The EMBO Journal vol.6 no.10 pp.3035–3041, 1987

Purification and characterization of a heat-shock element binding protein from yeast

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Communicated by H.R.B.Pelham

The promoters of heat shock genes are activated when cells are stressed. Activation is dependent on a specific DNA sequence, the heat-shock element (HSE). We describe the purification to homogeneity of an HSE-binding protein from yeast (*Saccharomyces cerevisiae*), using sequential chromatography of whole cell extracts on heparin–agarose, calf thymus DNA–Sepharose and an affinity column consisting of a repetitive synthetic HSE sequence coupled to Sepharose. The protein runs as a closely spaced doublet of ~150 kd on SDS–polyacrylamide gels; mild proteolysis generates a stable 70-kd fragment which retains DNA binding activity. The relative affinities of the protein for a range of variant HSE sequences correlates with the ability of these sequences to support heat-inducible transcription *in vivo*, suggesting that this polypeptide is involved in the activation of heat-shock promoters. However, the protein was purified from unshocked yeast, and may therefore represent an unactivated form of heat-shock transcription factor. Study of the purified protein should help to define the mechanistic basis of the heat-shock response.

Key words: heat shock/transcription factor/yeast (*Saccharomyces cerevisiae*)

Introduction

Cells of all organisms so far examined contain heat-shock genes whose transcription is activated following exposure to high temperature or to certain other stresses (for reviews see Lindquist, 1986; Craig, 1985). In eucaryotes, this response is mediated by a specific DNA sequence (the heat-shock element, HSE) that is found in multiple copies in the promoter regions of heat-shock genes (reviewed by Bienz and Pelham, 1987). HSE sequences confer strong heat-inducibility on heterologous promoters, and can act both as TATA-proximal elements and as enhancers (Pelham, 1982; Pelham and Bienz, 1982; Bienz and Pelham, 1986). They have been shown to be binding sites for a heat-shock transcription factor (Parker and Topol, 1984; Wu, 1984a; Wiederrecht *et al.*, 1987), and experiments with both *Drosophila* and HeLa cells indicate that an HSE-binding activity is induced when cells are heat shocked (Wu, 1984b; Sorger *et al.*, 1987; Kingston *et al.*, 1987). Since inhibitors of protein synthesis do not block the activation of heat-shock genes, this induction probably reflects the modification of an inactive form of the heat-shock transcription factor. Thus an understanding of the mechanism of heat-shock gene activation requires knowledge of the changes that occur to this factor upon heat shock.

We report here the isolation of an HSE-binding protein from yeast cells by means of DNA-affinity chromatography, a method that has been successfully used to purify a number of DNA binding proteins (e.g. by Rosenfeld and Kelly, 1986 and Briggs *et al.*, 1986). This protein binds tightly to functional HSEs, but only weakly to variant HSE sequences that have little or no function *in vivo*, which suggests that it is involved in the heat-shock response. However, in contrast to the situation with HeLa and *Drosophila* cells, the binding protein is readily detectable in extracts from unstressed yeast.

Results

Properties of the yeast HSE binding activity

To search for proteins that bind to the heat-shock element (HSE) in yeast, we used an assay in which crude extracts were mixed with labelled HSE-containing DNA fragments and subjected to gel electrophoresis (Fried and Crothers, 1981; Garner and Revzin, 1981). As a probe we routinely used a synthetic sequence containing two overlapping binding sites (HSE2; Figure 1). These sites consist of a perfect match to the HSE consensus sequence (CNNGAANNTTCNNG) previously deduced from a comparison of several heat-shock gene promoters (Pelham and Bienz, 1982). Overlapping HSEs occur frequently in such promoters in a wide variety of organisms and appear to be strong binding sites for the heat shock transcription factor (see Bienz and Pelham, 1987, for review). As shown in Figure 2, an HSE-binding activity is indeed present in yeast whole cell extracts. The protein–DNA complexes appeared on gel electrophoresis as a series of three closely spaced bands whose ratio changed with the concentration of the extract (Figure 2b, lanes 1 and 2). Multiple bands were

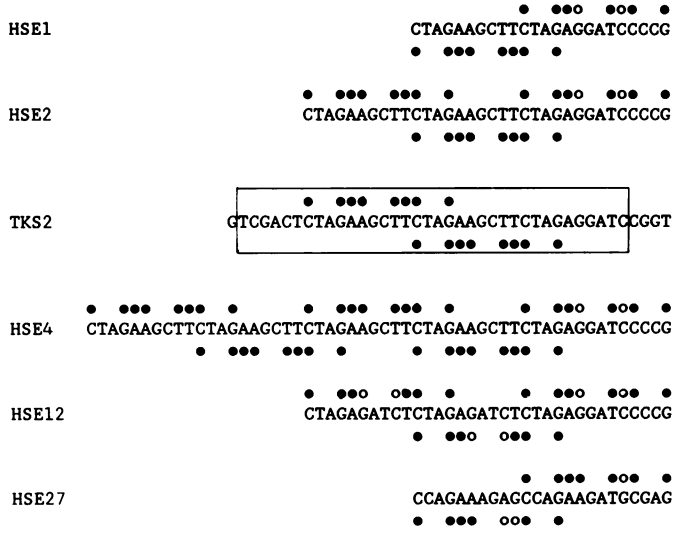

Fig. 1. HSE sequences. The relevant parts of the DNA fragments used for DNA binding and competition experiments are shown. Matches to the HSE consensus sequence CNNGAANNTTGNNG are indicated by filled circles, mismatches by open circles. The TKS2 fragment was used for footprinting (see Figure 4), and the protected region of the strand shown is boxed. The affinity column used to purify the binding protein contained long repeating sequences of the kind seen in HSE4. For details of the plasmid constructions see Materials and methods.

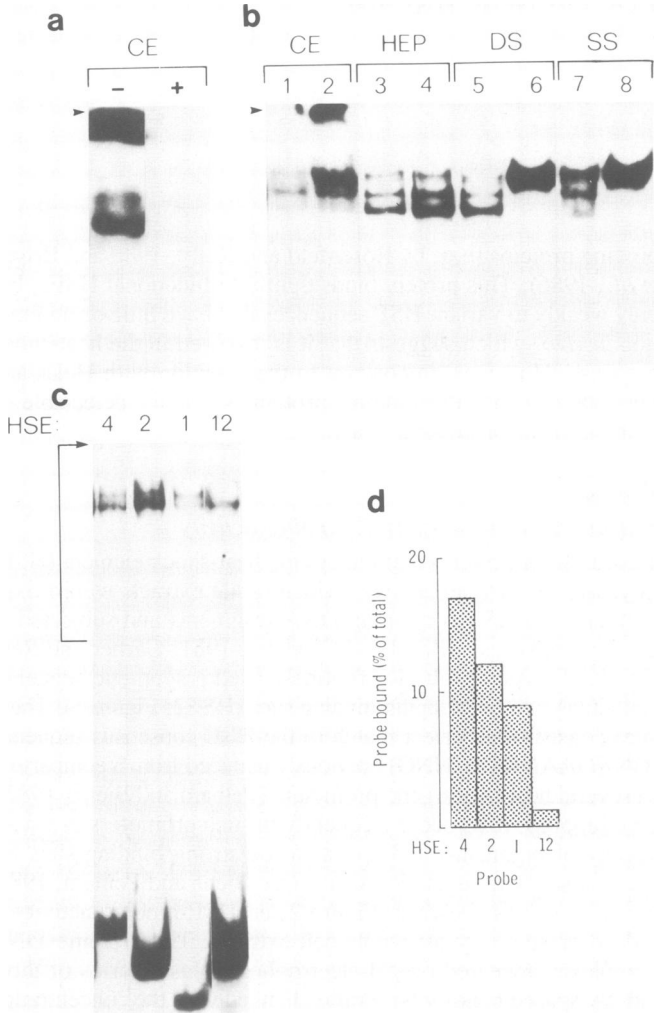

Fig. 2. Gel electrophoresis of protein–HSE complexes. **(a)** Crude extract with HSE2 probe, without (−) or with (+) unlabelled HSE2 competitor. This extract was prepared by quick-freezing cells in liquid nitrogen, grinding the frozen pellet and thawing in binding buffer. The arrow marks the top of the gel, where some complexes are found. Only the top portion of the gel is shown, as indicated by the bracket in **(c)**; no other bands were seen apart from the free DNA. **(b)** HSE2 probe with crude extract as used for purification (CE; **lane 2** has twice as much extract as **lane 1**), fractions from the heparin–agarose column (HEP; **lanes 3** and **4**), calf thymus DNA–Sepharose fraction (DS; **lane 6** has twice as much protein as **lane 5**) and affinity-purified protein (SS; **lane 8** has twice as much protein as **lane 7**). **(c)** DS fraction assayed with HSE4, HSE2, HSE1 and HSE12 probes. The entire gel is shown. **(d)** Quantitation of the binding shown in the previous panel.

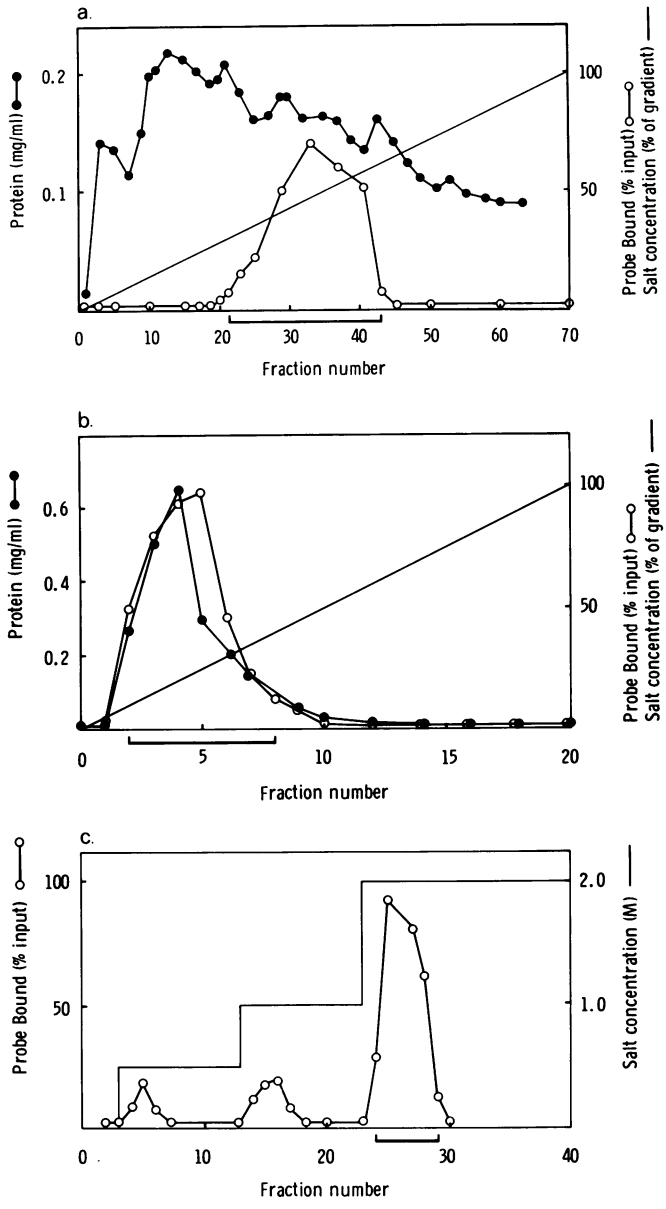

Fig. 3. Purification of the HSE-binding activity. **(a)** Elution profiles of activity eluted from the heparin–agarose column between 50 and 400 mM ammonium sulphate (indicated as 0–100% of gradient). Ten microlitres of each 10-ml fraction were assayed with 0.5 ng of HSE2 probe. The peak HEP pool is bracketed. **(b)** Elution profile of the calf thymus DNA–Sepharose column between 50 (0%) and 240 mM (100%) ammonium sulphate. Four microlitres of each 1-ml fraction were assayed with 0.5-ng probe. **(c)** Profile of the HSE DNA–Sepharose affinity column eluted with steps of 0.5 M KCl, 1.0 M KCl, and 2.0 M NaCl and assayed as in **(b)**.

observed using not only HSE2 as a probe but also a variety of other synthetic and naturally occurring HSEs with a wide range of binding affinities (see Figure 2c,d) but were not always well resolved. All bands were eliminated by the addition of an excess of unlabelled probe DNA (Figure 2a). By analogy with other systems, it seems likely that the lower mobility species represent complexes with multiple subunits of a single protein bound to each DNA molecule.

Purification of an HSE-binding protein

HSE-binding proteins were purified from whole cell extracts using DNA-affinity chromatography on a column consisting of Sepharose linked to concatenated synthetic oligomers (20–100 binding sites/molecule) containing overlapping HSE consensus sequences as shown in Figure 1. Gel retardation assays with HSE2 as a probe were used to follow binding activity during the purification. Extracts were made by mechanically disrupting late log phase cultures of a protease-deficient yeast strain (see Materials and methods) either with glass beads or by homogenization in liquid nitrogen, and a high speed supernatant prepared and concentrated with ammonium sulphate. This crude extract was loaded onto a heparin–agarose column and eluted with a linear salt gradient (Figure 3a). Peak fractions were pooled (HEP fraction), loaded onto a calf thymus DNA–Sepharose column and the column eluted with a similar gradient (Figure 3b). The

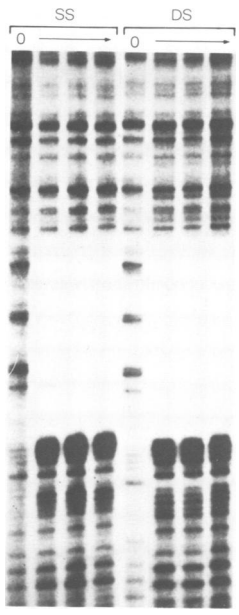

Fig. 4. Footprinting of HSE sequences by affinity-purified protein (SS) and the peak fraction from calf thymus DNA–Sepharose (DS). TKS2 probe was digested with DNase I after incubation without (0) or with increasing concentrations of protein fraction (as indicated by the arrows). Equivalent amounts of binding activity (0.2–1.0 U) were used with each fraction. The protected sequence is indicated in Figure 1.

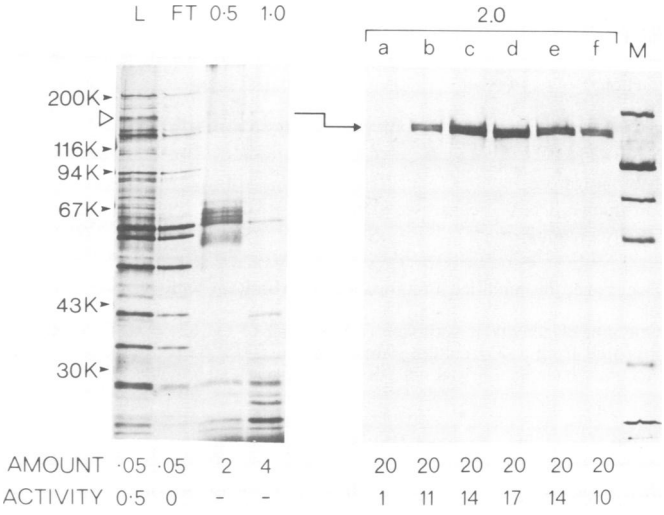

Fig. 5. Silver-stained SDS–polyacrylamide gels of fractions from the DNA affinity column. Samples are as follows: affinity column load (L) and flowthrough (FT), 0.5 M KCl eluate (0.5), 1.0 M KCl eluate (1.0), six successive fractions of the 2.0 M NaCl eluate (2.0, **a**–**f**) and markers (M). 'Amount' refers to the percentage of each sample loaded on the gel: 4 μl of 7 ml for L and FT, 100 μl of 5 ml for 0.5 M eluate, 200 μl of 5 ml for 1.0 M eluate and 200 μl of six 1-ml fractions from the 2.0 M eluate. 'Activity' indicates the number of units of binding activity (see Table I) loaded on the gel. Mol. wt markers are labelled in kilodaltons and the position of the 150-kd HSE-binding protein indicated by the open triangle.

HSE-binding activity eluted at low salt, between 50 and 200 mM ammonium sulphate (DS fraction), and was thus separated from proteins that bind strongly but non-specifically to DNA.

When DS fractions, in ~120 mM ammonium sulphate, were loaded onto the DNA-affinity column described above, >95% of the total protein flowed through the column, whereas HSE-binding activity was retained. The affinity column was eluted with successive steps of 0.5 M KCl, 1 M KCl, and 2 M NaCl; ~9% of the activity eluted with the first wash, 14% with the second and 75% with the last (SS fraction; Figure 3c). Altogether, nearly 100% of the applied activity was recovered from the affinity column. This high recovery was aided by the addition of either 0.1% NP-40 or 0.025% *n*-octyl-beta-D-glucopyranoside to both the loading and elution buffers. Detergents have also been found to aid purification of a protein that binds to the c-*fos* promoter (Treisman, 1987).

The HSE-binding activity eluted as a single peak from both the heparin and calf thymus DNA columns suggesting that the purification of only a single protein is being monitored. In agreement with this, the pattern of DNA–protein complexes on polyacrylamide gels remained constant at all steps in the purification (Figure 2b). The bands were less clear when the samples contained high concentrations of salt (as was the case with the SS fraction), but inspection of the autoradiograms revealed that the same three bands could be seen with material at all stages of purification. As with the crude extract, the relative amounts of the three bands were dramatically altered when the concentration of binding protein was varied (e.g. lanes 5 and 6 of Figure 2b). The stability of the complexes, as determined by their rate of dissociation in the presence of unlabelled competitor DNA, also remained comparable throughout the purification (data not shown). Furthermore, footprints of crude and purified extracts were identical, both giving strong protection of a 36-base region centred over the two overlapping HSEs (Figure 4; see also Figure 1).

The protein composition of the calf thymus DNA–Sepharose and of affinity-purified fractions was determined by SDS–polyacrylamide gel electrophoresis. As shown in Figure 5, the 2.0 M NaCl affinity column wash contains predominantly a doublet which migrates with an apparent mol. wt of 150 kd. On other gels it was found to comigrate with the large subunits of *Escherichia coli* RNA polymerase (151/155 kd; see Figure 7). Even in heavily silver-stained gels, no other proteins, with the exception of the 70-kd species discussed below, were consistently detected. The 150-kd doublet was also visible in the affinity column load (L), 0.5 M KCl and 1.0 M KCl washes but not in the flowthrough (FT). As indicated by the numbers below the gel in Figure 5, the intensity of the 150-kd band correlates well with the amount of binding activity.

In order to determine the native size of the binding protein, a sample of the affinity-purified material was applied to a calibrated f.p.l.c. Superose 12 column (Pharmacia). The HSE-binding activity eluted in a broad peak with sharp boundaries corresponding to an apparent mol. wt of 300–1200 kd (data not shown). Although gel filtration alone gives only a rough indication of mol. wt, the larger native size of the binding complex suggests that at least it consists of a dimer of 150-kd subunits and possibly even a tetramer or larger multimer.

The overall recovery of HSE-binding activity is summarized in Table II, but these values should only be considred a rough guide since our assay, especially in crude extracts, was not linear. The loss of activity following chromatography on calf thymus DNA–Sepharose was mostly due to overloading of the column, and additional activity could be recovered from the flowthrough material. The amount of protein recovered in the SS fraction in the purification shown in Table I corresponds to ~5 molecules/cell of a 150-kd protein. Subsequent larger-scale preparations, with larger columns and lower losses, have yielded up to 40 molecules/cell.

U.v. crosslinking

In order to confirm that the major 150-kd protein identified by DNA affinity chromatography is the same HSE-binding protein

Table I. Summary of purification

Fraction		Volume (ml)	Protein conc.[a] (mg/ml)	Total protein (mg)	Total activity (units)[b]	Specific activity (units/mg)	Yield (%)
Crude extract[c]	(CE)	400	18	7200	(3500)[d]	(0.5)[d]	
Heparin agarose	(HEP)	48	0.8	40	2600	65	=100
Calf thymus DNA−Sepharose	(DS)	22	0.2	4.5	1200	260	46
Affinity column	(SS)	6	0.0008	0.005	1050	210 000	40

[a]Determined by modified Bradford method (BioRad) with BSA as a standard except for the SS fraction, whose concentration was estimated from silver-stained gels.
[b]One unit of activity is sufficient to bind 1 ng of the 62-bp HSE2 probe under conditions of probe excess.
[c]Prepared from a 40-l culture, which yielded 500 g packed cells (4×10^{12} cells).
[d]Accurate quantitation was not possible in crude extracts due to the non-linearity of the assay.

Table II. Activity of variant HSE sequences *in vivo*[a]

HSE sequence	Beta-galactosidase activity[b]	
	Control	Heat shock[c]
HSE4	80	2200
HSE2	60	1600
HSE1	20	600
HSE27	100	500
HSE12	<5	<5
None	<5	<5

[a]HSE sequences were inserted into a disabled *cyc1−lacZ* vector which was transfected into the yeast as described in Materials and methods.
[b]Units are those used by Breeden and Nasmyth (1987).
[c]Yeast cells were grown at 23°C, heat shocked for 20 min at 39°C and allowed to recover for 1 h before assay.

that was detected in cruder extracts, an HSE2 probe containing bromodeoxyuridine (BUdR) and ^{32}P was photochemically crosslinked to proteins in the heparin (HEP), nonspecific DNA−Sepharose (DS) and affinity-purified (SS) fractions, and the labelled proteins analysed by SDS−polyacrylamide gel electrophoresis (Ogata and Gilbert, 1977; Chodosh *et al.*, 1986; Treisman, 1987). The substitution of BUdR for thymidine did not interfere with protein−DNA complex formation as judged by gel retardation assays (data not shown). It is customary to treat samples with DNase I following crosslinking, but this decreased the amount of label incorporated into discrete bands without reducing the background smear. We therefore omitted DNase treatment and either removed free probe by phenol extraction of irradiated samples followed by ethanol precipitation of the proteins from the organic phase, or in the case of the SS fraction simply precipitated the samples with trichloroacetic acid following irradiation.

As shown in Figure 6, the principal labelled species in the SS fraction was a band at 150−160 kd, corresponding to the protein detected by silver staining (the band at the bottom of the gel is free probe). The addition of 70 bases of DNA apparently has little effect on the electrophoretic mobility of this protein in the presence of SDS. A protein of the same size was also detected in the cruder DS and HEP fractions. When a 40-fold molar excess of unlabelled HSE2 probe DNA was added to the DS reactions, the strength of this band was greatly reduced (lane 2). In contrast, when a weak binding site was added as a competitor (HSE12; see Figure 2d and below), the strength of the 150−160 kd band was not greatly affected, confirming the binding specificity of this protein.

In addition to the expected HSE-binding protein, the HEP and DS fractions contained proteins in the 70−90 kd range that produced a labelled smear after u.v. crosslinking. This smear, unlike

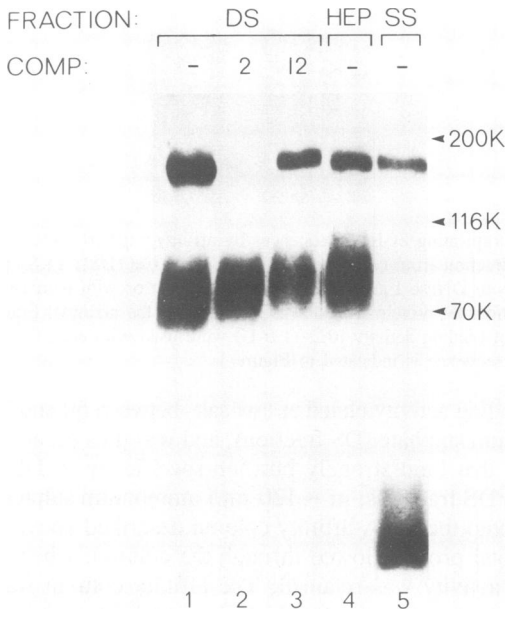

Fig. 6. U.v. crosslinking of HSE sequences to protein. Samples of protein from the calf thymus DNA−Sepharose column (DS; 2 μg protein per lane), heparin−agarose column (HEP, 8 μg protein) and the affinity column (SS, 6 μl) were cross-linked to 0.6 ng of labelled BUdR-substituted HSE2 probe. 'Comp' indicates the presence of a 40-fold molar excess of either HSE2 or HSE12 competitor DNA over probe. Reactions with HEP and DS fractions were phenol extracted and ethanol precipitated following crosslinking but the reaction with the SS fraction was TCA precipitated without extraction; unbound probe is therefore seen only in the SS lane. The positions and mol. wts of marker proteins are indicated.

the larger band, was not greatly reduced by the addition of excess unlabelled probe or HSE12 competitor. We presume that it represents protein(s) that bind weakly and/or non-specifically to DNA, and are so abundant that they cannot easily be saturated with competitor. This material is completely removed by the affinity-purification step, although there is no loss of binding activity as measured by the gel retardation assay, and no change in the electrophoretic mobility of the DNA−protein complexes (Table I; Figure 2). Conversely, no activity could be observed when the flowthrough material from the affinity column, which contains the 70−90-kd proteins, was assayed in this way. We therefore conclude that the 70−90-kd material does not contribute to the complexes seen on native polyacrylamide gels, and that the specific HSE-binding activity that we detect in crude extracts can be entirely accounted for by the 150-kd polypeptide.

Proteolysis of the affinity purified protein

In some preparations of binding protein, small amounts of a 70-kd protein were detectable by silver staining. Moreover, Wiederrecht

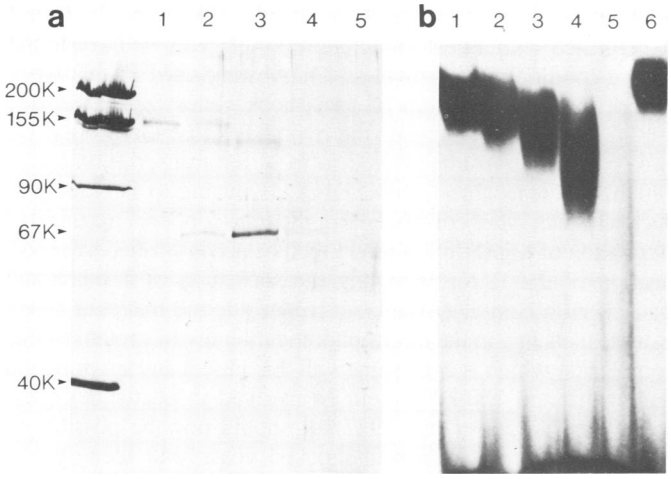

Fig. 7. Proteolytic digestion of affinity-purified HSE-binding protein. **(a)** Silver-stained polyacrylamide gel. Each lane contains an estimated 20 ng of protein digested with 0.5 ng papain for 0 min **(lane 1)**, 5 min **(lane 2)** or 30 min **(lane 3)** or with 10 ng papain for 5 min **(lane 4)** or 30 min **(lane 5)**. The 155-kd mol. wt marker is the largest subunit of *E. coli* RNA polymerase. **(b)** DNA binding assay performed with 2 ng of the same five samples with 0.4-ng probe or with untreated affinity-purified protein for comparison **(lane 6)**. The poor resolution of individual complexes on this gel (cf. Figure 2b) is caused by the high salt concentration of the affinity column eluate.

et al. (1987) have recently described the isolation of an HSE-binding protein with a mol. wt of 70 kd. Because of its variable yield, we suspected that the 70-kd protein was a proteolytic fragment of the 150-kd protein. To test this hypothesis, samples of the SS fraction were digested with low levels of papain. The results are shown in Figure 7 and indicate that proteolysis does indeed yield a relatively stable fragment of 70 kd. The digested samples were assayed for DNA binding by gel retardation, and concomitant with the decrease in size of the major protein band, there is a general increase in the mobility of the protein–DNA complexes (the broad bands seen in Figure 7b probably represent multiple species which are not well resolved; poor resolution was frequently observed when SS fractions containing 2 M NaCl were analysed in this way). This suggests that fragments of the 150-kd protein can bind DNA and that the migration of complexes containing HSE-binding proteins and DNA depends upon the size of the protein. In particular, samples containing no intact protein, but only the 70-kd fragment, retain DNA-binding activity (lanes 4). It thus seems possible that the protein isolated by Wiederrecht *et al.* is a fragment of the 150-kd protein that we have purified, and represents a DNA-binding domain that is relatively resistant to proteolysis.

Transcriptional and binding properties of variant HSEs

Because the affinity-purified protein binds to a synthetic HSE sequence, it is likely to be involved in the transcription of heat-shock genes. However, we have no functional assay for the purified material. To provide further evidence for the role of the protein, we tested a variety of HSE sequences for their ability to bind to the protein *in vitro* and to support heat-inducible transcription *in vivo* when linked to a suitable reporter gene. Three synthetic binding sequences with one, two and four overlapping 'perfect match' binding sites (HSE1, HSE2, HSE4; Figure 1), one synthetic mutant binding site with overlapping 6/8 matches (HSE12), and one pair of overlapping 6/8–7/8 binding sites from the *Drosophila* hsp27 promoter (HSE27) were tested for their ability to compete with an HSE2 probe in a DNA binding assay

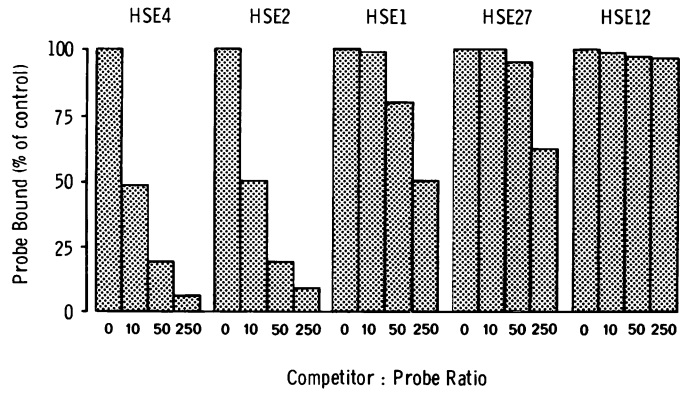

Fig. 8. Competitive DNA binding assays with variant HSEs. Parallel reactions with varying amounts of each competitor DNA, 1 μg of protein from the calf thymus DNA–Sepharose column (DS fraction) and 0.5 ng of HSE2 probe were analysed by gel electrophoresis, and the bound and free probe excised and counted. The total amount of DNA in each reaction was adjusted to 5.5 μg by addition of pUC19 carrier. The amount of probe bound is expressed as a percentage of that bound in the absence of competitor (50% of input), and represents the average of two determinations.

(Figure 8). The competitions were performed with calf thymus DNA–Sepharose fractions in the presence of a 2-fold excess of probe. Similar results were obtained with affinity-purified protein (data not shown). The relative strengths of the binding sites as competitors were HSE4 = HSE2 > HSE1 > HSE27 ⩾ HSE12. When DNA fragments containing these sites were used as probes in binding assays and the efficiency of binding estimated from the ratio of bound to unbound DNA, the same relative ordering was observed (Figure 2d). Similarly, HSE2 was a strong and HSE12 a very weak competitor in u.v. crosslinking assays (Figure 6).

The same five sequences were inserted upstream of a cytochrome *c1–lacZ* fusion gene from which the normal cytochrome *c* upstream activating sequences had been deleted (Guarente and Mason, 1983). These constructs were introduced into yeast cells and tested for beta-galactosidase production following heat shock at 39°C (Table II). The relative abilities of the binding sites to activate transcription in this *in vivo* assay were entirely consistent with their protein-binding affinities; HSE4 was the most powerful activating sequence, and HSE12 the weakest. It is striking that the change of four bases between HSE12 and HSE2 is sufficient to abolish both protein binding *in vitro* and transcriptional activation *in vivo*. This correlation supports the idea that the 150-kd HSE-binding protein is a heat-shock transcription factor.

Discussion

We have described the purification to homogeneity of a sequence-specific DNA-binding protein from yeast. The three-column procedure used was based on previous methods (Rosenfeld and Kelly, 1986; Kadonaga and Tjian, 1986; Shore and Nasmyth, 1987; Treisman, 1987) and proved simple and efficient. It is interesting to compare this procedure with that described by Kadonaga and Tjian (1986). The latter method involves passing proteins over a specific DNA-affinity column in low salt, in the presence of large amounts of nonspecific carrier DNA. Proteins partition between the carrier and the affinity resin; non-specific DNA-binding proteins are inevitable contaminants of the bound fraction, but can be eliminated by multiple passes over the column. There are two main differences between this procedure and

the one we used. First, we found that the addition of carrier DNA was not necessary; most DNA-binding proteins simply did not bind to the affinity column. This was probably because the sample was loaded in relatively high salt, but it also seemed that many proteins bound more weakly to the short repetitive sequence of the affinity column than to calf thymus DNA. These proteins presumably prefer a precise DNA sequence or geometry that can be found amongst the genomic sequences, but is different from that of the synthetic oligonucleotide.

A second difference involves the preliminary fractionation of the proteins on a 'non-specific' (calf thymus) DNA column, as recommended by Rosenfeld and Kelly (1986). This step removes proteins that bind very tightly but non-specifically to DNA. Proteins which subsequently elute from the affinity column at high salt must therefore show sequence specificity, in that their binding to this column is more salt-resistant than their non-specific binding to calf thymus DNA. In practice, this can eliminate the need for multiple passes over the affinity column and greatly reduces the problem of identifying the correct protein.

The efficiency of purification was undoubtedly increased by our use of a very tight binding site for the affinity column; this was provided by a repeating symmetric consensus-like sequence rather than by any naturally occurring heat shock promoter element. Dissociation of the HSE-binding protein from this sequence is very slow at moderate salt concentrations ($t_{1/2} \gg 1$ h). This is probably due to cooperative interactions between multiple protein subunits bound to overlapping sites on the DNA, because dissociation of protein from a single HSE sequence is much more rapid ($t_{1/2} \sim 15$ min).

The protein we obtained is large and protease-sensitive. We were able to avoid degradation during purification by using a yeast strain deficient in several major proteases, by including a variety of protease inhibitors in the buffers, and by disrupting the cells mechanically at low temperature, thus avoiding the proteases which contaminate commercial preparations of cell-wall-degrading enzymes. It is hard to rule out the possibility that *in vivo* the protein is even larger than the 150-kd polypeptide that we have isolated, but we see no signs of larger material, and u.v. crosslinking experiments indicate that the protein does not change substantially in size during purification. Our results differ from those of Wiederrecht *et al.* (1987), who reported the isolation of a 70-kd HSE-binding protein. We have found that mild proteolysis generates a 70-kd DNA-binding fragment from the 150-kd protein (Figure 7); it is possible that they have purified this proteolytic fragment.

It is likely that the protein we have purified is a heat-shock transcription factor. First, this protein accounts both quantitatively and qualitatively for all the HSE-binding activity detectable in crude extracts using the gel retardation assay, and we can detect no other protein with equivalent properties. Second, the affinity of the protein for variant HSE sequences, as determined by competition assays and by direct binding to various labelled probes *in vitro*, correlates well with the activity of the corresponding HSE sequences *in vivo*. The correlation is not quantitative, in that binding sites which show very high affinity *in vitro* stimulate transcription only slightly more efficiently than sites with moderate affinity. This is not unexpected: once the interaction between the HSE and the protein is tight enough to ensure a high occupancy of the site *in vivo*, an increase in binding affinity would have only a moderate effect on gene activity.

Although one would expect a heat-shock transcription factor to be active only in stressed cells, we did not have to heat shock the yeast to obtain binding activity. One explanation for this might be that the cells were stressed inadvertently. However, using yeast transformed with a heat-inducible reporter gene we have found tht the conditions of growth and harvesting used in this work do not induce expression of heat-shock genes (Sorger *et al.*, 1987). We therefore favour the hypothesis that the protein we have purified is an unactivated form of the transcription factor which, although able to bind DNA, is unable to interact productively with other components of the transcriptional machinery. Activation of transcription might then be accomplished by modification of the factor, allowing the formation of a functional transcription complex. We have recently found that heat shock induces a change in the electrophoretic mobility of protein–HSE complexes, which is consistent with this model and supports the notion that the 150-kd protein is involved in heat-shock gene activation (Sorger *et al.*, 1987). It should now be possible to purify the protein from heat-shocked yeast, and identify any modification that has occurred.

Materials and methods

Plasmids, probes and oligonucleotides

The variant HSEs pHSE1, pHSE2 and pHSE4 were constructed by ligating the adapter CTAGAAGCTT into the *Xba*I site of pUC19 as described for the construction of the pTKs plasmid series (Pelham and Bienz, 1982). pHSE12 consists of the *Sal*I–*Bam*HI fragment of pTKS12 inserted into the corresponding sites in pUC19. pHSE27 consists of bp −330 to +87 of the *Drosophila* HSP27 promoter inserted into the *Hin*cII site of pUC12. The HSE27 fragment used for binding assays and for insertion into the *lacZ* vector described below consists of bp −330 to −228 and contains the proximal overlapping HSP27 HSE (called HSE1 in Riddihough and Pelham, 1986). Probes for binding assays were made by filling in the unique *Eco*RI site in each of these plasmids followed by cutting with *Pst*I (or *Aha*III for HSE27) and purification on acrylamide gels. The sizes of the probes were 52 bp (HSE1), 62 bp (HSE2, HSE12), 82 bp (HSE4) and 134 bp (HSE27).

For u.v.-activated photocrosslinking experiments, probes were generated by primed synthesis on either strand of collapsed supercoiled templates (Chen and Seeburg, 1985) using 50 μM 5-bromodeoxyuridine trisphophate and [α-^{32}P]-dATP (800 Ci/mmol) followed by cleavage with *Eco*RI and *Hin*cII and purification on acrylamide gels. For DNase I footprinting experiments, the 425-bp *Bgl*II–*Nru*I fragment of pTKS2 was labelled by filling in the *Bgl*II site.

The *in vivo* activity of the various HSEs was tested by inserting them upstream of a disabled *cyc1* promoter/*lacZ* fusion gene in a yeast vector with a 2-micron origin of replication and the *ura3* gene as a selectable marker. HSEs were inserted as *Sal*I–*Bam*HI fragments (or *Aha*II–*Bam*HI for HSE27) into the unique *Xho*I–*Bgl*II sites of plasmid GA1695, kindly supplied by G.Ammerer. This plasmid was derived from pLGΔ-178 (Guarente and Mason, 1983) by insertion of the synthetic oligonucleotide TCGAGGCCTAATTANGAGATCTG (N = A, C, G or T) into the *Xho*I site at position −178; this reforms a single 5′ *Xho*I site and introduces a 3′ *Bgl*II site.

DNA binding assays

DNA binding assays were performed in a variety of conditions. Typically 125 μg of whole cell extract, 5–10 μg of heparin–agarose fraction, or 0.5–1 μg of calf thymus DNA–Sepharose fraction were incubated wih 0.25–1 ng probe, 20 μg BSA (Boehringer), and 0.5–5 μg pUC19 and/or poly[d(I-C)] (Boehringer) for 30 min at room temperature in 20 mM Hepes pH 7.9, 1 mM $MgCl_2$, 60 mM KCl, 12% glycerol and 1 mM DTT in a reaction volume of 25 μl, bromophenol blue added, and the sample electrophoresed on a 0.75-mm thick 4% acrylamide/0.1% bisacrylamide gel at 300 V for 1 h. The gel buffer was 22.5 mM Tris base, 22.5 mM boric acid and 0.63 mM disodium EDTA. For competition assays a 10–250 molar excess over the probe of unlabelled DNA was included prior to addition of the extract. Affinity-purified fractions (typically 2–5 μl) in 2.0 M NaCl were assayed as described above but in a reaction volume of 40–60 μl in binding buffer lacking KCl but supplemented with 0.1% NP-40 and 0.5 mM spermidine (final NaCl concentration 160 mM or less). Gels were dried and autoradigraphed, and binding activity quantified by counting excised bands.

For footprinting, standard binding reactions containing 2 μg poly[d(I-C)] were adjusted to 5 mM $CaCl_2$ and digested with empirically determined amounts of DNase I for 1.5 min. The reaction was stopped with 25 mM EDTA and 0.1% SDS (final concentrations), phenol extracted, ethanol precipitated, and the DNA fragments anlaysed on a 6% polyacrylamide gel containing 6 M urea.

U.v. crosslinking

Binding reactions contained 0.6 ng continuously labelled probe, 20 μg BSA,

2 μg poly[d(I-C)], 50 ng *Msp*I-cut pUC19 (or 800 ng *Msp*I-cut HSE2 or HSE12 competitor DNA) in the case of HEP and DS fractions and 40 μg BSA, 1 μg poly[d(I-C)] for SS fractions in the buffers described above. After incubation, these were diluted to 60 μl with buffer and spotted on SaranWrap laid on a UV Photoproducts TM20 medium wavelength (302 nm peak emission) lightbox and irradiated for 1–20 min at 4°C. HEP- and DS-fraction-containing samples were extracted with an equal volume of phenol, the organic phase and interface precipitated with 2.5 vol of ethanol on dry ice for 15 min and resuspended in SDS sample buffer. The SS-fraction-containing samples were precipitated by addition of trichloroacetic acid to 10% directly after irradiation. DNase I treatment was omitted as described the Results section.

Protein analysis and proteolysis

Samples of column fractions (50–200 μl) were mixed with up to 20 μg insulin as a carrier and precipitated with 10% trichloroacetic acid. They were then taken up in SDS sample buffer, electrophoresed on 10% polyacrylamide SDS gels, and the gels silver-stained using a kit from New England Nuclear. Mol. wt markers were: ovalbumin, 43 kd; BSA, 67 kd; phosphorylase b, 92 kd; beta-galactosidase, 116 kd; beta/beta′ subunits of *E. coli* RNA polymerase, 151/155 kd; and myosin, 200 kd. For proteolytic cleavage, samples of affinity-purified material (estimated by silver staining to contain 20 ng protein) were digested for varying times with either 0.5 or 10 ng papain in a total volume of 100 μl of 1 M NaCl, 25 mM ammonium sulphate, 20 mM Tris pH 7.5, 1 mM EDTA, 5% glycerol, 0.1% *n*-ocytl-beta-D-glucopyranoside, 5 mM DTT and 0.5 mM cysteine. Samples for protein gels were inactivated by addition of iodoacetic acid to 2.5 mM followed by addition of 2 μg insulin as a carrier and precipitated with 10% trichloroacetic acid. To avoid possible inactivation of binding activity by iodoacetic acid, the activity of digested samples (10 μl) was assayed in standard binding reactions containing 20 μg BSA to quench the papain.

Purification of an HSE-binding protein

Cultures of the multiply protease-deficient *Saccharomyces cerevisiae* strain BJ2168 (*leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* (gift of M.Lee) were grown to late log phase (1×10^8 cells/ml) in YEPD medium in either 10-l bottles, a 40-l fermenter or, more reently, a 400-l fermenter. In a procedure adapted from Klekamp and Weil (1982), harvested cells were resuspended in ~2 vol breakage buffer: 200 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 500 μM TPCK, 25 μM TLCK and 2 μg/ml Pepstatin A and disrupted with 0.5-mm glass beads in a 'Bead Beater' (Biospec Products, Bartlesville, OK, USA) chilled with dry ice. Recently, frozen cell paste has been broken in a blender cooled with liquid nitrogen and the powder thawed in 2 vol breakage buffer. Microscopic examination shows that this procedure results in disruption of >95% of the cells and lysis of their nuclei. Broken cells were extracted for 30 min at 4°C in the presence of 0.4 M ammonium sulphate and centrifuged at 45 000 r.p.m. for 1 h in a Beckman type 45Ti rotor. The supernatant was precipitated with 0.35 g ammonium sulphate/ml, resuspended in a minimum volume (typically 350 ml) of A50 buffer [50 mM $(NH_4)_2SO_4$, 1 mM EDTA, 20 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM DTT with the mixture of breakage buffer protease inhibitors at 1/10 dilution] and exhaustively dialysed against the same buffer. Forty litres of culture typically yielded 500 g of packed cells and 7.5 g of crude extract; the volumes given below are correct for this amount of extract (Table I). Dialysed crude extract (400 ml) was loaded onto a 250-ml heparin–agarose column prepared according to the method of Davidson *et al.* (1979) at a flow rate of 60 ml/h, washed extensively and eluted with a linear gradient of A50–A600 buffer (identical to A50 but containing 600 mM ammonium sulphate; 500 ml of each buffer was used). Activity was monitored using the gel assay with an HSE2 probe and the peak eluting at 150–300 mM salt is referred to as the HEP fraction; the flowthrough contained 25% of the total activity loaded. HEP fraction was concentrated 10-fold with ammonium sulphate as described above, dialysed against A50 and loaded onto a 10-ml calf thymus DNA–Sepharose column (made by coupling sheared DNA to CNBr-activated Sepharose CL-4B as described below for the affinity column) at a flow rate of 8 ml/min. Up to 40% of the binding activity flowed through the column but was fully retained on a second passage, indicating that the 10-ml column had insufficient capacity. The column was eluted with a 50-ml gradient of A50–A600.

The synthetic oligonucleotide CTAGAAGCTT (300 μg; Pelham and Bienz, 1982) was ligated to form double-stranded molecules with lengths of 200–1000 bp, and these were coupled to 2 ml of CNBr-activated Sepharose CL-4B essentially as described by Kadonaga and Tjian (1986). The final 1.7-ml column was estimated to contain 120 μg of DNA. Peak fractions from the calf thymus DNA–Sepharose column (DS fraction) were applied in A80 to this column; binding activity was quantitatively retained. The column was washed extensively with A50 and then eluted with 10-ml aliquots of A50 supplemented successively with 0.5 M KCl, 0.05% NP-40, 1.0 m KCl, 0.05% NP-40; and 2.0 M NaCl, 0.025% *n*-octyl-beta-D-glucopyranoside (Sigma). Peak fractions in the 2.0 M eluate were pooled to form the SS fraction.

The native mol. wt of affinity-purified HSE-binding protein was determined on an HR10/30 Superose 12 f.p.l.c. gel filtration column (Pharmacia) run in A50 containing 5% glycerol, but supplemented with 0.1% NP-40, at 0.2 ml/min. The column was calibrated with BSA, ferritin, catalase and thyroglobulin.

Beta-galactosidase assays

The activity of HSE–*lacZ* constructs was determined in yeast strain MC881 (MATa *ade2-101 ura3-52 suc2-5d9*, kindly provided by Marian Carlson). The heat inducibility of constructs varied with cell density and all cultures were assayed in early log phase at a density of $5-10 \times 10^6$ cells/ml; cultures were grown at 23°C. Beta-galactosidase activity was determined as described by Breeden and Nasmyth (1987) either in the absence of heat shock or after heat shock to 39°C for 20 min followed by recovery for 1 h at 23°C. Similar assays were performed with the strain used for protein purification to assess the effects of growth conditions and harvesting methods on the expression of heat-shock genes. The relative activities of the various HSE sequences was the same when they were assayed on a centromere-containing vector; thus the results are unlikely to reflect differences in copy number of the plasmids.

Acknowledgements

We thank David Shore for much advice, and for the kind gift of chromatographic materials. We also thank Richard Treisman for many helpful discussions, Mike Lewis and Guy Riddihough for the gift of plasmids and probes, Colin Young for assistance with yeast fermentation and Kim Nasmyth for encouragement at a crucial stage in the project. Above all, we would like to thank Gustav Ammerer for assistance, advice and materials without which this research would not have been possible. P.K.S. was supported by a Marshall Commission Scholarship.

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Received on April 21, 1987; revised on June 9, 1987

Note added in proof

We have recently found that the HSE-binding protein purified from heat-shocked yeast has an apparent mol. wt of more than 150 kd, and that this difference is due, at least in part, to phosphorylation of the protein in heat-shocked cells.