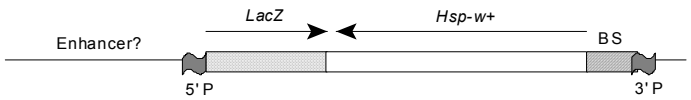
DETECTION OF GENE ENHANCERS BY *Drosophila* ENHANCER TRAP ANALYSIS

Enhancers: Transcriptional enhancers are DNA sequences that control expression of nearby genes and direct tissue-specific, positive regulation of those genes (Karp, 2002; Griffiths, *et al.*, 2002). The general mode of action of these sequences is that they associate with specific activator proteins (that are found in only certain tissues); this association of enhancer and activator facilitates transcription initiation at the target gene's promoter. Often located 1 kb or more from the target promoter, enhancers might be located upstream, downstream or even within the transcription unit that they control. Under the influence of one or more enhancers, a gene can be abundantly expressed in certain tissues of the organism (*i.e.*, cells in which the activator protein is found) and weakly or not at all expressed in other tissues. By identifying and studying enhancers, we learn how genes are regulated in time and space within multicellular organisms and we are able to use enhancers to "tag" tissues or even individual cells.

Enhancer trap strategy: Recently, a method for detecting and studying enhancers has been developed called enhancer trap mutagenesis. This method utilizes a recombinant transposon construct that can be inserted at essentially random sites within the genome and that, when inserted near an enhancer, will express a reporter gene product within the tissue or cells containing activator protein for that enhancer. The enhancer trap transposon contains a "basal" promoter element linked to a reporter gene that is inactive, except when influenced by a nearby enhancer. Because most enhancers direct tissue-specific gene expression, insertion of the enhancer trap transposon near an enhancer usually results in reporter gene expression within the particular tissue or group of tissues in which that enhancer normally functions. By mobilizing the transposon to diverse, essentially random chromosomal sites, one can detect diverse enhancers at those sites and study the tissue-specific gene control that they direct. Furthermore, enhancer trap transposons have been engineered to permit cloning of genomic DNA adjacent to the site of any particular insertion, enabling the isolation of the enhancer and its target gene from genomic DNA. This has proved to be a powerful system for studying enhancers and genes that direct highly specific cell expression (*e.g.*, genes that are expressed in individual cells that direct or reflect differentiation of those cells and their neighbors).

Specific transposon: The P[*lacW*] transposon (Fig. 1) consists of the open reading frame (ORF) for *E. coli lacZ* joined to the 5´ end of a P element, such that the P second exon and the *lacZ* ORF are fused in-frame (Bier, *et al.*, 1989). The resulting protein includes the first 123 amino acids of the P protein joined to the *lacZ* ORF. Expression of this fusion protein is controlled by the P element promoter that is expressed very weakly in all tissues, unless a nearby enhancer invokes abundant expression. The fusion protein is relatively unstable, permitting detection of both temporal and spatial control by the enhancer. P[*lacW*] also contains *Hsp-w*$^{+}$ sequences, a *Hsp70* promoter-*white*$^{+}$ gene fusion that provides a dominant *w*$^{+}$ marker with which we can detect the element in flies, and bacterial plasmid vector sequences. All of these sequences are located within inverted copies of the P element 31 bp inverted repeat sequences (denoted as dark triangles in Fig. 1) that will permit mobilization of the transposon within cells containing the necessary P element transposition protein called transposase.



DET Fig 1. The P[*lacW*] transposon (Bier, *et al.*, 1989). Extents of the *lacZ* and *Hsp-w* genes, the bacterial plasmid (BS), and P element terminal repeat sequences are indicated.

Genetic strains & markers: In the stock designated #102, the P[*lacW*] element is inserted into an X chromosome that carries the recessive marker *w* (white eye). A formal description of this stock is:

w P[lacW]AN365; +; +; + (stock #102)

where + denotes wild-type chromosomes 2, 3 and 4. Phenotypically, these flies are red-eye; the latter is due to expression of the *Hsp-w*$^{+}$ construct and is influenced by temperature at which the flies are grown (*e.g.*, more orange at temperatures below 25^{o}C).

To provide transposase for mobilization of the P[I*w*B] transposon, we will use a specially modified P element called P[Δ2-3] that cannot itself move and that abundantly provides transposase to all tissues of the fly. The terminal inverted repeat sequences of this element have been altered, blocking its transposition; transcription of the P element ORF is unusually abundant; modification of one intron results in translation of functional transposase in all tissues (rather than being limited to germ-line cells as is normal for P transposase)(Robertson, *et al.*, 1988).

In the stock designated #108, the P[Δ2-3] element is inserted into cytogenetic map region 99B of a balancer third chromosome carrying the marker *Sb* (dominant short bristles, recessive lethal). A balancer chromosome is a multiply inverted, multiply marked chromosome; the inversions inhibit crossingover in heterozygous animals. The other third chromosome in the #108 stock contains the dominant marker *Dr* (tiny eye). The X chromosome of the #108 stock carries the marker *w*. Phenotypically, these flies are white-eye, stubble-bristles, small-eye. A formal description of the #108 stock is

w; +; In(3LR)TMS, Sb P[Δ2-3]99B / Dr; + (stock #108)

A third stock, #143, is a simple *y w; +; +;* + strain that you will use to isolate new insertion lines. Animals from this stock are yellow-body (*y*) and white-eye (*w*).

***E. coli lacZ* as a reporter gene**: *lacZ* gene fusions are widely employed as reporters to measure transcriptional and translational regulation. *E. coli* β-galactosidase is an inherently stable protein and, as a foreign protein, it appears to lack signals borne by most eukaryotic proteins that govern their processing and longevity in eukaryotic cells. Differences in β-galactosidase activity in different tissues of an animal are reflections of rates of synthesis of the reporter protein in those tissues, which in turn is usually determined by the abundance of reporter mRNA. For a wide range of cellular systems, transgene β-galactosidase enzymatic activity is proportional to rates of transcription of the transgene copy. Another advantage of *lacZ* reporter systems is the highly sensitive and versatile array of detection techniques that have been devised during its long study and use in *E. coli*. In this exercise, you will use the artificial β-galactosidase substrate X-gal (5-bromo-4-chloro-indolyl-*D*-galactopyranoside) that, when hydrolyzed by the enzyme, produces a vivid blue dye. This provides a sensitive means of detecting and roughly quantitating reporter gene expression in whole tissue preparations.

You will perform β-galactosidase staining of tissues from *D. melanogaster* adults and larvae of the new strains you have generated that contain the P[*lacW*] transposon inserted at diverse genomic sites. Because these strains differ in their insertion sites and, therefore, expression of the weak P element promoter within the P[*lacW*] transposon might be influenced by novel enhancer elements, it will be necessary to stain a variety of tissues in each strain to ascertain the type(s) of enhancers effective in each strain.

DESCRIPTION OF FLY CROSSES

(see Fig. 2)

DET LAB 1: The Initial Cross

1. Mate virgin females from the #102 stock with males from the #108 stock. Because *Drosophila* females can store functional sperm and use it for several weeks, it is necessary to use virgin females for outcrosses. Virgins are obtained as females less than eight hours post-eclosion (newly eclosed females do not respond to courtship by males). We have isolated these virgins for you. Use approximately 12 pairs of flies per bottle. Each student should set one bottle of this cross, being careful to not let your flies become stuck in the medium.

2. Place the cultures in the 25^{o} incubator. At this temperature, development will take place rapidly and adult progeny will begin to emerge in ten days.

DET LAB 2: The Second Cross

3. Collect orange-eye, stubble-bristle males from the Lab 1 cultures. The transposon is "jumping" in these flies, producing prominent variegation of eye color (*i.e.*, clones of cells have been formed in which the transposon has relocated, resulting in varied expression of the *white* gene in the transposon). Mate these males with virgin *y w* females from stock #143. Again, use approximately 12 pairs of flies per bottle. Each student should set 4 bottles of this cross; place the cultures in the 25^{o} incubator.

DET LAB 3: Establishing Lines of Novel Transposon Sites

4. Examine progeny from the Lab 2 cross, seeking yellow-body, orange-eye males. Examine the flies of each Lab 2 bottle separately, estimating the number of animals that you examine (so that you can estimate the frequency with which "jumps" occur).

5. Each yellow, orange male should be placed in a fresh vial of medium, along with 2-3 virgin *y w* females from stock #143. Each such male probably contains the P[*lacW*] element at a novel and unique site, so *each male will be used to generate an enhancer trap line*. Remember that two or more males that emerge from the same bottle could possess the same insertion site, but males from different bottles must contain different insertion sites. *So, be sure to give each male a unique label and note the number of the Lab 2 bottle from which it emerged.* For example, name the four males from your first bottle 1a, 1b, 1c, and 1d. Males from the second bottle can be 2a, 2b, *etc.* ***Each student should isolate at least ten lines.***

6. Place the cultures in the 25^{o} incubator.

DET Fig. 2: Enhancer Trap Cross Scheme

Lab 1 ♀♀ ***w P[lacW]AN365; +; +; +*** **X** ♂♂ ***w; +; TMS, Sb P[Δ2-3] / Dr; +***

(stock #102) (stock #108)

Lab 2
(2 weeks later)

♂♂ ***w P[lacW]AN365; +; TMS, Sb P[Δ2-3] / +; +*** **X** ♀♀ ***y w; +; +; +***

(stock #143)

Lab 3
(3 weeks later)

1. Select orange-eyed, preferably non-stubble ♂♂
2. Mate each, individually, to create a subline containing the transposon at a novel location.

♂ ***y w; +; +; + P[lacW]*** **X** ♀♀ ***y w; +; +; +***

(single-male per vial; transposon inserted into chromosomes 2, 3 or 4) (stock #143)

Lab 4
(3 weeks later)

1. From each vial (line) select orange-eyed adult progeny
2. Dissect several & β-galactosidase stain
3. Cross several orange-eyed adults in a fresh vial to maintain the line

♂♂ ***y w; +; +; + P[lacW]*** **X** ♀♀ ***y w; +; +; + P[lacW]***

DET LAB 4: β-Galactosidase Staining

7. Verify that each line is an autosomal "jump" (*i.e.,* no longer sex-linked) and perform β-galactosidase staining of adult abdominal tissues. *Each student should dissect animals from at least four lines.* For dissecting and staining flies (and their viscera), you will need a dissecting microscope, a pair of watchmaker forceps, two depression dishes, a heat block set at 37°, microscope slides, coverslips and the solutions described below.

8. Anaesthetize animals from one vial (one line) and observe them under the dissecting microscope. Are half the males and half the females red-eye? If so, you have confirmed autosomal linkage of the transgene.

9. If the answer to 8 above is "yes", place ten pairs of red-eye flies into a fresh vial. This culture will be grown at 20° to propagate the line for later use.

10. Dissect and stain animals in a series of steps (modified from Glaser, *et al.*, 1986). First, place 400 µl Buffer A in a well of a depression dish and dissect an anesthetized fly directly in the fixative. Dissect up to three males and three females in the same well, discarding the carcasses.

Buffer A: 10 mM $NaPO_4$, pH 7.5
150 mM NaCl
1 mM $MgCl_2$

Fixative: 0.25% glutaraldehyde in Buffer A
Add 12.5 µl 70% glutaraldehyde to 3.45 ml Buffer A

Avoid contact with glutaraldehyde and avoid inhaling its fumes.

11. After dissecting all animals from one strain, transfer all of the viscera to 200 µl fixative in a well of a 9-well depression dish. Allow the sample to fix for 5 minutes, then transfer the tissue to a well of a 9-well depression dish containing 400 µl Buffer A. This washes away the fixative and hold the tissues until you have dissected all of your samples. Label or keep notes of which sample you put into which well!

12. Repeat steps 8-11 for at least four transposon lines *and with some white-eye flies from at least one of your lines.* The latter will serve as a negative staining control (transposon-negative).

13. After you have completed dissecting and fixing organs from each of your lines, draw off the Buffer A from each well and replace it with 200 µl of stain mix.

Stain Mix: 1.55 ml McIlvaine buffer (0.1 M $NaPO_4$ to pH 8 with citric acid)
0.2 ml 50 mM $K_3Fe(CN)_6$
0.2 ml 50 mM $K_4Fe(CN)_6$

Warm to 37° in a heat block
Add 50 :l 8% X-gal (in N,N-dimethylformamide)
Maintain at 37° to avoid X-gal precipitation

14. Place the dish in a sealed, humid box. Incubate at 37° overnight, to permit β-galactosidase staining to proceed.

15. To stop the staining, remove the stain mix from the well, removing as many X-gal crystals as possible with the stain mix. Replace the stain mix with Buffer A and return the depression dish to the sealed, humid chamber while the crystals attached to the tissue fragments dissolves. This will be done by the instructors on the day after Lab 4.

16. Transfer the stained adult tissue to a drop of mounting medium on a new microscope slide. Place all tissues from all animals of a single transposon strain onto a single slide.

Mounting Medium: 50% glycerol in Buffer A

17. Gently overlay the drop with a new coverslip. Under the microscope, gently press the coverslip over the tissue to splay tissues for observation.

Slides may be stored in a moist, sealed box and re-examined for several weeks, without deterioration of the tissues or fading of the staining pattern.

18. Observe the staining pattern in tissues of each line.

a. Use the provided anatomical diagrams to determine which tissues express β-galactosidase. Record your observations using the score sheets (next page).

b. Distinguish transposon-derived from endogenous fly β-galactosidase by observing the staining patterns in your control (white-eye) flies.

c. Note whether the patterns of expression in lines derived from the same Lab 2 bottle are the same or different.

References

Ashburner, M. (1989) *Drosophila. A Laboratory Handbook*. Cold Spring Harbor Press, NY.

Bier, E., H. Vaessin, S. Shepherd, K. Lee, K McCall, S. Barbel, L. Ackerman, R. Carretto, T. Uemura, E. Grell, L. Jan & Y. Jan (1989) Searching for pattern and mutation in the *Drosophila* genome with a P- lacZ vector. *Genes & Development* **3**:1273-1287

Glaser, R., M. Wolfner & J. Lis (1986) Spatial and temporal pattern of *hsp26* expression during normal development. *EMBO Journal* **5**:747-754.

Griffiths, A., W. Gelbart, J. Miller & R. Lewontin (2002) *Modern Genetic Analysis,* 2nd edition. W.H. Freeman, San Francisco. **[or another contemporary introductory genetics text]**

Karp, G. (2002) *Cell and Molecular Biology*, 3rd edition. John Wiley & Sons, Inc., NY. **[or another contemporary introductory cell biology text]**

Robertson, H., C. Preston, W. Phillis, D. Johnson-Schlitz, W. Benz & W. Engels (1988) A stable genomic source of P element transposase in *Drosophila melanogaster. Genetics* **118**:461-470.

BIO 410 $-galactosidase Staining of Tissues Group_____

Transgenic Subline		Intestine	Rectum	Ovary	Oviduct	Uterus	Testis	Ejaculatory duct	Accessory gland	Malpighian tubules		

Recommended scoring: - no stain
+ some stain
++ dark stain

Outline for Lab Report B: *Drosophila* Enhancer Trap Analysis

BIO 410

I. Introduction and Background (one-page limit)

Provide a brief definition of "enhancer" and briefly describe the enhancer trap approach to revealing enhancers.

II. Specific Aims

Concise list of the objectives of these experiments; can be a numbered list of single sentences.

III. Experimental Methods & Results

Describe what you did and present the results of those experiments. Experimental detail found in the experimental guide need not be repeated, although you should present data (numbers of animals handled, frequencies of phenotypes, and observations at each generation). Include figures and tables presenting your results (*e.g.*, diagrams of crosses, lists of lines obtained, staining patterns in each strain, and other observations on animals from those lines).

IV. Conclusions & Discussion (one and one-half page limit)

Self-explanatory. Do animals in a single line show the same expression pattern? Do the different lines you isolated show distinctive *lacZ* expression patterns? Which lines do you think have identical transposon insertion sites and which are clearly different lines? Give your rationales. How do you explain how a single enhancer trap line exhibits reporter gene expression in more than one tissue? A figure may be necessary to explain your model.

V. Recommendations for course improvement

Give us three recommendations of means by which these experiments or the course in general can be improved in the future; don't repeat recommendations you gave before.