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, 1980a; Velazquez *et al.* 1983; Lindquist, 1980b; 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,
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, Bugasisky & Lindquist, 1982a, 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 polyn linker (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 ry106 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 21 kb BamH 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/EcoR 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 [α-32P]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, 1.5, 2, 20, 40 or 80 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.
hsp70 expression following multiple heat shocks

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 (morpholino-propane sulphonic acid), 5 mM sodium acetate, 1 mM-EDTA pH 7.0, 19% 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 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 re-probing 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 3a, 4), although not to control values. After 30 min recovery at 25 °C however, RNA levels
levels detected 30 min after a single heat shock. Recovery following multiple heat shocks were less than larvae after repeated heat shocks.

Endogenous mRNA levels present after 30 min

Endogenous hsp70

Levels of endogenous (iii) mRNA in transgenic

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 3b, 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. 1982a, b) and multiple heat shocks lead to the progressive accumulation of heat shock proteins (DiDomenico et al. 1982a), 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
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 0 h 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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