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A germline transformation analysis reveals flexibility in the organization of heat shock consensus elements

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ABSTRACT

Maximal expression of the Drosophila heat shock gene hsp70 can be activated by a pair of heat shock consensus elements (HSE's) positioned close to the transcription start site. In contrast, required HSE's of other heat shock genes (i.e., hsp26, 27, 23) are located several hundred base pairs (bp) farther upstream of their start sites. Using germline transformation, we analyzed the requirements for HSE organization in the hsp70 and hsp26 regulatory regions. A 51 bp fragment containing the two proximal hsp70 HSE's was sufficient to rescue the heat shock response of an hsp26-lacZ gene devoid of its HSE's. Heat inducibility was restored with either orientation of the fragment relative to the hsp26 transcription start. In hsp70 gene constructions, relocation of hsp70 HSE's to more remote positions by inserting 127 or 331 bp into the regulatory region failed to substantially reduce expression. Thus, in contrast to their native configurations, the hsp26 promoter can be activated by HSE's solely in a proximal position and the hsp70 promoter can be activated by remote HSE's. In addition, a simple and sensitive assay for quantitative measurement of ß-galactosidase activity in crude fly extracts is described.

INTRODUCTION

Analysis of the regulatory regions of eukaryotic genes indicates that sequence elements that modulate transcription are often present in multiple copies (1-6). For example, sequence motifs involved in metallothionein regulation in mammals (3) or galactose regulation in yeast (6) are reiterated within the upstream regulatory regions. In some cases, it has been established that this multiplicity of elements has functional consequence since optimal expression requires two or more copies of the regulatory sequence (3,5-11). Although it is clear that many cis-acting sequence elements exert their effects by binding specifically to protein factors (6,12-15), the mechanism by which this influences expression is unknown. Similarly, the molecular basis of a requirement for multiple regulatory elements remains to be elucidated.

The heat shock genes of the fruit fly, Drosophila melanogaster, constitute an attractive model system for the study of reiterated regulatory elements. The transcriptional activation of these genes in response to environmental stress requires a cis-acting sequence element, the heat shock consensus element (16) (HSE,

consensus = CTNGAANNTTCNAG). Different members of the heat shock gene family possess from three to six copies of the HSE in their 5'-nontranscribed regions (see Fig.1). A protein factor that binds specifically to HSE's has been identified and partially purified (29). This heat shock transcription factor (HSTF) enhances the transcription of the heat shock gene hsp70 in vitro. A similar or identical factor has been shown to bind to HSE's of the heat shock gene hsp82 in chromatin (30). These in vitro experiments as well as in vivo competition experiments using an HSE polymer (31) indicate that HSE's mediate heat shock gene activation in a positive manner. These studies also demonstrate that the coordinate regulation of different members of the heat shock gene family is mediated through their HSE's.

Deletion analyses of hsp70 using Drosophila germline transformation (18,19) or transfection into Drosophila tissue culture cells (20) have shown that the region containing the two proximal copies (HSE1 and HSE2) is sufficient for optimal expression. Fifty to one hundred-fold less heat-inducible expression is observed from a 5' deletion that leaves only HSE1 intact (18). Binding studies with the hsp70 HSE's have shown that HSE1 has a 12.5-fold higher intrinsic affinity for HSTF than does HSE2 and binding to HSE1 facilitates binding to the adjacent HSE2 in a cooperative fashion (17) . In addition, these studies identified HSE3 and HSE4 as high affinity binding sites for HSTF. Although a two-fold enhancement of in vitro transcription was observed due to HSE3 and HSE4, the in vivo mutational analyses failed to detect a contribution to expression from these sites (18-20). Thus, although hsp70 possesses HSE's in relatively remote positions, the two proximal HSE's appear sufficient for optimal levels of expression.

A contrasting picture of the organization of required HSE's has emerged from mutational analysis of the small heat shock genes: hsp's 22, 23, 26 and 27. Hsp26 and hsp22 possess a pair of HSE's close to the transcription start site in positions similar to those of hsp70 (see Fig. 1). Mutational analyses demonstrate that HSE1 of hsp26 plays an important role in heat shock regulation (23) and suggest a role for both HSE1 and HSE2 of hsp22 (21). Unlike hsp70, however, 5' deletion analysis suggests that additional upstream sequence elements, presumably HSE's, are required for optimal heat-induced expression. Removal of HSE3 of hsp22 results in a 5-fold reduction (21) and a 25 to 50-fold reduction is seen in constructions that lack HSE's 4, 5 and 6 of hsp26 (24). As shown below (see Results), the bulk of this hsp26 reduction is most likely due to the loss of HSE6 at -350. Hsp23 and hsp27 lack HSE's in the proximal position (within 100 bp of the transcription start) and mutational analyses implicate important roles for HSE's positioned as far as 350-400 bp upstream from the start sites of these genes (22,23,25,26). In support of the mutational analyses of the small heat shock genes, heat shock-dependent changes in chromatin structure have

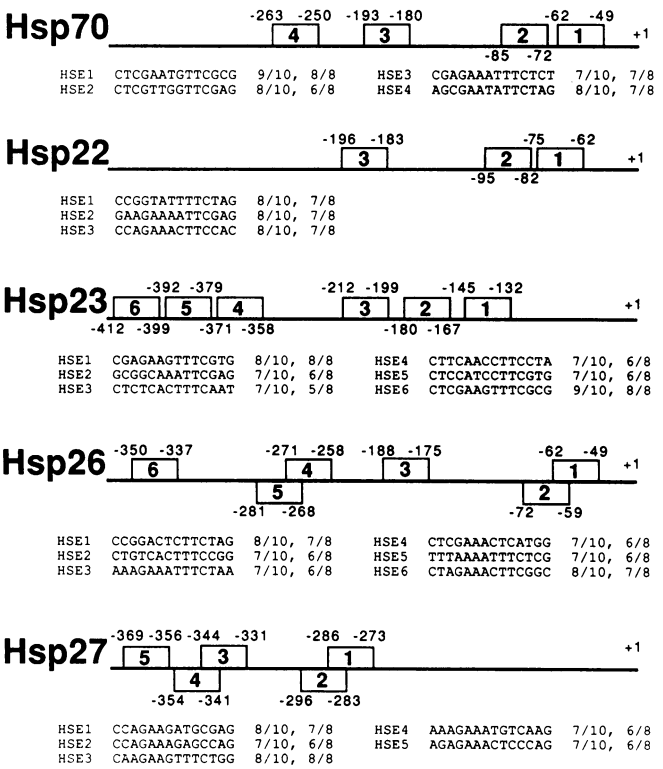


Figure 1. Organization of Heat Shock Consensus Elements in the Upstream Regions of Heat Shock Genes

The regions immediately upstream of the five heat shock genes that have been mutationally analyzed are shown. Numbered boxes represent identified matches to the heat shock consensus sequence (CTNGAANNTTCNAG) at 7 or more of the 10 specified positions. The positions of these HSE's relative to transcription start sites are indicated. The maps are not drawn precisely to scale. The nucleotide sequences of the HSE's are shown below the map for each gene. The match to the conserved 10 bp of the HSE sequence (16) is indicated as well as the match to an abbreviated 8 bp version (CNNGAANNTTCNNG) derived from HSTF binding studies (17). The figure was compiled from previous mutational analyses (17-26) with HSE's identified by these workers shown. The nucleotide sequences of the hsp70 HSE's are those for the first hsp70 copy in the clone 132E3 (27,28). The nucleotide sequence of HSE4 shown here deviates from that reported (17), reflecting sequence differences between these distinct copies of hsp70 (28).

been observed in the regions harboring remote HSE's (32). Thus, in contrast to hsp70, at least some of the HSE's critical for small heat shock gene expression are located in remote positions.

This difference in positioning of critical HSE's could be due to specialization of the

promoter regions (TATA box and start site) of distinct heat shock genes. That is, the hsp70 promoter region, by virtue of sequence or bound protein factors, may be specially adapted for interaction with HSE's positioned proximally whereas the small heat shock gene promoters may be adapted for both proximal and distal interactions. This model predicts that activation of the hsp70 promoter could not occur if critical HSE's were located in remote positions. Alternatively, the small heat shock gene requirement for distally positioned HSE's may simply reflect the positions of particularly high affinity HSE's. We note that distal HSE3 of hsp22, HSE6 of hsp26, HSE6 of hsp23 and HSE3 of hsp27 possess 7/8 or 8/8 matches to the consensus sequence. This argument would not explain why HSE3 and HSE4 of hsp70 are dispensable, however, since these are known to bind HSTF with high affinity (17).

Here we analyze requirements for the arrangement of HSE's using the heat shock genes hsp70 and hsp26. Quantitative analysis of expression in germline transformants demonstrates considerable flexibility in HSE organization.

MATERIALS AND METHODS

Plasmid constructions

The parental hsp26 transformation vector, cP26Z (illustrated in Fig. 2a), contains about 2 kb of hsp26 upstream sequence. cP26Z and cP26Z-Δ278 have been described previously (36). The cP26Z-Δ278 deletion endpoint breaks within HSE5 and recreates the consensus match by changing TTTAAAATTTCTCG to GTTAAAATTTCTCG. cP26ZΔX was constructed by deleting the XbaI fragment containing sequences between -350 and -52 in cP26Z (R. Glaser, unpublished.) cP26Z-70a and cP26Z-70b were constructed as follows. The 51 bp XhoI-SalI fragment from pXM1 (31) that contains HSE1 and HSE2 of hsp70 was inserted, in both orientations, into the XbaI site at the -350/-52 deletion junction of cP26ZΔX after filling in the sticky ends with Klenow polymerase. The "a" orientation is the same as in the wild-type hsp70 configuration.

The parental hsp70 transformation vector, c70Z (see Fig.2a), possesses wild-type hsp70 sequence from -194 to the lacZ fusion point at +260 and has the hsp70 poly (A)$^+$ signal region positioned downstream of lacZ. c70Z contains a novel version (H. Xiao and J.T.L., in preparation) of the fusion gene described by us previously (34) and it is expressed several-fold more efficiently in transformants. This hsp70-lacZ fusion gene yields a predicted fusion protein that contains only the first seven amino acids of hsp70. All remaining hsp70-lacZ transformation vectors contain the same fusion gene and vector background as c70Z except for mutations in the 5' flanking regions as follows. cBNΔHSE1 contains a 13 bp deletion from -63 to -51 generated by cleaving at BssHII (-67) and NruI (-50) sites, filling in the BssHII sticky end and religating. cBs-13Z contains a 13 bp deletion from -70 to -58 generated by cleaving at the BssHII site, overdigesting with S1 nuclease and religating. cBs+10Z contains a 10 bp insertion generated by cleaving the BssHII site, filling in the sticky end and inserting a 6 bp HpaI linker (GTTAAC). cBs+127Z and cBs+331Z contain a 117 bp ScaI-XmnI

pBR322 fragment or a 321 bp HincII-NruI pBr322 fragment, respectively, inserted into the HpaI site of cBs+10Z. A computer search demonstrated the absence of sequence elements with a 7/10 or better match to the heat shock consensus sequence within these pBR322 fragments. The DNA sequence in the mutated regions of cBNΔHSE1, cBs-13Z and cBs+10z was determined using the chemical (37) or dideoxy chain termination (38) methods.

P element-mediated transformation and establishment of transformed lines

Drosophila transformation was performed as described (19) using the rosy^{+} gene as a transformation marker and the Adh ^{fn6} cn; ry^{502} (ACR) strain as the injection strain. Transformants were made homozygous for their inserts as described (19) using a CyO; TM2 (Ubx, ry^{-})/ T(2;3) Ap^{Xa} balancer stock. The balancer crosses were also used to distinguish X-linked from autosomal insertions. Lethal insertions on chromosomes 2 or 3 were maintained over the CyO or TM2 balancer chromosomes. Since several transformants were available for each hsp70-lacZ construction, individual transformants that gave expression results deviant from others with the same construction, presumably due to chromosomal position, could be identified. Thus, transformants whose heat shock expression deviated by greater than two-fold from the average of the remaining transformants of that class were eliminated from the quantitative analysis. Of 29 hsp70-lacZ transformants, 4 failed to satisfy this condition. Two of the four were balanced lethals, one was a visibly unhealthy line and all four deviated to give anomalously low expression values. Since smaller numbers of hsp26-lacZ containing transformants were analyzed, all such transformants were included in the quantitative analysis.

ß-Galactosidase assays

Five adult females (hsp70 constructions) or males (hsp26 constructions) were heat shocked by immersing a glass vial containing the flies in a water bath at 29°C and then resetting the temperature for 36°C. The time of incubation was 2 hours from the time of immersion with the 36°C temperature attained in 20 min. Control flies were maintained at their normal growth temperature, 23°C. After heat shock, flies were etherized and transferred to 1.5 ml Eppendorf tubes. 100 µl of assay buffer (50 mM potassium phosphate, 1mM magnesium chloride, pH 7.5) was added and the flies were homogenized at room temperature by 20 strokes with a plastic pestle shaped to fit the tubes (Kontes Scientific). 900 µl of additional assay buffer was added and extracts were vortexed at medium speed for 30 seconds. Aliquots of this 1 ml extract were added to plastic cuvettes containing the substrate chlorophenol red-ß-D-galactopyranoside (CPRG, Boehringer Mannheim) in assay buffer. The final concentration of CPRG was 1 mM in a final reaction volume of 1 ml. Reactions were incubated at 37°C and OD at 574 nm was read at 0.5, 1 and 2 hours after extract addition. The rate of color development was constant throughout this time period. Control experiments with cP26Z and c70Z transformants indicated that color development varied linearly with the amount of extract used between 0.1 and 10 µl of heat shock extracts and between 10 and 50 µl of control extracts. For actual expression measurements, 50 µl of control extracts was used. 5 µl of heat shock extracts was used for hsp70-lacZ transformants and 10 or 20 µl was used for hsp26-lacZ transformants. ß-Galactosidase expression was measured in units of

OD units/hours reaction time/µl extract. ß-Galactosidase activity in these crude fly extracts was stable at 4°C for at least 5 hours. Background due to endogenous fly ß-galactosidase activity and light scattering material present in the crude extracts was measured by using extracts prepared in parallel from the nontransformed injection strain (ACR). In calculating ß-galactosidase levels in transformant extracts, this background was subtracted. For heat shock measurements of c70Z transformants, background was less than 2% of the signal. Expression values reported in Figures 3 and 4 are the average of assays repeated at least in triplicate except for cP26ZΔX transformants which were assayed in duplicate. The standard errors of the means of the triplicate determinations were less than 20% of mean expression levels for all heat shock samples that gave signals reproducibly above background.

RNA analysis

Adult females were heat shocked as described for ß-galactosidase assays except the incubation was for 1 hour from the time the water bath reached 36°C. RNA preparation was as described (19) except the homogenization buffer contained an equal volume of equilibrated phenol. RNA samples were electrophoresed through formaldehyde 0.8% agarose gels and transferred to nitrocellulose essentially as described (39) except 10 mM sodium phosphate pH 7.4, 2.2 M formaldehyde was used as electrophoresis buffer. Ethidium bromide staining prior to transfer revealed the presence of roughly equivalent amounts of RNA in all lanes.

RESULTS

Generation and processing of germline transformants

Germline transformation was employed to assay hsp70 or hsp26 genes with altered regulatory regions. The advantages of this transformation method over transient transfection assay systems include maintenance of the introduced segment in a defined and low copy number and residence of that segment in the chromosome rather than on a circular plasmid. In contrast to the relatively low inducibilities of transfected heat shock genes [3 to 20-fold (20,31,40)], magnitudes of heat induction closer to that of the endogenous genes can be obtained in germline transformants (18). Indeed, quantitative RNA analysis of a transformant possessing the c70Z construction demonstrated heat induction on the order of 200-fold (data not shown). A potential disadvantage of the germline transformation system is the tendency for expression to be quantitatively influenced by genomic position. The effect of chromosomal position was controlled by examining multiple (usually three or more) transformants for each construction.

Single-insert transformants were made homozygous for their inserts. A small number of lines were maintained as balanced lethals. Those transformant lines that behaved as true-breeding single-insert lines were analyzed by genomic Southern blots to verify copy numbers. An example of this analysis is shown in Figure 2b. Of the 40 transformant lines described here, 38 have defined copy numbers as determined

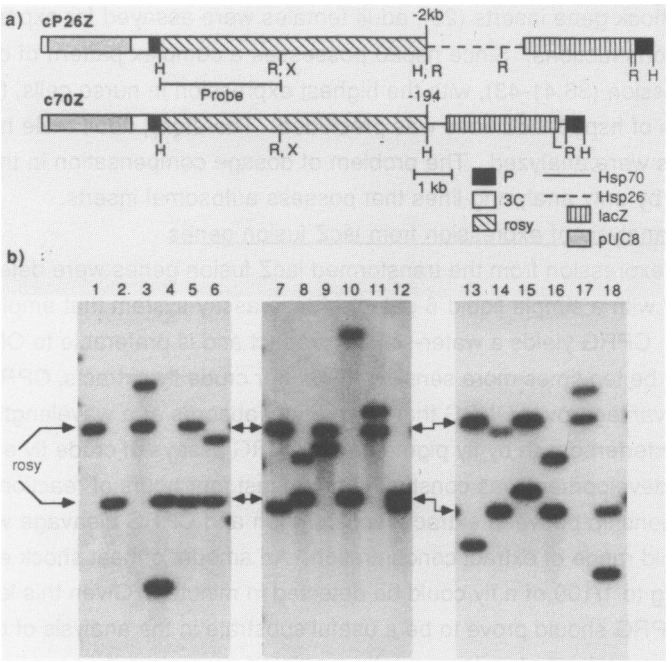


Figure 2. Southern Blot Analysis of DNA from Transformants

Maps of the parental hsp26 (cP26Z) and hsp70 (c70Z) constructions are shown in (a). H= HindIII, R= EcoRI and X=XhoI. Preparation of fly DNA and Southern blots (33) were performed as described (34). The hybridization probe used for the blots shown in (b) was a nick-translated (35) 3.2 kb SalI-XhoI fragment containing a 5' portion of the rosy gene. The extent of homology of this probe to the P element insertions is indicated in (a). Odd numbered lanes in (b) contain EcoRI-digested genomic DNA and even numbered lanes contain XhoI-digested genomic DNA. Lanes 1 and 2 contain DNA from the ACR injection strain. Each of these lanes shows hybridization to a single band derived from the endogenous rosy locus, 87D. The arrows indicate these species throughout the remaining lanes. All other pairs of lanes contain DNA from germline transformants with the number of hybridizing species per lane in addition to the endogenous rosy species indicative of insert copy number. All lanes contain DNA from homozygous single insert lines except lane pairs 7, 8 and 13, 14 which contain DNA from balanced lethal lines heterozygous for single inserts.

by Southern analysis or by in situ hybridization to polytene chromosomes. (The insert copy number of two transformants possessing the cP26ZΔX construction was not determined since these transformants failed to give heat-inducible expression above background [see below].) The insert copy number per haploid genome was one for 36 of these 38 transformants.

In order to avoid complications due to uncertainty about dosage compensation of X-

linked heat shock gene inserts (23), adult females were assayed for expression of hsp70-lacZ constructions. Since hsp26 possesses a complex pattern of developmental expression (36,41-43), with the highest expression in nurse cells, the use of adult females of hsp26-lacZ lines was precluded. Therefore, adult male hsp26-lacZ transformants were analyzed. The problem of dosage compensation in these males was avoided by only analyzing lines that possess autosomal inserts.

Quantitative analysis of expression from lacZ fusion genes

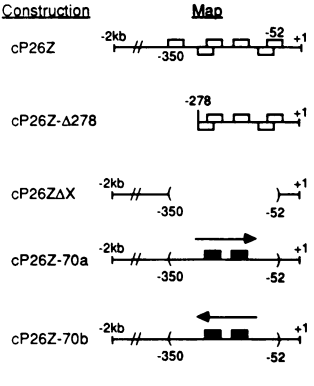
Levels of expression from the transformed lacZ fusion genes were determined quantitatively with a simple liquid ß-galactosidase assay system that employs CPRG as substrate. CPRG yields a water-soluble product and is preferable to ONPG since it is reported to be ten times more sensitive (44). For crude fly extracts, CPRG offers the additional advantage over ONPG that the product absorbs at a wavelength not significantly interfered with by fly pigments. In CPRG assays of crude fly extracts, the rate of color development was constant over at least four hours of reaction at 37°C and the relationship between extract concentration and CPRG cleavage was linear over a 100-fold range of extract concentration. An amount of heat shock extract corresponding to 1/100 of a fly could be detected in minutes. Given this level of sensitivity, CPRG should prove to be a useful substrate in the analysis of other lacZ fusion genes containing developmentally-regulated promoters by assaying extracts from whole animals or specific tissues.

In addition to ß-galactosidase assays, a number of transformant lines were examined quantitatively by RNA blots. These experiments showed that hsp70-lacZ mRNA accumulates in c70Z transformants to levels similar to that of endogenous hsp70 mRNA on a per gene basis (note probe homology differences in Figures 5,6 and see below). The good agreement between hsp70-lacZ mRNA levels and ß-galactosidase activity measurements (summarized in Figure 4) demonstrates that the enzyme activity measurement accurately reflects expression at the RNA level in these transformants.

Heat shock element organization of hsp26

The parental construction, cP26Z (Fig. 2a), contains approximately 2 kb of upstream sequence and contains six copies of the HSE (Fig.1). As has been shown previously (36), transformants harboring this construction produce ß-galactosidase activity in response to heat shock in all tissues. Three of these transformants were analyzed quantitatively for ß-galactosidase activity using the CPRG assay. The average heat induction of this activity was 90-fold. The average heat-induced level of expression from these cP26Z transformants was set to 100% and heat-induced expression from the remaining constructions is expressed as a relative percentage (Fig. 3).

The cP26Z-Δ278 construction removes all DNA upstream of HSE5. The deletion



Construction	Map	Transformant Name	Heat Shock Expression (% βgal Activity)	Average Heat Shock Expression (% βgal Activity)
cP26Z		26Z-36A	57	100
		26Z-49B	131	
		26Z-84D	112	
cP26Z-Δ278		Δ278-53F	4	3
		Δ278-65D	1	
		Δ278-78B	3	
cP26ZΔX		ΔX18	1	1
		ΔX54	1	
cP26Z-70a		X111a	65	40
		X124a	23	
		X125a	33	
		X129a	40	
cP26Z-70b		X220b	45	35
		X230b	36	
		X237b	24	

Figure 3. Heat Shock Expression in Hsp26-lacZ Transformants

All constructions have the plasmid background shown for cP26Z in Fig. 2a. The hsp26 upstream regions with depicted mutational changes are shown. Open boxes represent hsp26 HSE's as in Fig.1. Filled boxes represent HSE1 and HSE2 of hsp70. The arrows indicate the normal (cP26Z-70a) and reversed (cP26Z-70b) orientations of the hsp70 HSE's relative to the transcription start site. The maps are not drawn to scale. The average level of heat shock ß-galactosidase expression for the cP26Z transformants (.0481 OD units/hour/ul extract) was set to 100% and expression values per gene for individual transformants are expressed as a relative percentage. Transformants producing ß-galactosidase in response to heat shock at levels of 1% are not significantly above background in the CPRG assay system.

ends within HSE5 but only changes the first position of the consensus from one nonconserved nucleotide to another (see Methods.) Absolute heat-induced expression from the 26Z-Δ278 gene is reduced approximately 30-fold, but it is still several-fold inducible. This magnitude of reduction in expression is similar to that reported for a deletion to -236 (24) suggesting that, of distal HSE's 4, 5 and 6, HSE6 makes the greatest contribution to heat-induced expression. We note that HSE6 possesses the best match to the consensus sequence of these three HSE's (see Fig. 1.) Thus an HSE positioned as far as 350 bp upstream from the transcription start site appears to play an important role in heat activation of hsp26.

Sequences between -350 and -52 have been deleted in cP26ZΔX, thereby removing all six HSE's. A further reduction in expression is seen from this gene such that no reproducible heat shock induction above background in the CPRG assay system is observed. A similar result has been reported for the identical deletion in a different hsp26 fusion gene background (24).

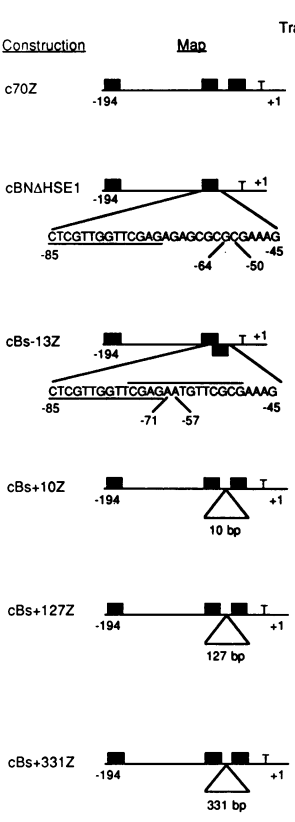
The cP26ZΔX gene then served as an uninducible gene from which cP26Z-70a and cP26Z-70b were built. These two constructions contain a 51 bp hsp70 fragment that

includes HSE1 and HSE2 inserted, in either orientation, into cP26ZΔX at the site where hsp26 HSE1 normally resides. The results in Figure 3 demonstrate that the insertion causes an approximately 35 to 40-fold increase in heat shock expression. Thus the hsp26 promoter can be activated by a pair of hsp70 HSE's solely in the proximal position. Although efficient heat induction of the wild-type hsp26 gene requires an HSE at -350, this result shows that an HSE in such a remote location is not an essential feature for induction. The result also suggests that no sequence elements critically required for heat induction exist in the region from -350 to -52 other than HSE's. Finally, binding studies have demonstrated that HSE1 of hsp70 is a 12.5-fold higher affinity binding site for HSTF than is HSE2 (17). Since similar expression levels are obtained with either insert orientation, some flexibility exists in the positioning of these high and low affinity HSE's relative to the transcription start site. Although heat-induced levels are substantially increased by the insertion, they are 2.5 to 3-fold lower than cP26Z levels. This effect may be due to alteration in the distance between the proximal HSE and the TATA box in these constructions relative to that for wild-type hsp26. Alternatively, quantitative contributions from several of six HSE's, or other sequence elements, may make the cP26Z heat shock promoter stronger than that created from the insertion of only two HSE's.

Heat shock element organization of hsp70

A map of the parental hsp70 construction, c70Z, is shown in Figure 2a. The fusion gene in c70Z contains hsp70 upstream sequence to -194 that includes HSE's 1, 2 and 3. RNA blot analysis of heat shock expression from c70Z in transformants is shown in Figures 5 and 6. The blot in Figure 5a was hybridized with a probe containing both hsp70 and lacZ sequences. Only the 2.4 kb hsp70 mRNA is detected in lane 2 which contains RNA from heat shocked nontransformants. In lane 4, RNA from a heat shocked c70Z transformant displays this hybridizing species as well as a higher molecular weight species that corresponds to hsp70-lacZ mRNA. When differences in probe homology and gene copy number are considered, the ratio of signal intensities between these species is consistent with similar levels of expression from the transformed hsp70-lacZ gene and the endogenous hsp70 genes. In an experiment of this type, using dilutions of a heat shock c70Z RNA sample, approximately 200-fold induction of hsp70-lacZ mRNA was observed. This value is in agreement with measurements of the endogenous hsp70 gene induction (45). ß-Galactosidase enzyme assays were performed on 3 c70Z transformants and their average level of heat shock expression was set to 100% for comparison with other constructions shown in Figure 4.

Deletion analyses strongly suggest a major role for HSE2 in heat shock regulation (18-20). Although biochemical studies (17,29) predict a role for HSE1 in vivo as well,



Construction	Map	Transformant Name	HS Expr. (% βgal)	Average HS Expr. (% βgal)	HS Expr. (% mRNA)	Average HS Expr. (% mRNA)
c70Z		Z205	115	100	ND	100
		Z217	73		84	
		Z243	112		116	
cBNΔHSE1		Z403	12	15	ND	14
		Z411	14		15	
		Z417	16		ND	
		Z424	9		12	
		Z425	24		ND	
cBs-13Z		Z810	123	123	84	78
		Z828	123		72	
cBs+10Z		Z614	146	98	ND	ND
		Z622	58		ND	
		Z626	92		ND	
		Z647	86		ND	
		Z657	107		ND	
cBs+127Z		Z717	43	53	67	68
		Z754	59		79	
		Z770	84		84	
		Z782a	38		62	
		Z782x	46		57	
		Z784	49		61	
cBs+331Z		Z121	76	73	84	78
		Z122	58		63	
		Z123	76		81	
		Z148	83		84	

Figure 4. Heat Shock Expression in Hsp70-lacZ Transformants

All constructions have the plasmid background shown for c70Z in Fig. 2a. The hsp70 upstream regions with depicted mutational changes are shown. Filled boxes represent HSE1, HSE2 and HSE3 of hsp70 as in Fig.1. T represents the hsp70 TATA box. The nucleotide sequences in the regions of the cBNΔHSE1 and cBs-13Z deletions are shown with the matches to the consensus sequence indicated by horizontal lines above or below the sequence. Triangles below the cBs+10Z, cBs+127Z and cBs+331Z maps represent insertions of the indicated sizes. The maps are not drawn to scale. The average level of heat shock ß-galactosidase expression for the c70Z transformants (.205 OD units/hour/ul extract) was set to 100% and expression values per gene for individual transformants are given as a relative percentage. The mRNA levels were determined by scanning preflashed autoradiograms of the RNA blots shown in Figures 5 and 6 using a BioRad Model 620 Video Densitometer. The areas under the peaks were determined for the hsp70-lacZ species shown in Figures 5a and 6a and for the hsp70 species shown in 5b and 6b. The ratio of these signals (hsp70-lacZ/hsp70) was determined for the c70Z transformants and their average was set to 100%. The same signal ratio was determined for individual transformants and the value relative to that for the c70Z transformants is presented as a percentage. ND = not determined.

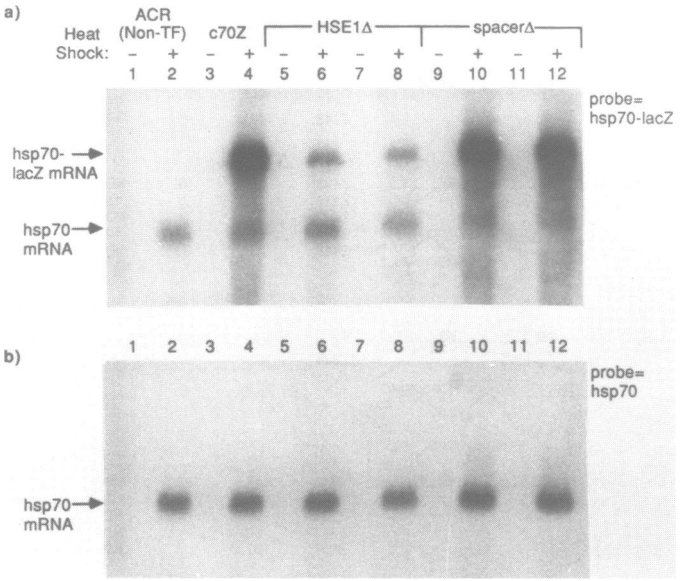


Figure 5. RNA Analysis of cBNΔHSE1 and cBs-13Z Transformants

Approximately 12 μg of total RNA was loaded per track. (-) and (+) indicate control and heat shock RNA samples, respectively. Arrows indicate the 2.4 kb hsp70 mRNA species and the 3.4 kb hsp70-lacZ mRNA species. Lanes 1 and 2 contain RNA from the nontransformant injection strain. Lanes 3 and 4 contain RNA from the c70Z transformant, Z243. Lane pairs 5,6 and 7,8 contain RNA from the cBNΔHSE1 transformants Z411 and Z424, respectively. Lane pairs 9,10 and 11,12 contain RNA from the cBs-13Z transformants Z810 and Z828, respectively. The blot shown in (a) was hybridized with a nick-translated (35) hsp70-lacZ probe (plasmid p-194.70ZT) that possesses 420 bp of hsp70 homology and 3 kb of lacZ homology. After removing this probe, the same filter was rehybridized with an hsp70 probe (a 0.9 kb BamHI-SalI fragment from the 3' portion of the hsp70 gene) lacking homology to hsp70-lacZ mRNA. The amount of hsp70 mRNA in these lanes serves as an internal control for differences in RNA loading and variation in heat shock treatment between samples. The trailing of signal apparent in lanes after hsp70-lacZ hybridization was reproducibly absent after rehybridization with the hsp70-specific probe in these experiments. This observation suggests that the hsp70-lacZ mRNA is not as stable as endogenous hsp70 mRNA.

analysis of mutations that affect HSE1 alone has not been described. The cBNΔHSE1 construction shown in Figure 4 is a 13 bp deletion that specifically removes HSE1 without affecting the composition of HSE2 or the TATA box region. As shown at the RNA level in Figure 5, lanes 6 and 8, and at the protein level in Figure 4, the deletion of HSE1 reduces expression to 15% of the parental level. The reduction is likely to be due to the loss of the HSE1 element but the deletion also changes the distance between HSE2 and the TATA box. In order to argue against some inhibitory feature of

this new spacing, transformants containing the cBs-13Z construction were analyzed. cBs-13Z possesses a distinct 13 bp deletion of nucleotides -70 to -58 such that HSE2 is unchanged (Fig. 4). HSE1 is changed only in nucleotide 2 of the consensus sequence and overlaps with HSE2 by 4 bp. The distance from HSE2 to the TATA box in cBNΔHSE1 and cBs-13Z is identical. The difference between these two constructions is that cBs-13Z has an excellent match to the heat shock consensus (8/10, 8/8) starting at -75 whereas cBNΔHSE1 has a weak 5/10 match at the same position. As shown in Figure 5, lanes 10 and 12 and in Figure 4, cBs-13z yields heat shock levels of expression similar to the parental construction. These data, taken together, provide in vivo evidence for the importance of the proximal element, HSE1.

Although the level of heat shock expression from cBNΔHSE1 is significantly reduced compared to the parental construction, the absolute level observed (15%) is surprisingly high. Since several members of this transformant group still displayed 15 to 40-fold induction, it is clear that the removal of HSE1 in this background still leaves substantial gene activity. It is unlikely that the low affinity site, HSE2, is solely capable of driving this level of expression for the following reasons. First, germline transformants that contain constructions with only the HSE1 high affinity site express these genes at 50 to 100-fold reduced relative to wild-type (18). Second, transformants that possess a single copy of a perfect match to the consensus sequence upstream of the same hsp70-lacZ fusion gene used here fail to produce heat-induced activity (Hua Xiao and J.T.L. in preparation). Given these considerations, an additional HSE present in cBNΔHSE1 could be interacting with HSE2 to provide the level of expression observed. A likely candidate for this HSE is HSE3 at -193 which is known to have high affinity for HSTF (17). In order for HSE3 to contribute, the hsp70 regulatory region must tolerate interactions between HSE's over distances greater than that seen in the wild-type case. An alternative candidate for a second HSE in cBNΔHSE1 is the weak 5/10 match at -75.

In order to address whether hsp70 could be activated from HSE's in remote positions, transformants containing the cBs+10Z, cBs+127Z and cBs+331Z constructions were analyzed. cBs+10Z has 10 bp, including a HpaI linker, inserted between HSE1 and HSE2. cBs+127Z and cBs+331Z contain 127 or 331 bp, respectively, of spacer DNA derived from pBR322 inserted into the same position. As shown in Figure 4, cBs+10Z levels of heat shock expression are similar to those of the parental construction, indicating that the 10 bp insertion is tolerated. This result and the cBs-13z deletion result indicate that the 9 bp spacer sequence between HSE1 and HSE2 is not required for the heat shock regulation of hsp70. The expression results for the larger insertions of 127 and 331 bp into the spacer are shown in Figures 4 and 6. At both the RNA and protein levels, expression is 50-80% relative to the parental

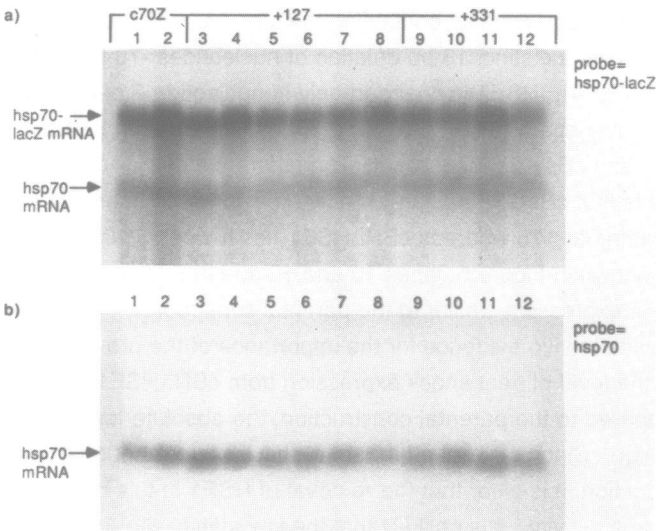


Figure 6. RNA Analysis of cBs+127Z and cBs+331Z Transformants
All lanes contain approximately 12 μg of total RNA from heat shocked flies. Arrows indicate the 2.4 kb hsp70 mRNA species and the 3.4 kb hsp70-lacZ mRNA species. Lanes 1 and 2 contain RNA from c70Z transformants Z217 and Z243, respectively. Lanes 3-8 contain RNA from cBs+127Z transformants Z717, Z754, Z782a, Z782x, Z784 and Z770, respectively. Lanes 9-12 contain RNA from cBs+331Z transformants Z121, Z122, Z123 and Z148, respectively. Panels (a) and (b) were hybridized with probes as described for Figure 5a and 5b, respectively.

construction. Thus the displacement of HSE2 and HSE3 to positions several hundred bp further upstream than the wild-type positions has little effect on the efficiency of heat shock expression. In addition, since HSE's are spaced at intervals of 100 bp or more in these constructions, there does not appear to be a requirement for two closely spaced HSE's for heat shock activation.

DISCUSSION

The results presented here demonstrate that there is considerable flexibility in the HSE organization of heat shock gene regulatory regions. HSE's from one heat shock gene are capable of promoting expression when substituted for HSE's of another heat shock gene, as shown here for the insertion of hsp70 HSE's into hsp26. Similar results have been reported in the case of hsp70 HSE insertion into hsp27 as analyzed by transfection assays (25). In addition, hybrid regulatory regions consisting of hsp70 plus hsp27 or hsp70 plus hsp23 HSE's have been shown to be functional (22,25). The ability to substitute and combine HSE's from different genes is in accord with the

idea that HSE's are the basis of coordinate heat shock gene regulation (31).

Flexibility in the physical distance between required HSE's was also observed. Although, the wild-type hsp26 gene is activated by HSE's distributed over several hundred bp, a pair of HSE's proximal to the TATA box was shown to be sufficient for activation. Conversely, the hsp70 gene can be activated by a pair of proximal HSE's but, as demonstrated here, can also be efficiently activated by HSE's positioned 100-300 bp farther upstream. The ability of HSE's to interact over large distances in germline transformants confirms similar observations that have been reported using transfection assays (22,25). Particularly striking was the report that HSE's can interact over a distance of at least 2 kb (25).

In experiments involving the insertion of the hsp70 fragment containing HSE1 and HSE2 into the hsp26 upstream region, flexibility in the orientation of this fragment was seen. Similar levels of expression were observed for each orientation even though the relative affinities of the two HSE's for HSTF are different. This result is intriguing in light of a recent report describing the molecular contacts between HSTF and these hsp70 HSE's (46). These workers demonstrate that when HSTF fills both the HSE1 and HSE2 sites, the pattern of DNA contacts within each site is quite similar. Thus, inverting the entire fragment may not significantly alter the structure of a symmetrical protein-DNA complex formed upon it. These results are consistent with the idea that the formation of the HSE1-HSE2 protein-DNA complex (complex B in [46]), but not the direction of sequential site filling, is important for activation.

A final observation concerning flexibility in HSE arrangment involves the cBs-13Z construction. This construction contains an HSE2-HSE1 hsp70 pair that overlap by 4 bp. It is conceivable that HSE's positioned in such a way might interfere with each other's function, yet the wild-type arrangment of HSE's in Drosophila hsp26, hsp27 and hsp82 and in a variety of heat shock genes from other organisms (47,48) include such overlapping elements. The level of expression from cBs-13Z implies that at least one of the two overlapping HSE's is functional. It cannot be determined whether both are fully functional since HSE3 may be contributing to expression in this construction. It will be interesting to examine expression from a cBs-13Z derivative lacking HSE3.

Why do the small heat shock genes require important contributions from remote HSE's whereas hsp70 does not require its remote HSE's for activation? The flexibility in HSE positioning observed here argues against the idea that the hsp70 heat shock promoter is specialized for activation from nearby HSE's. A possible explanation for the differences in hsp70 and small heat shock gene HSE organization may involve modes of expression other than the heat-induced mode. The small heat shock genes display a variety of developmentally-regulated modes of expression including ecdysone induction (36,41-43) whereas hsp70 does not. Mapping of cis-acting DNA

elements involved in these additional modes of expression reveals that they are localized either upstream of or interspersed with HSE's (21-26,36). Thus the placement of HSE's over relatively large distances in the regions upstream of the small heat shock genes may reflect the accomodation of other cis-acting elements that coexist in the same region. The relationship between HSE's and developmentally-regulated elements may be passive with the observed spacing designed to avoid conflict when disparate modes of expression are required. Alternatively, HSE's may interact with developmentally-regulated elements to contribute to those modes of expression. In fact, expression of the Xenopus hsp70 gene in such a mode in the absence of stress has been shown to require an HSE (49) and the possible involvement of HSE3 in the ecdysone regulation of hsp22 has been hypothesized (21).

Given that the hsp70 gene can be activated from remote HSE's, why do HSE3 and HSE4 appear dispensable? Although no contribution from these HSE's is apparent by analysis of 5' deletions, the observed heat inducibility of the cBNΔHSE1 construction in vivo may be due to an HSE3-HSE2 interaction. Further analysis of the remote hsp70 HSE's is necessary to clarify their roles in vivo.

A major unresolved issue in understanding heat shock regulation is the requirement for multiple copies of HSE. Similar requirements for multiple copies of a cis-acting element have been described for optimal expression of mammalian metallothionein (3,8) and glucocorticoid-regulated genes (10,11) as well as the yeast GAL1 and GAL10 (6,7) HIS3 (5) and HIS4 (9) genes. A possible basis for the requirement for both HSE1 and HSE2 of hsp70 has been recently described (46). These workers detect a conformational change in HSTF bound to HSE1 that is dependent upon HSE2 occupancy by HSTF. If this conformational change is critically required for activation, then the need for HSE1 plus HSE2 is explained. Alternatively, it may simply be the multimeric HSTF complex created by occupancy of two HSE's that is required. Regardless of what specifically comprises the activation complex, it seems clear that such a complex can form with a variety of DNA distances separating HSE's. One means by which such flexibility might be explained is for HSTF molecules bound to separate DNA sites to have a propensity for self-association, with the concomitant "looping out" of the intervening DNA. Such DNA looping has been observed in prokaryotic transcription regulatory systems (50,51) and has been hypothesized to occur in eukaryotic systems as well (51,52). Whether DNA looping permits HSE interaction over large distances remains to be determined.

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REFERENCES

1. McKnight, S.L., Kingsbury, R.C., Spence, A. and Smith, M. (1984) Cell 37, 253–262.
2. Everett, R.D., Baty, D. and Chambon, P. (1983) Nucl. Acids Res. 11, 2447–2464.
3. Stuart, G.W., Searle, P.F., Chen, H.Y., Brinster, R.L. and Palmiter, R.D. (1984) Proc. Nat. Acad. Sci. 81, 7318-7322.
4. Donahue, T.F., Daves, R.S., Lucchini, G. and Fink, G.R. (1983) Cell 32, 89-98.
5. Struhl, K., Chen, W., Hill, D.E., Hope, I.A. and Oettinger, M.A. (1985) Cold Spring Harbor Symp. Quant. Biol. 50, 489-503.
6. Giniger, E., Varnum, S.M. and Ptashne, M. (1985) Cell 40, 767-774.
7. West, R.W., Jr., Yocum, R.R. and Ptashne, M. (1984) Mol. Cell. Biol. 4, 2467-2478.
8. Stuart, G.W., Searle, P.F. and Palmiter, R.D. (1985) Nature 317, 828-831.
9. Hinnebusch, A.G., Lucchini, G. and Fink, G.R. (1985) Proc. Nat. Acad. Sci. 82, 498-502.
10. Kuhnel, B., Buetti, E. and Diggelmann, H. (1986) J. Mol. Biol. 190, 367-378.
11. Buetti, E. and Kuhnel, B. (1986) J. Mol. Biol. 190, 379-389.
12. Dynan, W.S. and Tjian, R. (1983) Cell 35, 79-87.
13. Graves, B.J., Johnson, P.F. and McKnight, S.L. (1986) Cell 44, 565-576.
14. Jones, K.A., Yamamoto, K.R. and Tjian, R. (1985) Cell 42, 559-572.
15. Hope, I.A. and Struhl, K. (1985) Cell 43, 177-188.
16. Pelham, H.R.B. (1982) Cell 30, 517-528.
17. Topol, J., Ruden, D.M. and Parker, C.S. (1985) Cell 42, 527-537.
18. Dudler, R. and Travers, A.A. (1984) Cell 38, 391-398.
19 Simon, J.A., Sutton, C.A., Lobell, R.B., Glaser, R.L. and Lis, J.T. (1985) Cell 40, 805-817.
20. Amin, J., Mestril, R., Lawson, R., Klapper, H. and Voellmy, R. (1985) Mol. Cell. Biol. 5, 197-203.
21. Klemenz, R. and Gehring, W.J. (1986) Mol. Cell. Biol. 6, 2011-2019.
22. Mestril, R., Schiller, P., Amin, J., Klapper, H., Jayakumar, A. and Voellmy, R. (1986) EMBO J. 5, 1667-1673.
23. Pauli, D., Spierer, A. and Tissieres, A. (1986) EMBO J. 5, 755-761.
24. Cohen, R.S. and Meselson, M. (1985) Cell 43, 737-746.
25. Riddihough, G. and Pelham, H.R.B. (1986) EMBO J. 5, 1653-1658.
26. Hoffman, E. and Corces, V. (1986) Mol. Cell. Biol. 6, 663-673.
27. Moran, L., Mirault, M.-E., Tissieres, A., Lis, J.T., Schedl, P., Artavanis-Tsakonas, S., and Gehring, W.J. (1979) Cell 17, 1-8.
28. Karch, F., Torok, I. and Tissieres, A. (1981) J. Mol. Biol. 148, 219-230.
29. Parker, C.S. and Topol, J. (1984) Cell 37, 273-283.
30. Wu, C. (1984) Nature 311, 81-84.
31. Xiao, H. and Lis, J.T. (1986) Mol. Cell. Biol. 6, 3200-3206.
32. Cartwright, I.L. and Elgin, S.C.R. (1986) Mol. Cell. Biol. 6, 779-791.
33. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
34. Lis, J.T., Simon, J.A. and Sutton, C.A. (1983) Cell 35, 403-410.
35. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
36. Glaser, R.L., Wolfner, M.F. and Lis, J.T. (1986) EMBO J. 5, 747-754.
37. Maxam, A.M. and Gilbert, W. (1980) Meth. Enzymol. 65, 499-560.

38. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Nat. Acad. Sci. 74, 5463-5467.
39. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) "Molecular Cloning" Cold Spring Harbor Laboratory.
40. Di Nocera, P.P. and Dawid, I.B. (1983) Proc. Nat. Acad. Sci. 80, 7095-7098.
41. Zimmerman, J.L., Petri, W. and Meselson, M. (1983) Cell 32, 1161-1170.
42. Mason, P.J., Hall, L.M.C. and Gausz, J. (1984) Mol. Gen. Genet. 194, 73-78.
43. Ireland, R.C., Berger, E., Sirotkin, K., Yund, M.A., Osterbur, D. and Fristrom, J. (1982) Dev. Biol. 93, 498-507.
44. Boehringer Mannheim Biochemicals, personal communication.
45. Lis, J.T., Neckameyer, W., Dubensky, R. and Costlow, N. (1981) Gene 15, 67–80.
46. Shuey, D.J. and Parker, C.S. (1986) J. Biol. Chem. 261, 7934-7940.
47. Russnak, R.H. and Candido, E.P.M. (1985) Mol. Cell. Biol. 5, 1268-1278.
48. Bienz, M. and Pelham, H.R.B. (1986) Cell 45, 753-760.
49. Bienz, M. (1986) Cell 46, 1037-1042.
50. Martin, K., Huo, L. and Schleif, R.F. (1986) Proc. Nat. Acad. Sci. 83, 3654–3658.
51. Griffith, J., Hochschild, A. and Ptashne, M. (1986) Nature 322, 750-752.
52. Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M., and Chambon, P. (1986) Nature 319, 121-126.