Demonstration of Heat Shock Induced Gene Activity in Transgenic *Drosophila melanogaster* with a Reporter Gene Fused to a *Heat Shock Promoter*

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Introduction

Sudden increase in surrounding temperature induces a cellular response, termed the HEAT SHOCK RESPONSE. Exposure of *Drosophila* larvae, adults or their tissues to temperature above 33°C for a short period (about 5 min or longer) causes induction of a specific set of genes, the HEAT SHOCK GENES, whose transcripts are rapidly translated to make the HEAT SHOCK POLYPEPTIDES (HSP, see the article on "Heat shock response in *Drosophila*" in this issue). The HSPs appear to protect cellular functions from thermal (and certain other stress-induced) damages. The selective induction of this set of heat shock genes is believed to be due to the presence of one or more HEAT SHOCK PROMOTER consensus sequence (CnGAAnnTTCnG) upstream of the transcription start point. The heat shock genes are induced to transcribe rapidly when a HEAT SHOCK TRANSCRIPTION FACTOR (HSTF) binds to the upstream heat shock promoter. The HSTF is activated by the thermal (and certain other) stress and only this activated HSTF can bind to the heat shock promoter.

In view of the specific interaction between the HSTF and the heat shock promoter, it is possible by recombinant DNA techniques to make any gene heat-inducible : this is achieved by joining *(in vitro)* the heat shock promoter to the other desired structural gene (whose own promoter has been removed). This structural gene is now also designated as "reporter" gene since it reports on the activity status of the given promoter. If this fusion gene (artificially synthesized *in vitro*) is put back in the genome (through germline transformation), this is also activated alongwith the other normal heat shock genes in response to the thermal stress. Two such reporter genes, viz. the *ADH* gene (coding for alcohol dehydrogenase) of *D. melanogaster* and the *lac-Z* gene (coding for beta-galatosidase) of *E.coli*, have been used.

The following describes use of both these reporter genes for studying heat shock induced gene activity. This experiment does not require any expensive equipments or other laboratory facilities. Therefore, this can be adapted for any class room. While doing this experiment, the students not only are able to follow the dramatic effect of heat shock on cellular transcription and translation, but can also understand the role of promoter sequences (and reporter genes), the application of recombinant DNA techniques and introduction of foreign genes into genome of an organism. These stocks of transgenic flies can be obtained from our laboratory.

I. The *HSP70 promoter - ADH* fusion gene of *Drosophila melanogaster*

A 70 kilodalton polypeptide (HSP 70) is most abundantly induced by heat shock in *Drosophila melanogaster*. By recombinant DNA techniques, the promoter region (heat shock promoter) of the HSP 70 gene of *Drosophila melanogaster* was isolated and joined with the coding region of the gene for ALCOHOL DEHYDROGENASE (*ADH*) to produce *HSP 70-ADH*-fusion gene. Using P-element mediated

germline transformation procedure, this *in vitro* synthesized fusion gene was inserted in *Drosophila melanogaster* embryos which were homozygous for a mutant allele of the usual *ADH* gene (*ADH* null allele, not making a functional ADH enzyme). In this way transgenic *Drosophila melanogaster* flies were produced which had their own *ADH* gene inactive (due to null alleles) but had a functional *ADH* gene under the control of HSP 70 promoter. The genotype of this stock, relevant to the present experiment is : Adh^{fn6}/Adh^{fn6}; Adh^{hs61c}/Adh^{hs61c}.

(Adh^{fn6} - a null allele of Adh on chromosome 2; Adh^{hs61c} - the fusion gene carrying HSP70 promoter upstream to the ADH coding region and inserted at 61C region of chromosome 3, as seen in salivary gland polytene nuclei).

These individuals do not make ADH constitutively, since their normal Adh genes are mutated to null alleles but would make ADH in response to heat shock due to activation of the *HSP 70-ADH* fusion gene (for further details, see Bonner *et al*, 1984).

Detection of ADH activity in tissues/cells by histochemical staining

Alcohol dehydrogenase uses ethanol or 2-butanol as substrate and as its name shows, it removes hydrogen ions from the substrate. The released hydrogen ions are accepted by NAD and transferred to tetrazolium salts. During ADH staining, Nitroblue tetrazolium (NBT) is provided alongwith the substrate and NAD. The reduced NBT causes a colored formazan deposit which can be seen at the site of enzyme activity.

Materials required

1. Late 3rd instar larvae of Adh^{fn6}; Adh^{hs61c} stock
2. Incubator or a water bath set of 37°C and at 24°C (or room temperature if the ambient is around 24°C)
3. Dissection instruments and Dissection microscope
4. Microscope slides and coverglasses (22 or 24 mm^2)
5. *Drosophila* Ringer's soln, ADH activity stain, acetomethanol, glycerol, DPX

ADH activity stain (to be prepared fresh)

0.05M Sodium Phosphate Buffer pH 7.5	2.0 ml
5 mg/ml Nitroblue Tetrazolium	0.5 ml
NAD (50 mg/ml)	0.1 ml
2-Butanol	0.1 ml
2 mg/ml Phenazine Methosulfate	0.05 ml

Procedure

1. Heat Shock

Healthy late 3rd instar larvae (grown under uncrowded at 20°C-24°C) are washed free of adhering food etc with water and some larvae are transferred to a small petridish lined with a moist filter peper. The petridish with larvae is put in an incubator maintained at 37°C for heat shock for 60 min. If a water bath is used for heat shock, the larvae are transferred to a small glass or plastic tube (lined with a moist filter paper) plugged with cotton and carefully floated in the water bath (water bath provides more uniform and rapid transfer of heat; when an incubator is used for heat shock, a water-filled beaker may be kept in the incubator maintained at 37°C to function as water bath). A parallel set of larvae is kept at 24°C as control.

2. ADH Staining

After the period of heat shock, the control and heat shocked larvae are dissected in Ringer's soln and unwanted tissues like cuticle, fat body etc are removed. Excess Ringer's soln is drained out by carefully tilting the slide and a small drop of ADH activity stain is added. The slide is kept in dark and staining allowed to proceed for 15-20 min after which the stain soln is drained out. Working with fine needles, the different organs are properly arranged on the slide and a few drops of aceto-methanol fixative are added to fix the tissues and to remove non-specific stain. Excess fixative is wiped with filter or tissue paper and the slide is allowed to be semi-dry. A drop of 50% glycerol is added and a coverglass is carefully mounted avoiding

bubbles. The edges of coverglass may be sealed with DPX for permanency.

Observations

The heat shocked tissues stain bluish-black while the control tissues show very little ADH activity. If the heat shocked larvae are allowed to recover for 1 or 2 hours at 24°C prior to ADH staining, a much more intense staining is seen.

II. The *HSP 70 - LAC Z* fusion gene of *Drosophila melanogaster*

By recombinant DNA techniques, the promoter region (heat shock promoter) of the HSP 70 gene of *Drosophila melanogaster* was isolated and joined with the coding region of the *E.coli* gene for BETA-GALACTOSIDASE *(Lac Z)* to produce the *HSP 70-Lac Z*-fusion gene. This *in vitro* synthesized fusion gene was inserted in *Drosophila melanogaster* embryos by P-element mediated germline transformation. In this way transgenic *Drosophila melanogaster* flies were produced in which the *Lac Z* gene functioned under control of an HSP 70 promoter (for further details, see O'Kane and Gehring, 1987). One such stock is named *Bg9* in which the *Hsp 70-Lac Z* fusion gene is inserted at the 9B region of X-chromosome.

Detection of beta-galactosidase activity in tissues/ cells by histochemical staining

Beta-galactosidase normally uses galactose as substrate. However, a chromogenic compound, X-gal (5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside), also acts as a good substrate. The colourless X-gal is converted by beta-galactosidase into its indoxyl derivative which in turn is oxidized to the blue dye 5,5′-dibromo-4,4′-dichloro-indigo. Potassium ferricyanide is used as a catalyst for oxidation of the indoxyl product. Potassium ferrocyanide is also added in equal amount to prevent over-oxidation to a colourless compound. Without the added oxidizing agents, initial products of beta-galactosidase reaction, the indole monomers, appear to diffuse out of the cell before they could be oxidized and dimerized by molecular oxygen.

Requirements

1. Late 3rd instar larvae of *Bg9* stock
2. Incubator or water bath set at 37°C and at 24°C (or room temperature if the ambient is around 24°C)
3. Dissection instruments and Dissection microscope
4. Microscope slides and coverglasses (22 or 24 mm^2)
5. *Drosophila* Ringer's, X-gal stain, acetomethanol, 2.5% Glutaraldehyde in 50mM Phosphate buffer (pH 8.0), Wash buffer (50mM Phosphate buffer pH 8.0), 50% Glycerol, DPX

1M Phosphate Buffer (pH 8.0)

1M Na_2HPO_4	93.2 ml
1M NaH_2PO_4	6.8 ml

Wash Buffer (50mM Phosphate Buffer pH 8.0)

1M Phosphate Buffer	50 ml
Dist. Water	950 ml

X-Gal stain

Stock soln	Amount	Final Conc.
5% X-Gal (in Dimethyl-Formamide)	60 ul	0.3%
100 mM $K_3[Fe(CN)_6]$	20 ul	2 mM
100 mM $K_4[Fe(CN)_6]$	20 ul	2 mM
1M Phosphate buffer (pH 8.0)	50 ul	50 mM
Dist. Water	850 ul	

Procedure

1 Heat Shock

Heat shock to larvae is given as described in the previous experiment.

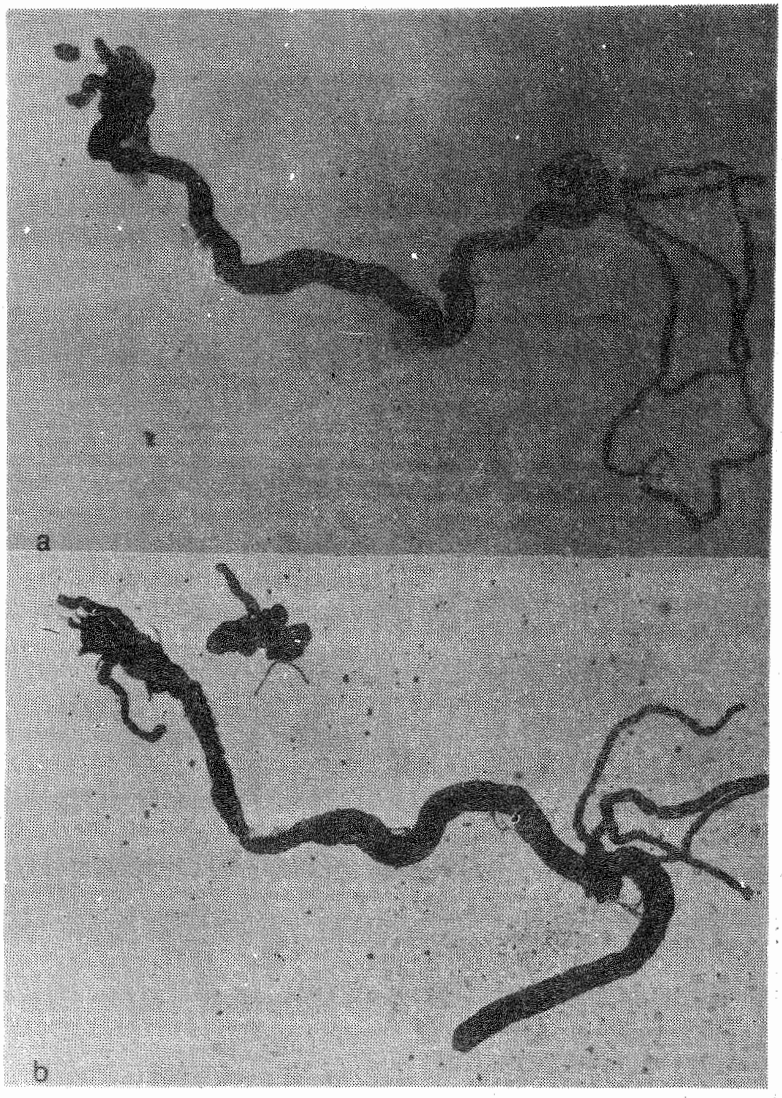

Fig. 1. Histochemical staining patterns in gut and associated tissues of *Bg*9 :
a. CONTROL and b. HEAT SHOCKED for 60 min at 37°C.
Note the intense blue-black staining after heat shock.

2 X-Gal Staining

The control and heat shocked larvae are dissected in Ringer's soln and unwanted tissues like cuticle, fat body etc are removed. The desired tissues are transferred in Ringer's soln to a fresh cavity slide. Exces Ringer's soln is removed and the tissues are fixed i 2.5% Glutaraldehyde for 10 min at room temperatur The fixative is removed and tissues washed 2x wit

wash buffer. The wash buffer is removed and 50 ul of X-Gal staining solution is added. The cavity slide is covered with coverglass and kept overnight in a moist chamber in dark at 37°C. The X-Gal stain is removed, the tissues washed with wash buffer and briefly refixed with aceto-methanol. The tissues are transferred to a fresh slide in a drop of 50% glycerol and spread as desired. They are carefully mounted with a clean coverglass. The edges are sealed with DPX mountant.

Observations

The heat shocked tissues stain bluish-black while the control tissues show very little beta-galactosidase activity (examples of staining patterns seen in gut and associated structures in control and heat shocked larvae are shown in figures 1a and b). Recovery of heat shocked larvae for 1 or 2 hours at 24°C results in more intense beta-galactosidase staining.

Points for Discussion

1. Why do heat shocked larvae show increased ADH or beta-galactosidase activity in these cases? (hint : hsp70 promoter linked to the *ADH* or *Lac Z* gene)
2. Why some staining may be seen in control tissues? (hint : normal developmental activity or larvae already mildly stressed)
3. Does *Drosophila* beta-galactosidase activity interfere with the results in the case of larvae transgenic for *hsp70-lac Z* fusion gene? (hint : pH optima of *the Drosophila* beta-galactosidase is in acidic range, whereas for the *E.coli* enzyme, pH 8.0 is optimal)
4. In the case of *hsp70-ADH* fusion gene, why was this gene inserted in ADH null genotype (hint : to prevent normal developmental expression of ADH activity)

References

Bonner, J.J., C. Parks, J. Parker-Thornburg, M.A. Mortin and H.R.B. Pelham 1984. The use of promoter fusions in *Drosophila* genetics: isolation of mutations affecting the heat shock response. *Cell* 37 : 979-991.

O'Kane, C.J. and W.J. Gehring 1987. Detection in situ of genetic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 84 : 9123-9127.