of the paragonial gland rather than in the testes and the ejaculatory bulb. The secondary cells express male specific genes, msts 355a and 355b (45) independent of the main cells (51). This results in some questions. Either, is the enhancer-trap

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expressed in secondary cells of the paragonial gland target gene causing male sterility? or does the enhancer-trap pattern possibly mirror another gene's expression? Another possibility is that the enhancer-trap only report part of the gene's expression pattern.

The Relationship Between Expression Patterns

The enhancer-trap P[GAL4]c682 was expressed in various tissues at different developmental stages. The line shows expressions in the following;

1- Longitudinal glia and some other cell body staining on midline in late embryogenesis
2- Giant fibre circuit expression pattern in adult fly
3- In the male, secondary cell expression in the paragonial gland
4- Terminal filament (8-9 disc shaped somatic cells) expression of each ovariole in the female.

The enhancer-trap mirrored the embryonic nervous system expression, however tissue distribution of Kısır expression whether in the giant fibre system or male genitalia has not been determined. These expression patterns may be produced by the same gene or the giant fibre expression could be represented by another locus trapped by P[GAL4]c682. A similar argument could be used for the genitalial expression of the enhancer-trap. This may suggest that either there is link between the enhancer-trap expression in the paragonial gland and the sterility phenotype or the enhancer-trap does not represent the complete expression pattern of kısır. Additionally, P[GAL4] is not well expressed in the female germline (personal communication; A. Brand, Halder G, Moffat KG). No data has been reported for the male germline.

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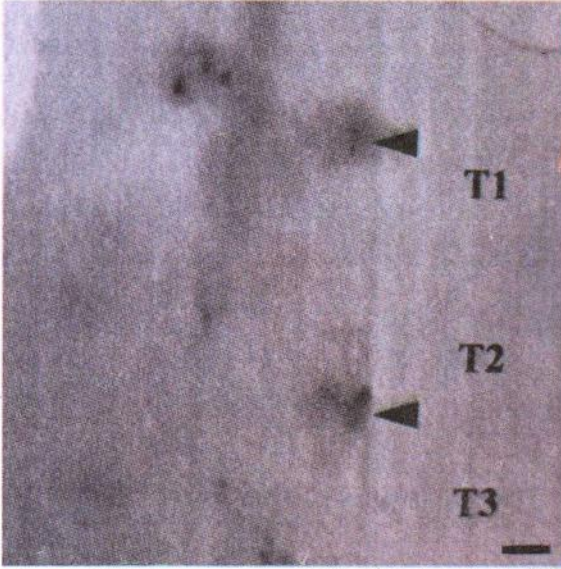
Figure 2. Enhancer trap of P[GAL4]c682 in the adult nervous system.
Resim 2. P[GAL4]c682'nin yetişkin sinir sisteminde inhasır trap lokalizasyonu. Abbreviations; GIC: The giant commisural interneuron, T1, T2 and T3: Thoracic neuromere 1, 2 and 3.
Scale bar: 25 μm
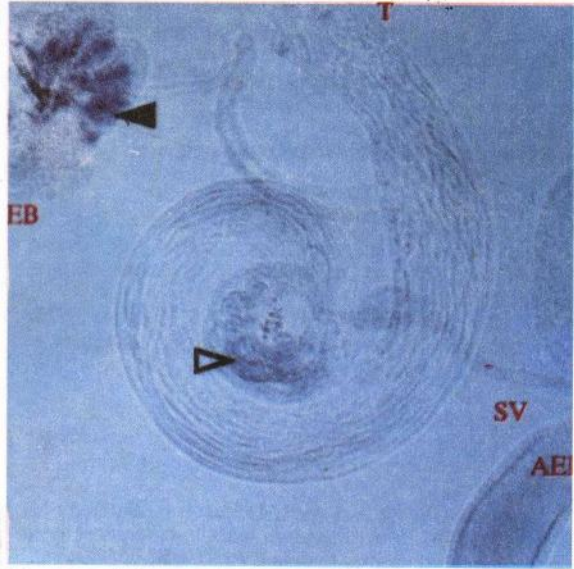
Figure 4. The enhancer trap pattern of P [GAL4]c682 in the male genitalia
Resim 4. P[GAL4]c682'nin erkek üreme sisteminde lokalizasyonu. Abbreviations; T: testes, PG: paragonial gland, SV: seminal vesicalis, AED: anterior ejaculotory duct, EB: ejaculotory bulb.
Scale bar: 100 μm
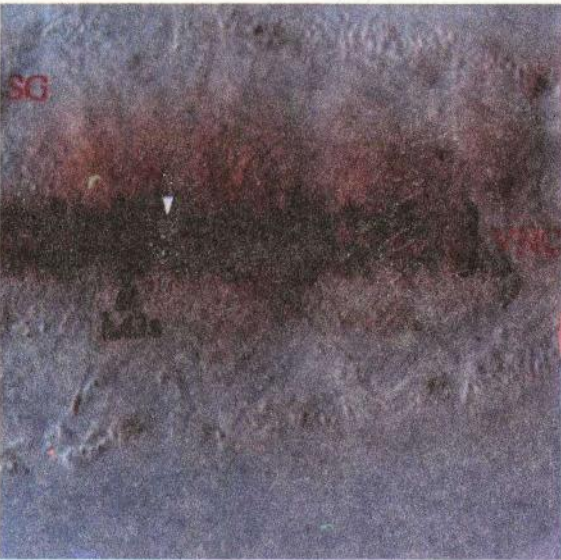
Figure 3. Embryonic expression pattern of P [GAL4]c682
Resim 3. P[GAL4]c682'nin embryonik lokalizasyonu. Abbreviations; SG: salivary gland, LGs: longitudinal glial cells, VNC: Ventral nerve cord, Scale bar: 50 μm
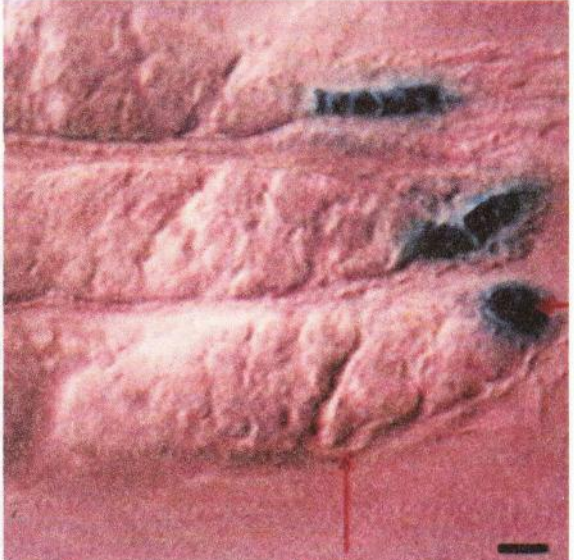
Figure 5. The enhancer trap pattern of P [GAL4]c682 in the female genitalia
Resim 5. [GAL4]c682'nin dişi üreme sisteminde lokalizasyonu. Scale bar 10 μm