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# Induced expression of a *Drosophila hsp70* promoter-fusion transgene is reduced after repeated heat shocks.

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**Summary**

Levels of transcripts produced by a heat shock protein 70 (*hsp70*)-antisense *white* transgene in *Drosophila* were measured after single and multiple heat shocks to determine whether the *hsp70* promoter could produce sustained high levels of transgene transcripts. A single heat shock resulted in typical highly inducible levels of RNA, but the amount of antisense RNA was substantially reduced after multiple heat shocks. Endogenous *hsp70* mRNA levels were also less abundant after multiple heat shocks as compared to a single heat shock. The *hsp70* promoter is unsuitable for use in fusion gene constructs for long term expression studies where repeated heat shocks are required.

## 1. Introduction

The choice of promoters is a crucial factor in designing fusion gene constructs for use in transgenic animals. Ideally, a promoter should allow conditional expression (either temporal or spatial) of the gene, allow low constitutive and high induced levels of expression, as well as being controlled by an agent that does not affect the normal physiology or development of the organism and whose action is reversible.

The *Drosophila* heat shock protein 70 (*hsp70*) promoter possesses many desirable features that make it an ideal choice to drive transcription of transgenes. It is the strongest promoter known in *Drosophila* (Velazquez *et al.* 1983) and is fully active in fusion transgenes. It has little constitutive activity (Velazquez *et al.* 1983; Steller & Pirrotta, 1984), but massive transcription is induced by heat shock at 37 °C for both the endogenous *hsp70* gene and for the fusion transgenes under its control (Steller & Pirrotta, 1985; Lindquist, 1980*a*; Velazquez *et al.* 1983; Lindquist, 1980*b*; Graziosi *et al.* 1980). It is inducible at blastoderm as well as at later stages in development (Dura, 1981; McGarry & Lindquist, 1986) in most cell types. Furthermore, the induced level of activity can be varied both in proportion to temperature in the range 29–37 °C and in proportion to the duration of the heat shock (Velazquez *et al.* 1983). In fusion transgenes, the promoter is relatively insensitive to position effect (Bonner *et al.* 1984).

The promoter most widely used for the expression of fusion genes in transgenic *Drosophila* has been the *Drosophila hsp70* promoter. It has been used to drive transcription of the *Drosophila* genes *fushi tarazu* (Struhl, 1985), *sevenless* (Basler & Hafen, 1989), *white* (Gehring *et al.* 1984; Steller & Pirrotta, 1985; Klemenz, Weber & Gehring, 1987), antisense ribosomal protein (Qian, Hongo & Jacobs-Lorenza, 1988), *Adh* (Petersen & Lindquist, 1989; Dudler & Travers, 1984; Klemenz, Hultmark & Gehring, 1985), and antisense *hsp70* and *hsp26* (Lindquist, McGarry & Golic, 1988; Pelham, 1987), and the *E. coli lacZ* gene (Lis, Simon & Sutton, 1983; Simon *et al.* 1985; Xiao & Lis, 1989), and chloramphenicol acetyl transferase gene (Petersen & Lindquist, 1989). The *hsp70* promoter has also been used to drive transcription in mammalian cells (Bonner *et al.* 1984) and in plants (Spena *et al.* 1985).

In most cases where *hsp70*-transgenes have been used with success (see above), a single heat shock resulted in levels of transcripts sufficient to produce the desired phenotype. However, it is unclear whether sustained transcription can be achieved with the *hsp70* promoter. Such sustained transcription is crucial where antisense genes are used to inhibit expression of endogenous genes, or where the gene product is required throughout life. In fact, an *hsp70*-antisense *white* transgene failed to regulate the expression of the endogenous *white* gene in an earlier experiment (Hunt,

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1991), and unsustainable levels of antisense RNA was a possible cause.

While considerable work has been done on regulation of the endogenous *hsp70* gene in cultured cells, mostly indirectly by measurement of protein levels (Petersen & Lindquist, 1989; Velazquez & Lindquist, 1984; DiDomenico, Bugaisky & Lindquist, 1982*a*, *b*; Petersen & Lindquist, 1988; Craig, 1985; Lindquist, 1986), a detailed temporal study of the activity of the *Drosophila hsp70* promoter at the mRNA level has not been reported for either adults or larvae.

The aims of this work were to assess the long-term effectiveness of the *hsp70* promoter in transgenes by carrying out temporal studies of the expression of an *hsp70*-antisense *white* transgene following single and multiple heat shocks in *Drosophila* adults and larvae. Although steady state levels of antisense *white* RNA reached typical highly inducible levels following a single heat shock, these induced levels were not achieved after repeated heat shocks.

## 2. Materials and methods

### (i) *Fly culture conditions*

Fly stocks were maintained at 25 °C in vials containing F (Claringbold & Barker, 1961) or PS medium (Frankham, Yoo & Sheldon, 1988).

### (ii) *The* hsp70-*antisense* white *transgene*

The *hsp70*-antisense *white* transgene was made by fusing a 3·3 kbp fragment of the *white* gene coding sequence, in the antisense orientation, to an 850 bp *hsp70* promoter fragment; the DNA fragments were obtained from the plasmid pw8 (Klemenz *et al.* 1987). The 850 bp promoter fragment contains the *hsp70* promoter, 216 bp of the *hsp70* gene untranslated leader sequence, as well as 196 bp from the 5′ untranslated region and 268 bp from the 5′ end of the *white* gene (positions +3707 to +3243) (O'Hare *et al.* 1984). The 3·3 kbp *white* gene coding sequence contains exons 2–6, 161 bp of the first intron, introns 2–5 and 525 bp of the 3′ untranslated region (positions +3243 to +3170, +432 to −2758) (O'Hare *et al.* 1984). The fusion construct was subsequently inserted into the polylinker (*Hpa* I/*Sal* I sites) of the Carnegie 20 P element vector (Rubin & Spradling, 1983) which contains a $rosy^{+}$ marker gene.

### (iii) *Transformation of* Drosophila

The final construct was introduced into a $ry^{506}$ background by germline transformation as previously described by Spradling (1986). Two separate transformed lines were used in this study, one with a single autosomal insertion (A) and the other with a single insertion on the X chromosome (X).

### (iv) *Radiolabelling of DNA probes*

Single-stranded M13mp19 DNA, containing a 2·1 kb *Bam*H I/*Sal* I fragment of the *white* gene derived from the plasmid pG2wSB (Howells, personal communication), was used as an antisense *white* probe. The plasmid pDm238 (Roiha & Glover, 1981) was used as a probe for rRNA. An *Xba* I/*Eco*R I *hsp70* gene fragment from the plasmid 'hst' (supplied by J. Ewer) was used as a probe for endogenous *hsp70* mRNA. DNA (60–100 ng, single- or double-stranded) was labelled with [$\alpha$-$^{32}$P]dATP using an OLK-50 kit supplied by Bresatec (South Australia) and used according to the manufacturer's instructions.

### (v) *Heat shock treatments*

Temporal changes in levels of *white* antisense RNA isolated from larvae and adults representing transgenic lines A and X were followed during an eight hour recovery period at 25 °C after a single 30 min heat shock treatment at 37 °C. The larvae (96 h old) and adults were snap-frozen at 0, 0·5, 1·0, 1·5, 2·0, 4·0 or 8·0 h after the end of the treatment.

The effects of multiple heat shocks on the levels of *white* antisense RNA were determined. Temporal changes in levels of *white* antisense RNA were determined during a four hour recovery period at 25 °C following one or six heat shocks (30 min each at four-hourly intervals). RNA was isolated from line X larvae (starting age 96 h old) snap frozen after 0, 0·5, 1, 2 or 4 h recovery at 25 °C following one or six heat shocks. The effects of one, two, four or six heat shocks on the levels of *white* antisense RNA were also determined in a similar manner, except that larvae from lines A and X were snap frozen either immediately or after 0·5 h recovery at 25 °C. RNA isolated from transgenic larvae maintained at a constant 25 °C was used as control. To control for the possibility that RNA levels might vary with the developmental stage of the larvae, RNA isolated from larvae subjected to a single heat shock at 120 h was also included in the study. To determine whether endogenous *hsp70* mRNA levels were similarly affected by multiple heat shocks, these were assayed independently in RNA samples isolated from transgenic line A.

Heat shocking was carried out by partially immersing vials containing developing larvae or adults in a 37 °C water bath. Between heat shocks the water temperature was maintained at 25 °C.

### (vi) *Determination of RNA levels*

RNA was isolated from larvae and adult flies as previously described by Chomczynski & Sacchi (1987). The isolated RNA was redissolved in diethylpyrocarbonate treated distilled water and the concentration was determined spectrophotometrically.

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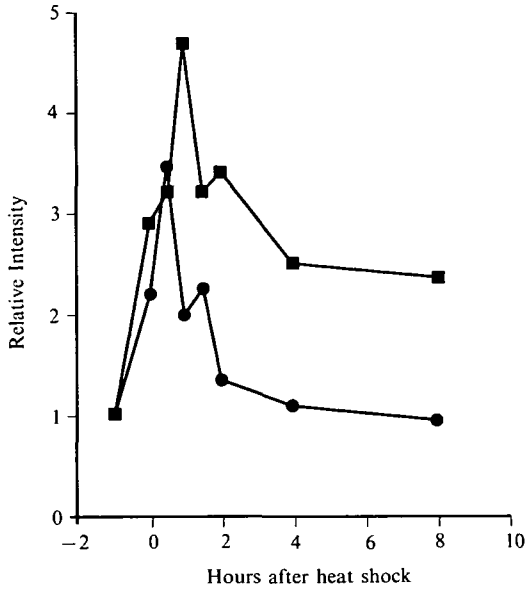


Fig. 1. Effect of a single heat shock on the levels of antisense *white* RNA from adults (■) and larvae (●) of line X. Relative intensity values were obtained by densitometric scanning of an autoradiogram of a northern blot (data not shown). The integrated values for each sample, adjusted for differences in track loadings, are expressed relative to the non-heat shock control value (time − 1) arbitrarily set at 1.

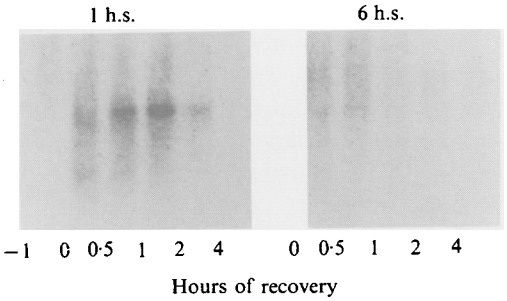


Fig. 2. Northern blot analysis of antisense *white* RNA from larvae of line X heat shocked one (1 h.s.) or six (6 h.s.) times at four-hourly intervals. RNA levels were determined after 0, 0·5, 1, 2 or 4 h recovery. The control (time − 1) represents transgenic larvae that were not heat shocked.

RNA was separated by electrophoresis in a 1·2% agarose gel made up in 20 mM-MOPS (morpholinopropane sulphonic acid), 5 mM sodium acetate, 1 mM-EDTA pH 7·0, 1·9% formaldehyde (Fourney *et al.* 1988). After electrophoresis at 4 V $cm^{-1}$ for 16 h, the gels were prepared for blotting onto a nylon membrane (Zeta Probe, Biorad) by soaking for 20 min in 0·05 M-NaOH, 1 × SSC; followed by two 20-min periods in 10 × SSC with gentle shaking. The RNA was transferred in 10 × SSC by capillary action and fixed to the membrane by a 5-min treatment with 50 mM-NaOH, followed by baking in an 80 °C vacuum oven for 1 h. The membrane was pre-hybridized for 1–4 h at 65 °C in 5 × SSPE, 7% SDS, 5 × Denhardt's solution (Reed & Mann, 1985), after which the probe was added to the mixture and allowed to hybridize for 16-24 h at

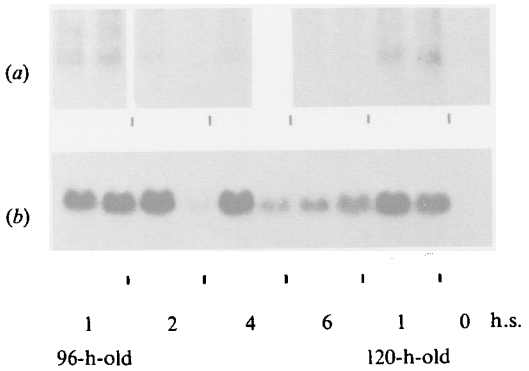


Fig. 3. Northern blot analysis of antisense *white* RNA (*a*) and of endogenous *hsp70* mRNA (*b*) from larvae of line A. Larvae (starting age 96 h old) were heat shocked one (1 h.s.), two (2 h.s.), four (4 h.s.) or six (6 h.s.) times at four-hourly intervals and RNA levels were determined after 0 or 0·5 h recovery (1st and 2nd track respectively in all except 0 h.s. categories). Transgenic larvae that were not heat shocked (0 h.s.), and 120-h-old transgenic larvae heat shocked once were used as controls.

42 °C. The membrane was washed for two 15-min periods in 2 × SSC, 0·1% SDS at room temperature with shaking followed by one wash in 1 × SSC, 0·1% SDS for 15 min at 65 °C and a high stringency wash in 0·1 × SSC, 0·1% SDS for 10 min at 65 °C. The hybridization signals were quantified by densitometric scanning (LKB 2202 laser densitometer) of autoradiograms obtained from two exposures of each filter. The integrated values for antisense *white* RNA and *hsp70* mRNA were adjusted for differences in track loadings as assessed by reprobing the filters with pDm238 DNA.

## 3. Results

### (i) *Temporal changes in antisense* white *RNA levels in adults and larvae following a single heat shock*

A 30-min treatment at 37 °C strongly induces the *hsp70*-antisense *white* transgene in transgenic adults and larvae of line X (Fig. 1). In adults, RNA levels peaked 1 h after the treatment and declined gradually with significant levels of RNA still being present after 8 h. In contrast, antisense RNA levels in larvae peaked about ½ h after heat shock and declined relatively rapidly, reaching control levels after 4 h. Results for line A and for repeat experiments were similar (date not shown).

### (ii) *Antisense* white *RNA levels in transgenic larvae following repeated heat shocks*

Induced levels of antisense *white* RNA in larvae subjected to six heat shocks were much less than in larvae heat shocked once (Fig. 2). The levels of antisense RNA detected immediately after 1–6 heat shocks (0 h recovery) gradually decreased with increasing number of heat shocks in both transgenic lines (Figs 3*a*, 4), although not to control values. After 30 min recovery at 25 °C however, RNA levels

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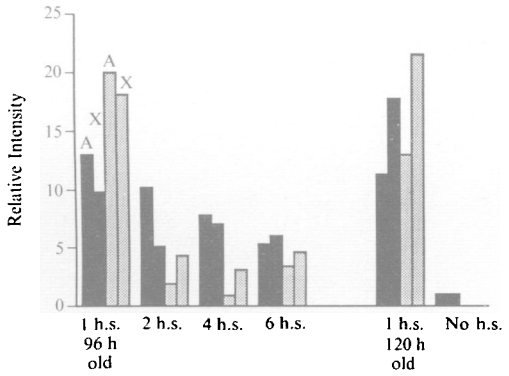


Fig. 4. Relative antisense *white* RNA levels following single and multiple heat shocks in larvae (starting with 96-h-old larvae) from lines A and X. The relative intensity values were obtained by densitometric scanning of the autoradiograms (line A shown in Fig. 3*a*, line X data not shown) as described in the legend to Fig. 1. Values are shown for both 0 h (■) and 0·5 h (▨) recovery following one (1 h.s.), two (2 h.s.), four (4 h.s.) or six (6 h.s.) four-hourly heat shocks. Values for 120-h-old transgenic larvae heat shocked once are shown as a control for any developmental effect.

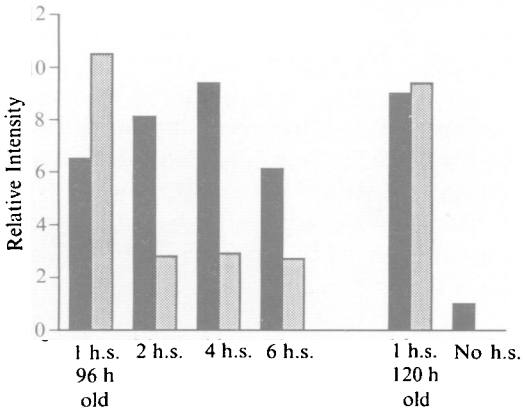


Fig. 5. Relative *hsp70* mRNA levels following single and multiple heat shocks in larvae from transgenic line A (starting age of 96 h). The relative intensity values were obtained by densitometric scanning of the autoradiogram in Fig. 3*b* as described in the legend to Fig. 1. Values are shown for both 0 h (■) and 0·5 h (▨) recovery following one (1 h.s.), two (2 h.s.), four (4 h.s.) or six (6 h.s.) four-hourly heat shocks. Values are also shown for 120-h-old transgenic larvae heat shocked once.

had dropped to virtually non-heat shock control levels in larvae that had been heat shocked two or more times. The observed changes in antisense RNA levels were not related to larval development since the response to a single heat shock in 120-h-old larvae is similar to that in 96-h-old larvae (Figs 3*a*, 4). Results from a repeat experiment were similar (data not shown).

(iii) *Levels of endogenous* hsp70 *mRNA in transgenic larvae after repeated heat shocks*

Endogenous *hsp70* mRNA levels present after 30 min recovery following multiple heat shocks were less than levels detected 30 min after a single heat shock. However, the magnitude of the effect was less than in the case of the transgene. Further, there was no consistent variation in the levels detected immediately after each heat shock (Figs 3*b*, 5). Changes in *hsp70* mRNA levels were not associated with larval age. Results from a repeat experiment were similar (data not shown).

## 4. Discussion

Multiple heat shocks substantially affect the level of antisense RNA detected after each heat shock in the transgenic *Drosophila* larvae. This resembles the observation that endogenous heat shock mRNA levels in plants (soybean) fall progressively with successive rounds of heat shock (Key *et al.* 1985). In the case of the transformants presented here, it is not possible to distinguish between the effects of multiple heat shocks on the *hsp70* promoter function and the effects on transcript degradation. However, the net effect of transcription shut down and RNA degradation is the relevant factor in the context of using the *hsp70* promoter in fusion transgenes.

Transcription of the antisense *white* transgene was induced to relatively high levels following a single heat shock in both larvae and adults, in accord with previous results for the endogenous *hsp70* gene (Petersen & Lindquist, 1989) and for other transgenes driven by the *hsp70* promoter (see Introduction). Depletion of antisense RNA during recovery after a single heat shock was much more rapid in larvae than in adults. In the case of the larvae, these kinetics were similar to those of transcripts of the endogenous *hsp70* gene in *Drosophila* cultured cells heat shocked at 36 °C (Petersen & Lindquist, 1989). This is in contrast with results reported for the endogenous *hsp70* gene in *Drosophila* cultured cells heat shocked at 38 °C and an *hsp70-Adh* transgene in cultured cells heat shocked at either 36 or 38 °C. In both cases, levels of transcripts remained high for 4 h following a single heat shock. The reasons for these differences are not clear.

Do endogenous *hsp70* mRNA levels vary in a similar manner to those of fusion transgene transcript levels following multiple heat shocks? Levels of *hsp70* mRNA detected after 30 min recovery from two or more heat shocks did decline. However, no significant change was observed in the mRNA levels detected immediately after each heat shock. Since the *hsp70* protein is known to exert negative feedback control over its own mRNA levels through repression of transcription and the destabilization of existing transcripts (DiDomenico *et al.* 1982*a*, *b*) and multiple heat shocks lead to the progressive accumulation of heat shock proteins (DiDomenico *et al.* 1982*a*), the observed effects of multiple heat shocks on levels of *hsp70* mRNA are probably not unexpected.

Why do the endogenous gene and the transgene respond differently? It is unlikely that position effects due to DNA sequences flanking the transgene caused

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different responses, since two transgenes located on different chromosomes behaved similarly if not identically. Any similarity in behaviour of the endogenous gene and the transgene (as observed in the 30 min effect) may have been due to the presence, in the antisense transgene, of 210 nucleotides of *hsp70* leader sequences containing regulatory elements responsible for repression of *hsp70* mRNA transcription (Bonner, 1985; Lindquist & DiDomenico, 1985; Rougvie & Lis, 1990). The differences in response of the endogenous gene and the transgene (as observed in the 0 h effect) may have been due to differences in RNA stability. Since the transgene lacked polyadenylation and transcription termination signals, the antisense transcript may have been less stable than the *hsp70* mRNA.

In summary, the *Drosophila hsp70* promoter is not suitable to drive transgenes where high levels of activity are required over extended periods of time, such as is the case with housekeeping genes and antisense genes.

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