The EMBO Journal Vol.1 No.10 pp.1279–1285, 1982

Regulation of heat-shock genes: a DNA sequence upstream of *Drosophila hsp70* genes is essential for their induction in monkey cells

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Communicated by A.Tissières
Received on 13 September 1982

Heat-shock genes coding for heat-shock protein 70 (HSP70) in *Drosophila melanogaster* were subcloned into an SV40/plasmid recombinant capable of replication in permissive monkey COS cells. Following transfection of COS cells, no significant amount of *Drosophila hsp70* RNA was detected at 37°C. In contrast, a heat-shock at 43°C or arsenite poisoning at 37°C induced the massive production of *Drosophila hsp70* RNA of correct size and faithful 5′ ends. After heat-shock, the efficiency of *hsp70* transcription in COS cells containing 2–4 x 10^4 gene copies was found to be 15–30% of that measured in *Drosophila*, on a per gene basis. By testing a series of 5′ deletion mutants in this inducible transcription assay it was found that a sequence <70 bp long, directly upstream of the *hsp70* gene, was essential for the heat or arsenite induction of transcription.

Key words: heat-shock/SV40/*Drosophila*/gene transfer/transcription

Introduction

Many organisms from *Escherichia coli* to man, including plants, respond to heat or other environmental stress conditions by the vigorous synthesis of a characteristic set of heat-shock proteins (HSPs) apparently involved in homeostasis (see, *Heat-Shock from Bacteria to Man,* 1982). The heat-shock response has been extensively studied in *Drosophila melanogaster* in which it was first discovered (Ritossa, 1962). One remarkable feature of the response is the rapid activation of the HSP-coding genes upon brief exposure to elevated temperature. This rapid switch in gene expression provides an attractive experimental system in which induction of transcription can be studied.

All major *D. melanogaster* heat-shock genes have been cloned, analysed in detail, and sequenced. The *hsp70* genes coding for the major 70 000-dalton heat-shock protein are found as two variants occurring in multiple copies at duplicate chromosomal loci, 87A7 and 87C1. These genes have no introns and are flanked by a 350-bp long conserved element Z_{nc} adjacent to the start of *hsp70* transcription. These sequences, however, are not located in front of other heat-shock genes but are found interspersed within repeated sequence elements ($\alpha\beta$) whose transcription is heat inducible (Artavanis-Tsakonas *et al.*, 1979; Ish-Horowicz *et al.*, 1979; Lis *et al.*, 1981). Does this conserved element Z_{nc} or any other adjacent DNA regions contain sequences required for heat-shock transcription regulation?

We report here that accurate transcription of *Drosophila hsp70* genes is efficiently induced in monkey cells by heat-shock or arsenite. Plasmid vectors containing an SV40 origin of replication were used to transfect COS cells (Gluzman, 1981) in which the plasmid DNA replicates to high copy number as circular episomes (Lusky and Botchan, 1981; Mellon *et al.*, 1981). Transcription of the *Drosophila* gene in these cells was assayed 24–48 h after transfection, thus providing a quick and convenient means for screening a large number of recombinant or deletion mutants generated *in vitro*. We have found that a sequence of <70 bp adjacent to the start of transcription is required for the induction of *hsp70* genes.

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Results

Construction of vectors containing Drosophila hsp70 genes which replicate in COS cells

Hsp70 gene variants from both chromosomal loci 87A7 and 87C1 in *D. melanogaster* were subcloned into the plasmid pSVO-Kan as shown in Figure 1. This cloning vector is a recombinant of pML2-RIIG, a plasmid which carries an SV40 origin of replication and replicates efficiently in COS cells (Lusky and Botchan, 1981) plus a segment of the bacterial transposon Tn5 that confers resistance to kanamycin (Rothstein *et al.*, 1980). None of these clones contain the full 72-bp repeat unit required for viral transcription (Benoist and Chambon, 1981) and which can enhance the transcription of neighbouring genes (Banerji *et al.*, 1981). Furthermore, the *hsp70* genes were inserted in an opposite polarity to that of the truncated SV40 early promoter in order to avoid any possible leakiness that might interfere with studies of the heat-shock promoter.

Replication of the pSVOri vectors in COS cells was analysed by hybridization of DNA blots to ^{32}P-labeled plasmid probes (Figure 2). Most pSVO-plasmid DNA found in these cells migrated in agarose gels as supercoiled DNA (lowest bands in Figure 2A). Replication of this DNA was demonstrated by its complete sensitivity to *Mbo*I restriction. In contrast, the transfecting DNA isolated from a *dam*$^+$ *E. coli* strain was resistant to *Mbo*I due to adenine methylation at its cleavage sites (Figure 2B). Supercoiled plasmid DNA from COS cells was titrated with increasing amounts of pSVO-H8 DNA for standardisation (Figure 2A). This DNA accounted for ~0.05% of the total cellular DNA 24 h after transfection and up to 0.3% after 48 h. If 5–10% of the cells were effectively transfected (this is the percentage of T-antigen positive nuclei we observed in control transfections of CV-1 cells with SV40 DNA) these quantities would correspond to 20–40 x 10^3 plasmid copies per transfected COS cell 24 h after transfection. As expected, little supercoiled DNA was associated with CV-1 cells which do not support the replication of pSVOri-vectors. These plasmid titrations and copy numbers are in good agreement with previous reports (Mellon *et al.*, 1981). They are used here to evaluate the transcription efficiency of the *Drosophila hsp70* genes in the COS cells.

Drosophila hsp70 genes are stress-inducible in COS cells

Transcription of *hsp70* genes on pSVOri-episomes was investigated in both COS and CV-1 cells. One or two days after

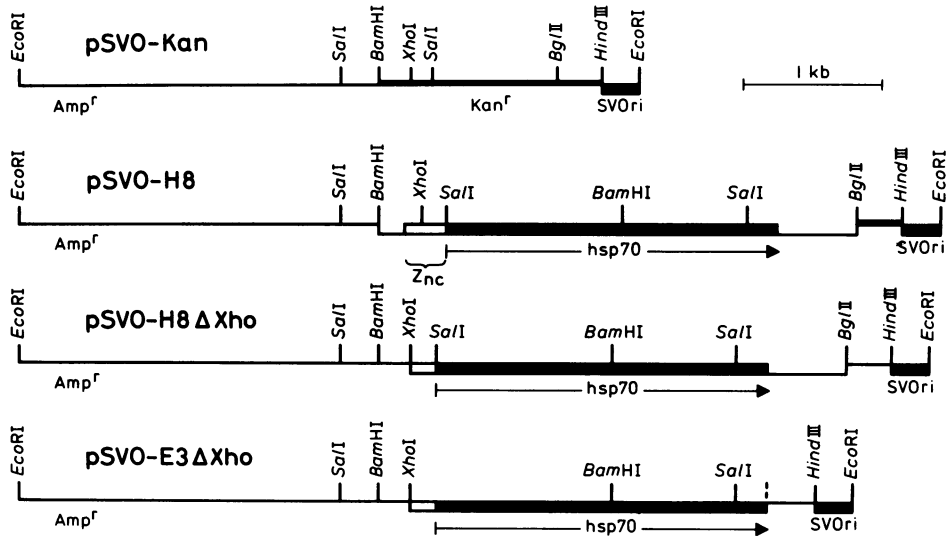

Fig. 1. Linear maps of an SVOri-plasmid vector and of derivatives carrying *hsp70* genes. The cloning vector pSVO-kan was constructed by joining the *Bam*HI-*Hind*III fragment of Tn5 that confers resistance to kanamycin (Rothstein *et al.*, 1980) to the large *Bam*HI-*Hind*III fragment of plasmid pML2-RIIG, a pBR322 derivative containing an SV40 origin of replication (SVOri) and deleted of a pBR322 sequence inhibitory to replication in COS cells (Lusky and Botchan, 1981). Plasmids pSVO-H8 and pSVO-H8ΔXho, which contain one *hsp70* gene copy of the chromosomal site 87A7 in *D. melanogaster,* have an *Xho*I-*Bgl*II segment of clone 56H8 (Moran *et al.*, 1979) inserted at the corresponding sites in pSVO-kan. The small *Bam*HI-*Xho*I segment of pSVO-H8 derives from clone 122 (Goldschmidt-Clermont, 1979); pSVO-H8 thus contains a complete Z_{nc} element adjacent to *hsp70*. Plasmid pSVO-E3ΔXho has one *hsp70* variant from site 87C1 in the *Xho*I-*Bgl*II fragment of clone 132E3 (Moran *et al.*, 1979; the *Bgl*II-*Hind*III segment of pSVO-kan is replaced by a *Bam*HI-*Hind*III segment of pBR322 that generated a hybrid site *Bgl*II/*Bam*HI shown as vertical dots).

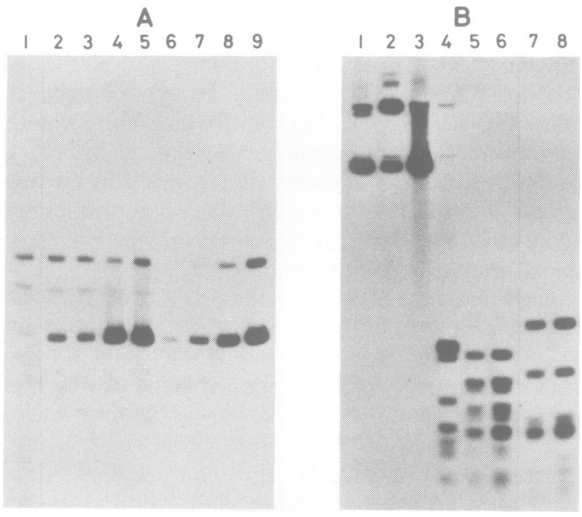

Fig. 2. Replication of pSVO-H8 in COS cells. **(A)** Total DNA (0.6–1.2 μg) from cells transfected by pSVO-H8 was fractionated by electrophoresis on a 0.7% agarose gel, blotted and hybridized with ^{32}P-labeled pSVO-H8 DNA. The filter was exposed to Kodak XR-5 film for 7 h with an intensifying screen. **Lane 1**: CV-1 cells, 24 h post-transfection; **lanes 2** and **3**: COS cells, 24 h post-transfection; **lanes 4** and **5**: COS cells, 48 h post-transfection (DNA in **lanes 2** and **3**, **4** and **5** is from cells in duplicate Petri dishes); **lanes 6–9**: 0.1, 0.3, 1, and 3 ng pSVO-H8 DNA, with carrier COS cell DNA (1 μg). **(B)** Restriction enzyme analysis. Following digestion, the DNA was fractionated in a 1.2% agarose gel, blotted and hybridized as above. The autoradiographic exposure was 20 h. **Lane 1**: pSVO-H8 (1 ng), undigested; **lane 2**: pSVO-H8 (1 ng), *Mbo*I-digested; **lane 3**: DNA from pSVO-H8 transfected COS cells, 48 h post-transfection (1 μg), undigested; **lane 4**: as in **lane 3**, but *Mbo*I digested; **lane 5**: pSVO-H8 (1 ng), *Hpa*II-digested; **lane 6**: as in **lane 3**, but *Hpa*II-digested. **lane 7**: pSVO-H8 (1 ng), *Hha*I-digested. **lane 8**: as in **lane 3**, but *Hha*I-digested.

transfection, the cells were heat-shocked (1 h at 43°C, followed by 1 h at 37°C) or maintained at the normal growth temperature (37°C). A massive synthesis of endogenous heat-shock protein (HSP70 and HSP89) was induced in the monkey cells under the shock conditions used here (data not shown). Transcription of the *Drosophila* genes was first assayed by hybridization of a ^{32}P-labeled *hsp70* probe to Northern blots of denatured total RNA and the results are shown in Figure 3. Strong signals corresponding to *hsp70* transcripts co-migrating with genuine 2.5-kb *Drosophila hsp70* RNA were detected in pSVO-H8 transfected COS cells following heat-shock. In contrast, no significant amount of *hps70* RNA was detected in non heat-shocked cells nor was endogenous monkey *hsp70* RNA detected with that probe under the stringent hybridization conditions used. The 50- to 100-fold increase in *Drosophila hsp70* RNA following heat-shock 24 h after transfection was not significantly higher after 48 h even though the gene copy number increased 6-fold in this interval (Figures 2 and 3). Thus, the system appears to be saturated by 4 x 10^4 gene copies or less per cell. *Hsp70* transcription was also inducible at low gene copy number in CV-1 cells, or COS cells 4 h after transfection (Figure 3, lanes 5–7). Transcription or induction was not saturated under these conditions.

Arsenite was reported to induce *hsp70* synthesis in chick embryos (Levinson *et al.*, 1979; Johnston *et al.*, 1980), *Drosophila* cultured Kc cells and monkey COS cells (Mirault, unpublished data). *Drosophila hsp70* genes introduced in COS cells are also inducible by arsenite treatment at 37°C (Figure 3). The amount of *hsp70* RNA accumulated during 4 h in arsenite treated cells was comparable to that induced by heat-shock (1 h at 43°C + 1 h at 37°C). During a 2-h poisoning period, however, the amount of RNA synthesized was much lower than in heat-shocked COS cells (data not shown).

In several instances an inverse correlation has been found between cytosine methylation in DNA and gene expression in eukaryotes (reviewed by Razin and Friedman, 1981). By restriction analysis, 5-methylcytosine was not detected in the plasmids replicated in COS cells (Figure 2B) whether shocked or not. Furthermore, transcription of *Drosophila hsp70* was

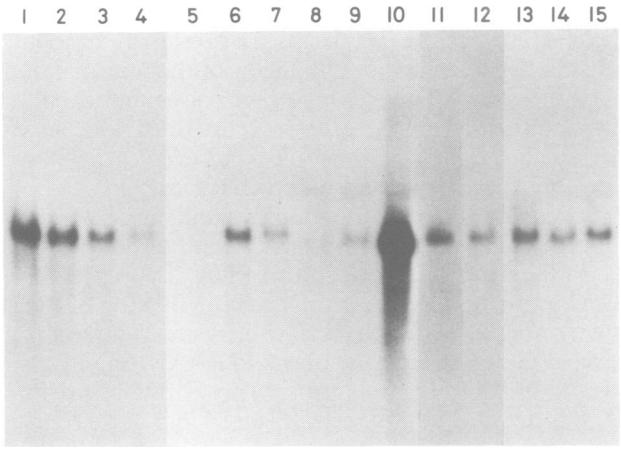

Fig. 3. Induced transcription of a *Drosophila hsp70* gene in monkey cells. Total cellular RNA after n-propanol/2 M NaCl precipitation (1.6 μg RNA unless specified) was denatured by formaldehyde (Lehrach *et al.*, 1977) and fractionated by electrophoresis on 1.2% agarose gels containing formaldehyde (2.2 M). The gels were then equilibrated with 20 x SSC (pH 6.8; 2 x 45 min, with one buffer change), blotted to nitrocellulose filters and hybridized to a ^{32}P-labeled *hsp70* probe (2-kb *Sal*I fragment of pSVO-H8). The filters were exposed to Kodak XR5 films with an intensifying screen. **Lanes 1–4**: Kc cells heat-shocked 1 h at 37°C (1.6; 0.8; 0.4; and 0.2 μg RNA, respectively); **lanes 5** and **6**: CV-1 cells 24 h after transfection by pSVO-H8, maintained at 37°C or heat-shocked; **lanes 7–15**: pSVO-H8 transfected COS cells; **lane 7**: heat-shocked, 4 h post-transfection; **lane 8**: no shock, 24 h post-transfection; **lanes 9** and **10**: heat-shocked, 24 h post-transfection, 0.04 and 1.6 μg RNA, respectively; **lanes 11** and **12**: heat-shocked, 24 h and 48 h post-transfection; **lanes 13–15**: 4 h incubation with 50 μM arsenite (**lane 15**), in the presence of 100 μg/ml hemetine (**lane 13**) or incubated as in **lane 15** but for 1 h more at 37°C in the presence of 5 μg/ml actinomycin D (**lane 14**). **Lanes 1–12** are from two filters hybridized in the same bag. Exposure times: **lanes 1–4, 11** and **12**: 2 h; **lanes 5–10**: 20 h; **lanes 13–15**: 7 h. **Lanes 10** and **11** are different exposures of the same lane.

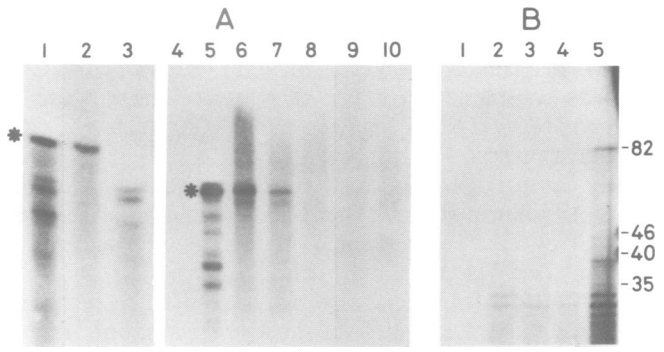

Fig. 4. S1 nuclease analysis of *Drosophila hsp 70* RNA from Kc and COS cells. **(A)** Size of the DNA protected by *hsp70* RNA. The analysis was performed according to the procedure of Favarolo *et al.* (1980). 5 ng *Eco*RI-cut pSVO-H8 DNA was hybridized with 3 μg total cellular RNA (DNA excess conditions). S1-resistant DNA was fractionated on alkaline 1.5% agarose gels, blotted, and hybridized to an *hsp70* probe as in Figure 3. Autoradiographic exposure was 15 h with an intensifying screen. **Lanes 1** and **5**: heat-shock RNA from Kc cells; **lanes 2** and **3**: heat-shock RNA from COS cells transfected by pSVO-H8 and pSVO-E3 *Xho*, respectively; **lane 4**: *E. coli* RNA; **lane 6**: RNA from pSVO-H8 transfected COS cells heat-shocked 1 h at 43°C and further incubated at 37°C for 1 h; **lane 7**: same as **6**, but no further incubation at 37°C; **lane 8**: same as **6**, but no shock; **lanes 9** and **10**: RNA from heat-shocked and control COS cells, respectively; **lanes 1–3** and **4–10** are from two independent experiments. The asterisk indicates the protected DNA of 2.4 kb. **(B)** High-resolution mapping of the 5′ end of *Drosophila hsp70* RNA from COS cells. This analysis was performed according to Weaver and Weissmann (1979). The 82-bp *Nru*I fragment of pSVO-H8 was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. This fragment was hybridized with total RNA from COS cells isolated 48 h after transfection with pSVO-H8, incubated with S1 nuclease and electrophoresed on a 20% acrylamide/urea gel. End-labeled DNA fragments of 46, 40, 35, and 25 bp were run on the same gel as markers. **Lane 1**: 10 μg RNA from control cells (no shock); **lanes 2–5**: 2 μg (in **2**, **3**, and **4**) and 10 μg (in **5**) of heat-shock RNA. **Lane 3**: RNA from cells transfected by pSVO-H8Δ5, a deletion mutant described in Figure 6. The RNA in **lanes 2** and **4** was from two independent transfections.

normally repressed and inducible in COS cells transfected 48 h in the presence of 10 μM 5-azacytidine (data not shown), a cytidine analog that is incorporated into DNA and RNA but cannot be methylated (Cihak, 1974).

Drosophila hsp70 genes are faithfully and efficiently transcribed in COS cells

The accuracy of transcription was investigated in two ways by S1 nuclease protection experiments (Berk and Sharp, 1977). Firstly, we looked for full size DNA protected by hybridization to *hsp70* transcripts (*Drosophila hsp70* genes have no introns). As seen in Figure 4A, *Drosophila* heat-shock RNA protects a major 2.4-kb DNA fragment of pSVO-H8, plus several smaller fragments. An equivalent single fragment of 2.4 ± 0.05 kb was protected by heat-shock RNA from pSVO-H8 transfected COS cells (the origin of some additional faint bands, not reproducible in different digestions, is unknown). The full transcript of an *hsp70* gene from chromosomal site 87A is expected to be 2390 nucleotides long (Török and Karch, 1980; Ingolia *et al.*, 1980; Karch *et al.*, 1981; Török, personal communication). Little, if any, protection was detected with RNA from COS cells transfected but not shocked, or from untransfected cells. These results confirm the Northern data. The smaller fragments of pSVO-H8 protected by *Drosophila* heat-shock RNA were also generated by Sl when pSVO-H8 DNA was hybridized to heat-shock RNA from COS cells transfected with pSVO-E3ΔXho, which contains the other gene variant (Figure 4A, lanes 1 and 3). A similar digestion pattern was observed in converse experiments in which pSVO-E3ΔXho DNA was hybridized to heat-shock RNA from pSVO-H8 transfected COS cells (data not shown). These fragments probably originate from mis-matched hybrids formed between transcripts of one variant gene with the DNA of the other variant. Indeed, significant regions of sequence divergence are found in the 5′ untranslated leader of the two variants and the 3′ ends are totally divergent (Karch *et al.*, 1981). Thus, these structural differences were used to distinguish between the transcripts of both *hsp70* variants in co-transfected cells by S1 mapping (see below).

Secondly, the 5′ ends of the *Drosophila* transcripts made in COS cells were examined in more detail using the procedure of Weaver and Weissmann (1979). For this, an 82-bp *Nru*I fragment spanning the cap site of the *hsp70* gene in pSVO-H8 was ^{32}P-labeled at its 5′ end, hybridized to heat-shock RNA from pSVO-H8 transfected COS cells, digested by S1 nuclease, and analysed on sequencing gels. The autoradiograph shown in Figure 4B reveals three to four prominent fragments one nucleotide apart, with some background varying from experiment to experiment. The same four bands were found protected by *Drosophila* heat-shock RNA (data not shown). The strongest band migrated as a fragment 33 bp long. Since the start of *hsp70* transcription in the clone 56H8 is located 32 ± 1 nucleotides upstream from the cleavage site used to label our probe (Török and Karch, 1980), we conclude that a significant fraction of the *hsp70* RNA made in

COS cells on *Drosophila* templates has 5′ ends indistinguishable from those of authentic *Drosophila hsp70* RNA.

The *hsp70* transcription efficiency in *Drosophila* and COS cells was compared on a per gene basis. The amount of *Drosophila hsp70* RNA made in pSVO-H8 transfected COS cells during 1 h at 37°C following a heat-shock at 43°C was directly compared to that made in Kc cells during the same time at 37°C (heat-shock). The relative transcription efficiency in COS cells was measured in several independent experiments (see Materials and methods). Typically, 24 h after transfection, an *hsp70* gene carried by a pSVO-H8 episome in a COS cell yielded 15–30% of the average amount of RNA made by a single *hsp70* gene in *Drosophila* Kc cells. However, this figure dropped to 2.5–5% 48 h after transfection, at a time when the number of plasmid copies had increased several fold with no corresponding increase in induced RNA. If saturation was reached before 24 h with 20–40 x 10^3 gene copies per cell, then a 15–30% RNA yield could well be a minimum estimate. In any event, the data indicate that the RNA yields of *Drosophila hsp70* genes induced in COS cells are relatively high. Intracellular plasmid DNA could not be accurately measured in cells with a low gene copy number; the supercoiled DNA signal was eclipsed by residual extracellular DNA, hence preventing an estimation of the maximal transcription efficiency. Thus, at high gene copy number either a significant fraction of the genes was activated with an efficiency similar to that in *Drosophila*, or every gene was activated and transcribed with reduced efficiency.

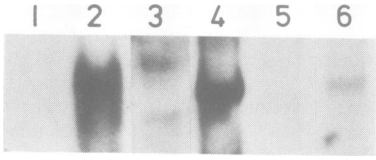

Fig. 5. Transcription of *hsp70* deletion mutants. RNA (2 μg) of control (no shock, **lanes 1, 3, 5**) and heat-shocked COS cells (**lanes 2, 4, 6**) isolated 48 h after transfection by pSVO-H8 (**lanes 1** and **2**), pSVO-H8 5 (**lanes 3** and **4**) and pSVO-E3ΔNru (**lanes 5** and **6**), was analysed as described in Figure 3. The description of the recombinants pSVO-H8Δ5 and pSVO-E3ΔNru is given in Figure 6.

Following heat-shock or arsenite induction, *Drosophila hsp70* RNA does not seem to have a high turnover in COS cells. At the most, 20–30% of this RNA disappeared in 1 h at 37°C in the presence of actinomycin D (Figure 3). In cells that were not shocked, we found no evidence for nascent synthesis and therefore turnover of *hsp70* RNA, using S1 assays to detect small RNA fragments. Since virtually no *hsp70* RNA was detected in these cells (Figure 3 and 4) we conclude that the *hsp70* genes are transcribed very inefficiently under normal conditions. Thus, the accumulation of *hsp70* RNA induced by heat-shock appears to involve an activation of the transcription rates by two orders of magnitude. We presume that the rate-controlling step is initiation of transcription.

DNA sequences required for hsp70 induction

Deletion mutants were generated *in vitro* and assayed for *hsp70* transcription inducibility in the COS cell system. Similar yields of *hsp70* RNA were obtained with the three vectors shown in Figure 1, irrespective of which *hsp70* variant was assayed and whether *Drosophila* DNA sequences upstream from position −186 (*Xho*I site; +1 being the start of transcription) were replaced by plasmid sequences in pSVO-H8ΔXho (Mirault *et al.*, 1982). Thus, the first, distal half of the 350-bp element Z_{nc} which is conserved adjacent to all copies of the *hsp70* gene in *D. melanogaster* (Artavanis-Tsakonas *et al.*, 1979; Mirault *et al.*, 1979; Ish-Horowicz *et al.*, 1979) does not appear to be required for induction, either

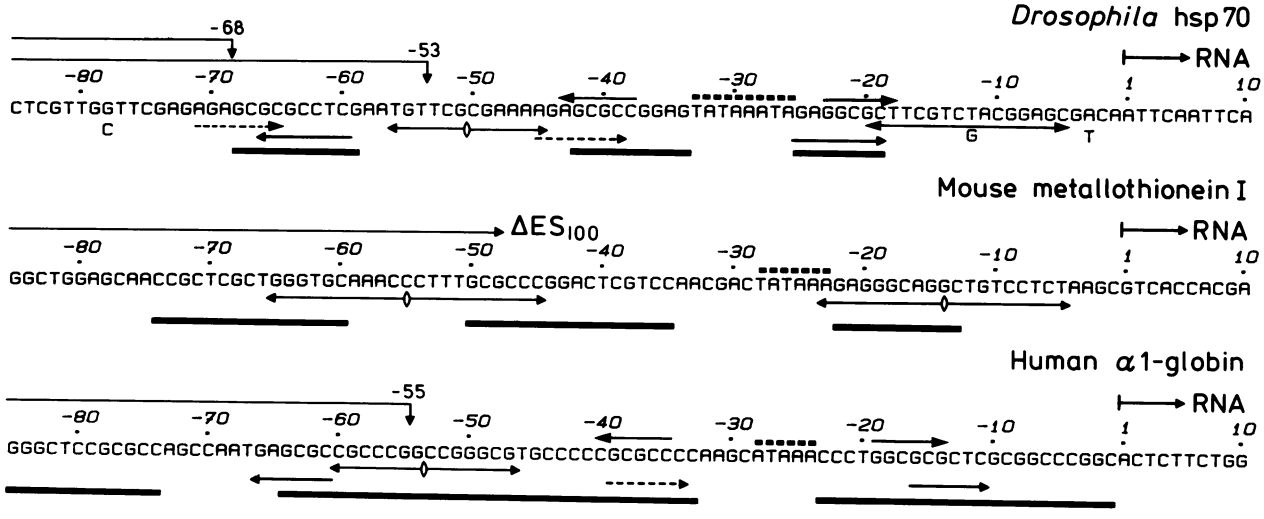

Fig. 6. 5′ Deletion mutants affecting the transcription of *Drosophila hsp70*, mouse metallothionein I, and human α-globin genes. The *hsp70* sequence is from an 87C1 variant; the three base substitutions observed in an 87A7 variant (Karch *et al.*, 1981) are shown below. The arrows from the left above the sequence indicate critical deletion mutants. The transcription properties of the mouse MT-I and human α1-globin deletion mutants have been published by Brinster *et al.* (1982) and Mellon *et al.* (1981), respectively. The *hsp70* [−68]5′ deletion mutant (pSVO-H8Δ5) was generated by *Bal*31 nucleolytic trimming of *Xho*I-cut pSVO-H8ΔXho and religation. The DNA sequence on each side of the ligation point was determined by the method of Maxam and Gilbert (1977). The replacement sequence upstream of −68 is: ...CAGGCGGTAACCGGCCTCTTCATCGGGAAT(−68). The *hsp70* [−53]5′ deletion mutant (pSVO-E3ΔNru) was obtained by removing a *Nru*I restriction fragment of ~1 kb in pSVO-E3ΔXho; a single *Nru*I site was reconstituted by ligation in pSVO-E3ΔNru at position −53 to −48. The replacement sequence upstream of this recognition site is: ...TCATAATGGGGAAGGCCATCCAGCCTCGCG(−53), as in pBR322. The TATA boxes are indicated by dotted lines, GC-rich stretches (≥7 bp, ≥85%G+C) by black bars. The arrows show direct or inverted repeats (perfect or not). The pattern but not the sequence of some of them is conserved in front of different genes, e.g., *hsp*70, −66 to −59/−25 to −18, *hsp*68, −68 to −61/−16 to −9, (Holmgren *et al.*, 1981), human αI-globin gene, −66 to −60/−39 to −33/−16 to −10. A 6-bp repeat is also found inverted on each side of the TATA box of *hsp70* (−43 to −37 and −23 to −17) and of the αI-globin gene (−40 to −34 and −19 to −13).

by heat-shock or arsenite. DNA was then progressively digested by *Bal*31 nuclease from the *Xho*I site of pSVO-H8ΔXho and pSVO-E3ΔXho to yield a series of 5′ deletion clones. A number of these were selected and assayed for *hsp70* transcription in COS cells. The deletion point of a critical clone was eventually determined by direct DNA sequence analysis. All recombinants with deletions upstream to position −68 (Figure 6) showed essentially normal inducibility. Transcription induction with pSVO-H8Δ5 (a −68 deletion) was nevertheless slightly lower than with pSVO-H8 or pSVO-H8ΔXho (Figure 5), and even lower in arsenite treated cells (data not shown). In contrast, deletions extending to position −53 (Figure 6) and beyond were found to reduce drastically the inducibility of transcription (Figure 5). Trace amounts of *Drosophila hsp70* RNA were nevertheless detected with pSVO-E3ΔNru following heat-shock. In control experiments using S1 mapping to distinguish between the transcripts of two *hsp70* variants in co-transfected cells (Figure 4A), we found that the unmodified gene of pSVO-H8 was strongly induced by heat-shock whereas the −53 deletion mutant was not and that both plasmids had replicated to the same extent in these cells (data not shown). Thus, we conclude that heat-shock induction of the *D. melanogaster hsp70* gene involves an important sequence element, the upstream boundary of which is located between position −68 and −53 (Figure 6).

Discussion

Drosophila hsp70 genes are heat inducible when introduced into COS or CV-1 monkey cells by means of a vector derived from SV40. A rise in temperature from 37°C to 43°C was sufficient to bring about the abundant synthesis of *Drosophila hsp70* RNA in these foreign cells. In contrast, no significant amount of this RNA was detected at 37°C, even though this temperature is optimal for *hsp70* induction in *Drosophila*.

Similar heat inducibility of *hsp70* genes from *Drosophila* has been observed in several other heterologous systems. Transformation of mouse cells by *D. melanogaster* heat-shock genes was first reported by Corces *et al.* (1981). Heat-shock of one of several transformants obtained by these authors, induced the synthesis of *Drosophila hsp70* RNA. Similar results were obtained recently with transformed rat cells (Burke and Ish-Horowicz, 1982). Heat-induced transcription of *Drosophila hsp70* genes injected into *Xenopus* oocytes has also been reported (Voellmy and Rungger, 1982; Pelham and Bienz, 1982). All these observations imply that the mechanism of induction, which is part of the response to stress, has been conserved throughout evolution. We show that not only heat, but also arsenite induced *hsp70* genes in COS cells, as was observed with the cells of *Drosophila*. Moreover, induction by either treatment did not require *de novo* protein synthesis (Figure 3). Thus, if heat-shock, arsenite, or amino acid analogues (Kelley and Schlesinger, 1978) have a common target involved in the repression or induction of the gene, it is likely to be a specific protein normally present in the cell. The function of this hypothetical protein, structural or catalytical, would appear to be conserved in quite divergent species. A negative control of the *hsp70* genes by means of a structural repressor seems unlikely since a tight repression of *hsp70* transcription was still observed in COS cells containing a large number of gene copies (100 000 at least). If an enzyme was required to maintain repression, its alteration could result in transcription induction. A positive activation might involve the chemical modification of a chromatin component or of an RNA polymerase factor. The observation that the *Drosophila hsp70* genes are efficiently activated within cells as distantly related as monkey, and at high copy number, favours the hypothesis of a common chemical modification being involved in induction. If a specific protein were to be modified so that it could interact with a particular DNA sequence in the heat-shock promoter, then such a DNA binding protein would have to be sufficiently abundant and conserved in *Drosophila* and African Green monkey. Whether a negative or positive control is operative on heat-shock genes, an essential signal in the induction mechanism is expected to reside in the DNA structure at or around the gene promoter itself.

A sequence of only 68 bp upstream from the start of *hsp70* transcription was found to be sufficient to preserve normal inducibility of an intact *hsp70* gene in COS cells. In contrast, the further removal of 15 bp drastically impaired transcription capacity, although a low level of *hsp70* RNA was still detected in heat-shocked cells (Figures 5 and 6). Essentially the same results were obtained independently by Pelham (1982). In addition, this author also found that the sequences essential for the induction of *hsp70* are confined from −66 to −10 since replacement of *hsp70* by the herpes thymidine kinase gene downstream of position −10 did confer heat-inducibility to this gene. As shown in Figure 6, a short imperfect dyad element (Ingolia *et al.*, 1980; Holmgren *et al.*, 1981) centered about two helical turns upstream of the TATA box is flanked on both sides by short GC-rich blocks. The 5′ deletion that impairs *hsp70* induction (−53) removes the distal block and truncates part of the central AT-rich inverted repeat. Imperfect dyad elements are also found in front of other *Drosophila* heat-shock genes, namely *hsp84, 68, 27, 26,* and *22* (Holmgren *et al.*, 1981; Ingolia and Craig, 1981; R.Southgate, in preparation). In comparing all these dyads, Pelham (1982) has deduced an apparent consensus sequence which overlaps the main palindrome. Site-directed mutagenesis in this region, eventually with different heat-shock genes, will be needed to find the significance of such dyads.

The presence of palindromic sequences in front of the TATA box is not unique to heat-shock genes. As shown in Figure 6, the mouse metallothionein I and the human αI-globin genes, for example, both have a dyad element centered about three helical turns upstream from the TATA box. Interestingly, as for *Drosophila hsp70*, 5′ deletions that truncate these dyads drastically reduce their transcription capacity: heavy metal inducibility of the metallothionein I gene is lost in mouse eggs (Brinster *et al.*, 1982) whilst constitutive expression of the αI-globin in COS cells is curtailed (Mellon *et al.*, 1981). In addition to these dyad elements, we note several repeats or pseudo-repeats whose pattern, but not their sequence, is conserved in front of several genes (Figure 6). For example *hsp*70 of *Drosophila* and the mouse metallothionein I gene both have a large imperfect palindrome between the TATA box and the start of transcription. The significance of these observations is so far unknown. It is of interest that the expression of the herpes thymidine kinase gene injected into *Xenopus* oocytes was reduced 10- to 20-fold when either element of a 6-bp GC-rich inverted repeat (−103 to −97/−55 to −49) was disrupted by site-directed mutagenesis (McKnight and Kingsbury, 1982). Inverted repeats or dyad elements are obviously not specific to heat-shock genes. They

may, however, play an essential role in transcription promotion. They could, for example, facilitate dynamic changes in chromatin conformation and strand separation at the polymerase entry site during transcription initiation. In this respect, the AT-rich DNA that is found to overlap the main dyad elements of *Drosophila* heat-shock genes might further contribute to destabilize the entry site.

Comparative studies of *hsp70* and metallothionein gene induction should be particularly relevant since both genes are inducible by sulfhydryl-binding metals (Levinson *et al.*, 1979) but only *hsp70* by heat. In addition, the mouse metallothionein I gene also retains its inducibility when introduced into human (Mayo *et al.*, 1982) or monkey cells by means of SV40 recombinants (Hamer and Walling, 1982). An understanding of the mechanism of induction of these genes will probably require knowledge of the spatial conformation of the DNA in the promotor region in both the repressed and induced chromatin. In particular, we wonder whether induction of heat-shock genes could be brought about by a local transition in DNA conformation (reviewed by Dickerson *et al.*, 1982) at the promoter site.

Finally, *hsp70* transcription studies should be carried out in homologous cells. Long range regulation by sequences remote from the transcription start in *hsp70* may well have been overlooked in COS cells. The reduced inducibility of transcription observed here with the −68 5′ deletion mutant already suggests that sequences upstream in Z_{nc} may contribute to *hsp70* transcription, once it is induced. Furthermore, if *hsp70* genes are feedback regulated as suggested by Lindquist *et al.* (1982), such regulation might involve other specific DNA sequences. Furthermore, the heat-shock genes of *Drosophila* are not expressed only under stress conditions but also at specific developmental stages (Sirotkin and Davidson, 1982; P. Mason, personal communication). Regulation of transcription in this case is likely to involve some different mechanisms.

Materials and methods

Cell transfection

CV-1 and COS-7 cells were propagated as described by Gluzman (1981). Subconfluent cells were transfected with plasmid DNA according to Sompayrac and Danna (1981) at 1 μg/ml DNA and 200 μg/ml DEAE-dextran in Tris-buffered saline (TBS) for 8–15 h at 30°C, followed by a 3 min incubation in TBS-20% glycerol. The cells were rinsed with TBS and incubated in complete medium with 10% foetal serum, at 37°C. The cells were washed 3 x with ice cold isotonic Tris saline prior to lysis and nucleic acid isolation.

Nucleic acids isolation and analysis

Subcloning, plasmid DNA preparation, restriction enzyme analysis, agarose gel electrophoresis, and ^{32}P-labeling of DNA by nick translation have been described previously (Moran *et al.*, 1979). Total nucleic acids from *Drosophila* or monkey cells were prepared by lysis and digestion in the presence of proteinase K and SDS, followed by phenol/chloroform and ether extraction (Favarolo *et al.*, 1980). DNA was selectively precipitated by gentle mixing with 2 volumes of absolute ethanol at room temperature and the resulting DNA clot was immediately centrifuged down at 2000 *g* for 3 min. RNA was precipitated from the supernatant by addition of 1/20 volume NaCl 5 M, left for at least 2 h at −20°C and centrifuged at 10 000 *g* for 15 min. The RNA pellet (from one confluent 10-cm Petri dish) was dissolved in 0.2 ml TES [10 mM Tris pH 7.4, 1 mM EDTA and 0.1% SDS] and transferred into a 1.5 ml Eppendorf tube; following addition of 0.09 ml n-propanol and 0.3 ml NaCl 4 M while vortexing, the RNA was left overnight in ice for precipitation (contaminating DNA remains soluble). After a 10 min centrifugation at 13 000 *g* the RNA was dissolved in TES and stored at −20°C. The DNA pellet was dissolved in TE and reprecipitated twice with ethanol as above to get rid of RNA contaminations.

Nucleic acid blots and hybridization

DNA was blotted from agarose gels onto nitrocellulose (Scheicher and Schüll BA85, 0.45 μm) according to Southern (1975) as modified by Wahl *et al.* (1979): partial acid depurination and hybridization with dextran sulfate was performed overnight at 43°C. The final wash of the filter was in 0.1 x SSCP at 50°C for 15 min. RNA was denatured in 2.2 M formaldehyde for 15 min at 50°C (Lehrach *et al.*, 1977) and electrophoresed on 1.2% agarose gels containing 10 mM sodium phosphate pH 6.8, 5 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde, without formaldehyde in the running buffer. The gels were then equilibrated in 20 x SSC pH 6.8 (2 x 45 min, one buffer change) transferred directly onto nitrocellulose, and hybridization was performed as described by Thomas (1980) in 50% 3 x recrystallised formamide (BDH, AnalR), 5 x SSC, 40 mM Pipes pH 6.4, 1 M EDTA, and 5–10% dextran sulfate, overnight at 43°C. The final washes were in 0.1 x SSC, 0.1% SDS, 2 x 15 min at 50°C.

The relative transcription efficiency of *Drosophila hsp70* genes in COS and *D. melanogaster* KC cells was estimated as follows: RNA from both sources was titrated so as to achieve comparable *hsp70* hybridization signals on RNA blots; the mass of total RNA loaded on the gel was converted into the cellular equivalent mass of DNA; the mass ratio of *Drosophila hsp70* genes present in total COS DNA was estimated from plasmid titrations (Figure 2); the number of *hsp70* genes in the diploid Kc cells being six per haploid genome of 165 000 kb (Mirault *et al.*, 1979), the mass ratio of 2.4-kb long *hsp70* genes to Kc DNA should be 8.73×10^{-5}; transcription efficiency is inversely proportional to these DNA mass ratios. Thus, for equivalent amounts of *hsp70* RNA detected by hybridization, the relative transcription efficiency in COS cells was taken as the *hsp70* mass ratio in the *Drosophila* Kc cells divided by that measured in transfected COS cells. This estimation, based on nucleic acid contents rather than cell numbers, is independent of transfection efficiencies which are usually unknown.

Acknowledgements

We thank Chia-Hwa Chen-Tonka for excellent technical assistance, O.Jeni for drawings and plates, Y.Gluzman for providing the COS-7 cell line, M.Botchan for plasmid pML2-RIIG, H.Türler for SV40 DNA, and P.Spierer for critical reading of the manuscript. We are particularly grateful to Alfred Tissières for his continued interest and encouragement in our work. This work was supported by the Swiss National Science Foundation (grant No. 3.079.81 to A.Tissières) and the E. and L. Schmidheiny Foundation.

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