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" INTERACTION OF HUMAN HSF-1 WITH PROMOTER DNA "

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Sincerely,

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1. INTRODUCTION

1.1 HEAT SHOCK PROTEINS

Prokaryotic and eukaryotic cells respond to potentially damaging stimuli such as elevated temperature by the rapid, vigorous and transient acceleration in the rate of expression of a small number of specific genes, known as heat shock genes. Consequently the products of these genes collectively known as heat shock proteins (HSPs) or stress proteins increase and accumulate in cells to reach fairly large concentrations. Several of the major heat shock proteins are members of gene families that include proteins normally present in the cell and, in most cases, essential for cell function.

The heat shock proteins can be classified into several major families according to their molecular mass: HSP60, HSP70, HSP90 and a small polypeptide called ubiquitin. Various members of these families are localized to specific compartments of the cell: the mitochondrion, the chloroplast, the nucleus and the endoplasmic reticulum. The studies on these proteins revealed that the heat shock proteins proved to be incredibly highly conserved among widely divergent organisms. For example, the major heat shock protein, hsp70, has about 50% of its sequence conserved between E. coli and human, and some domains are 96% similar (Schlesinger, 1990).

Not only have heat shock proteins been highly conserved in nature at the amino acid sequence level, but they also appear to

carry out similar functions, such as binding to other polypeptides and allowing them to either reach their correct intracellular destination in the cell, transporting other proteins across membranes, or preventing other proteins from misfolding. Studies on hsp70 revealed that the protein is composed of two major domains, the N-terminal ATPase domain and the C-terminal substrate interaction domain, and the hsp70 uses the energy of ATP to perturb the structure and influence the folding and assembly of many different protein substrates. The discovery of the function of heat shock proteins provided the data for the chaperone model. (Molecular chaperones are defined as a family of unrelated classes of protein that mediate the correct assembly of other polypeptides but that are not components of the functional assembled structures). Recently, one of the studies revealed a requirement for an HSP90 chaperone for folding protein by preventing non-native proteins from participating in unproductive intermolecular interaction, and the action of HSP90 does not depend on the presence of ATP, therefore, unlike HSP70, HSP90 may represent a class of molecular chaperones which uses a novel molecular mechanism to assist protein folding (Wiech et al, 1992).

Heat shock proteins not only mediate assembly processes but also promote the disassembly of proteins that have been damaged as a result of stress (Pelham, 1986.). It was suggested that in stressed cells, the heat shock causes proteins to denature and form incorrect aggregates, while heat shock proteins inhibit these process by binding to the interactive surfaces exposed by high temperature and

other stresses (Ellis, 1991). It is believed that the function of HSPs at some stressful conditions is to protect cells from the ill effects of stress, allowing them to recover and survive.

Many studies have shown that a pre-shock treatment can render a biological system more resistant to a subsequent stress and that this protective effect is transient. In most cases increased expression of hsps is accompanied by increased tolerance to extreme temperatures. This phenomenon is called 'induced thermotolerance'. A number of studies have shown a strong correlation between the expression and decay of thermotolerance, and the induction, accumulation and degradation of heat-shock proteins. The abundance of the major 70-kDa heat-shock protein (HSP70) shows the best relation with thermotolerance and is found to be involved in the protection of transfected CV1 monkey cells against a lethal heat treatment (Angelidis et al, 1991). In fruit fly embryos, HSP70 is also a critical thermotolerance factor and is beneficial to the fly embryo during heat shock (Linquist et al, 1993). While in yeast cells, the major protein responsible for thermotolerance is HSP104; HSP104 is also required for other forms of stress in fermenting cells (Sanchez et al 1992).

In medicine, hsp responses have been observed in almost every cell type looked at with a few interesting exceptions, such as certain neurons and testis cells. They are involved in immune responses and serve as important antigens in human nonviral infections. Antigens from a wide variety of infectious agents have been identified as members of stress protein families. The major

stress protein antigen recognized by antibodies in bacterial infections is HSP60. "Common antigen", an immunodominant protein antigen long known to be shared by most bacterial species, turned out to be HSP60 protein that are immunologically cross-reactive with their mammalian counterparts. Stress proteins have also been identified as immune targets in most major human parasite infections. Antibodies to HSP70 have been identified in the sera of patients suffering from malaria and trypanosomiasis. HSP90 is also a target of antibodies in trypanosomiasis. Owing to their extreme conservation, HSPs could also provide a clue to certain autoimmune diseases. For example, rheumatoid arthritis is thought to involve autoimmune processes, the antigen recognized by the arthritogenic T cells was shown to be mycobacterial HSP60 which shows partial homology with the mammalian cognate (Hogervorst et al, 1992). The structural homology between microbial and human stress protein has been postulated to be a basis for autoimmunization in chronic inflammatory disease.

1.2 THE HEAT SHOCK RESPONSE ELEMENT

Heat shock can have dramatic effects at all levels of gene expression. However, induction of hsps involves transcriptional activation of their genes. This regulation is mediated by a common, positive, cis-acting sequence, the heat shock element (HSE), that is present in multiple copies upstream of the transcriptional start site. The HSE was first identified as a short DNA sequence responsible for the heat shock induced expression of transfected hsp70 genes in mammalian cells (Corces et al, 1981; Pelham, 1982; Mirault et al,

1982). Insertion of a short oligonucleotide containing this sequence into a heterologous promoter is sufficient to confer heat inducibility to that promoter (Bienz and Pelham, 1982; 1986). This represents the first occasion by which an enhancer element has been identified using this approach. Mutational analysis and DNA-protein binding studies have revealed cis-acting elements responsible for basal levels of transcription as well as the element responsible for conferring heat inducibility. Sequence comparisons of eukaryotic promoter regions has revealed the presence of a well conserved dyad symmetrical sequence containing the 8 base pair core sequence, CNNGAANNTTCNNG. This so-called Pelham box was found in as many as three overlapping copies in certain promoters (Pelham, 1982). This element is present in varying configuration and copy number in the 5'upstream region of every hsp gene examined.

However, analyses of 5'deletion mutants of the Drosophila hsp70 gene in Drosophila cells (Amin et al, 1985) or germ-line transformants (Dudler and Travers, 1984; Simon et al, 1985) revealed that the promoter-proximal HSE alone, conferred only approximately 1% of the wild-type level of heat-induced expression. The minimal additional upstream sequences that were required to specify the wild-type level of heat-induced hsp70 expression contain an additional partial match to the 14-bp HSE consensus sequence as first noted by Dudler and Travers (1984). Therefore, the idea of the so-called Pelham or 14-bp consensus sequence as a valid description of the HSE has been questioned. Analyses of hsp70 promoter/lacZ hybrid constructs transformed into the Drosophila germ line reveal

that half of a HSE is able to complement a full HSE in terms of transcriptional activation. The HSE has been revised as a contiguous array of the 5-bp module -GAA- (or its complement -TTC-), where each module is inverted relative to the immediately adjacent modules. Examination of the regulatory regions of other hsp genes reveals the presence of tandem arrays of these alternating units (Xiao and Lis,1988; Amin et al, 1988). Alternation of GAA and TTC segments repeated at two bp intervals is the key feature of the HSE with a minimum of three GAA/TTC blocks required for functional in vivo expression in Drosophila.

1.3 HEAT SHOCK TRANSCRIPTION FACTOR

Evidence that HSEs are the binding sites for a specific transcription factor came from in vitro transcription experiments with extracts of Drosophila cells (Parker, 1984; Wu, 1987). The HSE has since been proposed as the site of binding for the transcription factor responsible for heat induced expression of hsp genes. Heat shock transcription factor HSF has been identified and characterized in Drosophila (Parker and Topol, 1984; Topol et al., 1985; Shuey and Parker, 1986), in Hela nuclear extract (Kingston et al.,1987; Morgan et al., 1987) as well as in yeast (Sorger et al., 1987; Slater and Craig, 1987). Given the rapid response of hsp expression, HSF is thought to preexist as an inactive form in unstressed cells. In addition, studies with protein synthesis inhibitors in Drosophila (Zimarino and Wu,1987), Xenopus (Zimarino et al., 1990), and in human cells (Kingston et al., 1987) indicate that de novo protein synthesis is not

required for heat shock induction. This supports the notion that HSF is present in equal amounts in both induced and non-induced states.

HSF from both S.cerevisiae (Sc-HSF) and Drosophila (D-HSF) are protein trimers in solution and when bound to DNA (Perisic et al., 1989; Sorger and Nelson, 1989). Each subunit of a D-HSF multimer is thought to bind to a single nGAAn unit, and the binding to successive units is highly cooperative (Xiao et al., 1991). The binding of trimers to adjacent sites is also highly cooperative (Shuey and Parker, 1986): in vitro, the dissociation rate from DNA of a complex of two DNA-bound trimers is more than three orders of magnitude lower that of a single trimer (Xiao et al., 1991).

HSF has been purified to apparent homogeneity in Drosophila (Wu et al., 1987; Wiederrecht et al., 1987), in yeast (Wiederrecht et al., 1987; Sorger and Pelham, 1987), and partially purified from Hela cells (Goldenberg et al., 1988). The gene encoding yeast S.cerevisiae HSF has been cloned and sequenced independently (Sorger and Pelham, 1988; Wiederrecht et al., 1988). Since then the corresponding data on HSF genes have been provided for several other organisms, including Drosophila (Clos et al, 1990;), humans (Rabindran et al,1991; Schuetz et al,1991), mouse (Sarge et al, 1991), the yeast K.lactis (Jakobsen and Pelham,1991), and tomato (Scharf et al,1990). Surprisingly, there are three different HSF genes in tomato, two of which are themselves heat stress-inducible (Scharf et al., 1990). But humans and mouse have two HSFs with obvious structural and functional differences. Sequence comparison and functional analysis have shown that despite the strong phylogenetic

conservation of heat shock proteins and the heat shock element sequence, the predicted amino-acid sequence of the cloned HSFs are highly divergent, except for the DNA binding domain of 90 to 100 amino acid residues in the N-terminal part of all HSFs, two to three hydrophobic repeats of the leucine-zipper-type required for oligomerization of HSF subunits and C-terminal activation domains. Further analysis of the activation domains reveals the presence of distinction: one involved in transcriptional repression during non-heat shocked (constitutive) conditions and the other responsible for transcriptional activation upon heat shock (Sorger, 1990; Nieto-sotelo et al., 1990).

Since it is evident that inducible hsp70 expression is controlled by transcriptional activation via HSF, the question that remains is how the heat shock signal is transduced to an inactive preexisting HSF protein. In Drosophila, human, and S. pombe cells, heat shock factor binds to DNA only after heat shock (Gallo et al., 1991). This suggests a simple mechanism for induction in which acquisition of the ability to bind DNA allows heat shock factor to interact with and activate heat shock promoters. In S.cerevisiae, however, Sc-HSF is bound to DNA both before and after heat shock. This suggests that different mechanisms of activation may exist for yeast and animal cells despite the evolutionary conservation of the HSE and the heat shock response in general. Both hu-HSF and Sc-HSF become highly phosphorylated following heat shock. Heat shock factor prepared from unshocked animal cells can be induced to bind DNA in vitro by exposing cell extracts to elevated temperatures or to reagents that

favor the dissociation and denaturation of protein complexes (Larson et al., 1988). However, recombinant D-HSF produced in E.coli binds to heat shock elements with high affinity in the absence of treatment with heat or denaturants (Clos et al., 1990). This suggests that heat shock factor may interact with one or more negative regulators found in eukaryotic cells and that the interactions can be disrupted in vitro by conditions that mimic the effects of heat shock (Clos et al., 1990).

Recently, there are several speculative possibilities that could be responsible for the activation of HSF:

1: Protein conformational change: HSF activation, as analyzed in vitro by gel electrophoresis retardation, is brought about by high temperature, chaotropic agents, pH shifts, or detergent, thus indicating that a conformational transition is responsible for promoter recognition (Mosser et al. 1990).

2. Removal of a negative regulation: the demonstration of a direct interaction between HSP70 and HSF in Hela cells (Voellmy, 1991) supports the speculation that HSP70 acts as an inhibitory subunit of the HSF. It is supposed that hsps bind to and repress heat shock factor activity under normal growth conditions. During heat shock, competition with high levels of thermally damaged proteins for binding to hsps causes the dissociation of heat shock factor-HSP complexes and a consequent increase in DNA-binding affinity of the factor (Morimoto, 1993)

3. Oligomerization of HSF subunits, which involves the hydrophobic repeats, is required for function. By interaction with the hs promoter, HSF trimers found in solution are assembled into hexamers (Rabindran et al, 1993).

4. Phosphorylation is not primarily involved but may contribute to the sustained response under high stress conditions.

There is some evidence to support the above speculations, but more experiments are needed to test these ideas.

2 AIM OF THIS STUDY

Regulatory regions of eukaryotic genes usually contain multiple DNA sequence elements to which regulatory proteins bind. These elements often act synergistically to provide a level of transcription that greatly exceeds the additive effects of single elements.

The natural heat shock regulatory regions usually contain multiple short arrays or a single long array of 5bp units. Arrays of 5bp units have been shown to act synergistically on the in vivo expression of heat shock gene, and the activity of two or more trimeric binding site is required for the transcriptional induction of heat shock genes. The mechanism underlying this requirement is not clear. Recently Xiao et al explained that highly cooperative binding of Drosophila HSF to HSE is presented. Although this explanation appears to be valid in Drosophila assays, it is not clear that it is also functional in human HSF.

The aims of the research presented in this thesis are threefold. The first aim of this study was the establishment of a purification scheme of human HSF1 from an expression vector in E.coli cells. This HSF preparation would subsequently be used during the analysis of protein/DNA interactions. Secondly, a purified hu-HSF1 was used to analyze detailed DNA/protein interactions, especially the cooperativity of the binding, using constructed HSEs as probes. The data obtained from this part was further quantitatively analyzed with a model appropriate for studying the cooperativity, and the

resulting parameters were compared. Finally, the oligomeric state of purified HSF was also investigated to help explain DNA/protein interaction on the basis of protein structure.

3 MATERIALS AND METHODS

3.1 PLASMID CONSTRUCTS

The 2.0 kilobasepair Xho I-Bgl II fragment corresponding to the human HSF-1 amino acids 1-529 contains the coding region and 3'-nontranslated sequences (Rabindran et al, 1991). This fragment was inserted at the Xho I site of plasmid pGEX-KG vector, which is a derivative of vector pGEX-2T with new polylinker sequence. The vector, pGEX-KG, can direct the expression of foreign polypeptides as fusion proteins with glutathione S-transferase (GST) and allow the thrombin cleavage of the fusion protein more efficiently (Guan and Dixon,1991). The complete HSF-1 cDNA, starting with the Met codon, was placed in the correct reading frame downstream of the Arg-Gly thrombin cleavage site. Because of the introduction of a linker located immediately following the thrombin cleavage site, the resulting protein should contain a short Gly-rich leader (GSPISGGGGGILDSMGRLE) preceeding the first codon of HSF-1.

3.2 EXPRESSION OF THE CLONED HU-HSF1

For expression, the recombinant pGEX-KG plasmids were transformed into E. coli strain JM109 (Yanisch-Perron et al. 1985). Cultures (2000ml of LB media with 100ug/ml ampicillin) were inoculated with 100ml of an overnight culture and grown at 37°C with shaking (200rpm) until bacteria reached a density of OD600=1.2. HSF-GST expression was then induced with final concentration of 0.1mM IPTG at 37°C overnight. Bacteria were harvested by centrifugation at 5500rpm (5000x gav) for 15 minutes

at 4°C and the cell pellet was lysed in 1x lysis buffer (60mM KCl, 20mM HEPES (pH7.9), 1mM EDTA, 5mM $MgCl_2$, 2mM dithiothreitol (DTT), 0.1mM phenylmethylsulfonyl fluoride (PMSF), 2mg/ml of benzamidine, 1ug/ml of pepstatin A, 4µg/ml of leupeptin, 10µg/ml of aprotinin, and 20ug/ml of soybean trypsin inhibitor) with two consecutive cycles of freezing (-80°C) and thawing (37°C). After a 45 min spin at 50,000rpm (Beckman, Ti70 rotor), the pellet was resuspended in 100ml of 4M guanidine-HCl (Bethesda Research Laboratories) and placed on a rotating wheel for 30 min on ice. Insoluble material was separated by centrifugation for 30 min at 50,000rpm (Beckman Ti70 rotor). The guanidine-HCl was removed from the extract by stepwise dialysis at 4°C in 1xPBS (137mM NaCl, 2.7mM KCl, 10.1mM Na_2HPO_4, 1.7mM KH_2PO_4) with 1mM DTT (Waterman et al., 1991). The white precipitate which appeared during the dialysis was removed by spinning down the sample at 2000rpm. Then the supernatant was loaded at room temperature to a 3.5ml column of glutathione-Sepharose beads (Pharmacia) which is balanced with 1xPBS/1% Triton X-100. Afterwards the column was washed with 30ml PBS/DTT and 20ml thrombin cleavage buffer (2.5mM $CaCl_2$, 50mM Tris·Cl pH7.5, 150mM NaCl). GST carrier was removed by adding 10ml of 1ug/ml thrombin in thrombin cleavage buffer. The column was sealed well and shaken at room temperature for 45 min. Released protein was recovered by washing beads with 15ml of H(7.0)G5ED NaCl100 buffer (20mM Hepes pH7.0, 5% glycerol, 0.1mM EDTA, 100mM NaCl, 1mM DTT) at 4°C. The recovered HSF was concentrated by Centricon membrane centrifugation (Amicon) at 4°C and then stored in aliquots at -80°C. The protein concentration was

determined by protein assay with Coomassie blue protein assay reagent (Pierce).

3.3 SDS-PAGE ANALYSIS

The protein content of the samples taken from every step of HSF purification was examined by adding SDS sample buffer (62.5mM Tris· HCl pH6.8, 10% glycerol, 2% (w/v) SDS, 5% 2-mercaptoethanol, 0.012%(w/v) bromophenol blue) to the sample and boiling for 2.5 minutes. The denatured samples were immediately loaded on discontinuous (Laemmli,1970) gel consisting of a separating (lower) gel (7.5% acrylamide, 37:1 acrylamide:bis, 0.375M Tris·HCl pH8.8, 0.1%(w/v) SDS, 0.05% ammonium persulfate, 0.05% TEMED) and a stacking (upper) gel(3.9% acrylamide, 37:1 acrylamide:bis, 0.05% ammonium persulfate, 0.1% TEMED) along with high molecular weight protein size markers (Bio-Rad) and electrophoresed in running buffer (3mg/ml Tris base, 14.4mg/ml glycine, 1mg/ml SDS) at 200 volts for 45 minutes. After staining with 0.1% Coomassie blue R-250 in fixative (40% methanol, 10% HoAC) for 15 minutes and destaining with 40% methanol/10% HoAC to remove background, gel was dried between cellophane at 60°C for 0.5 hour.

3.4 PREPARATION OF SYNTHETIC OLIGONUCLEOTIDES

Synthetic double stranded oligodeoxyribonucleotide AGAACGTTCTAGAAC corresponds to genetically idealized HSE (Cunniff, N.F.A., and W.D. Morgan. 1993) and was purified by preparative gel electrophoresis on a thick 20% polyacrylamide (19:1 acrylamide:bis) gel containing 8.3M urea. The most prominent band

(as visualized by UV shadowing) was excised and eluted by diffusion in TE/0.1M NaCl overnight following by two ethanol precipitations in 0.3M sodium acetate pH5.0,10mM $MgCl_2$. DNA was dissolved in TE and the concentration was determined spectrophotometrically at wavelength 260nm using the relationship $1A_{260}U$= 30ug/ml.

3.5 LIGATION OF SYNTHETIC OLIGONUCLEOTIDES

2.0ug of purified synthetic 15-mer oligonucleotide was incubated with cold 1.5ul 10mM ATP, 10units T_4 polynucleotide kinase in kinase buffer I (50mM Tris·HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA) in a 10ul volume. The reaction was incubated at 37°C for 1 hour. To ensure that the kinase reaction was carried out completely, 1ul of 10mM ATP, 7.5units of T_4 polynucleotide kinase in kinase buffer I were added to the reaction again and incubated for another 1 hour at 37°C before heat inactivation at 65°C for 15 minutes. The reaction was then added with 1ul (10ug/ul) glycogen, 1ul 1M $MgCl_2$, 10ul 3M NaoAc pH5.0 and precipitated with 300ul of 100% ethanol. The pellet was redissolved in 20ul TE buffer and incubated with 4.5ul 10mM ATP, 24units T_4 DNA ligase (Pharmacia) in ligase buffer (20mM Tris·HCl pH7.6, 5mM $MgCl_2$, 5mM dithiothreitol, 50ug/ml bovine serum albumin) in a 45ul volume at 0°C for only 2 minutes. The reaction was stopped by phenol/chloroform, chloroform extraction, and followed by twice ethanol precipitation. The pellet was then resuspended in 20ul TE/0.1M NaCl pH8.0.

3.6 TRANSFORMATION OF RECOMBINANT PLASMIDS INTO E· COLI

The oligonucleotide pellet resuspension was ligated with dephosphorylated pUC118-Sma I vector in reaction containing ATP, T4 DNA ligase in ligase buffer (20mM Tris·HCl pH7.6, 5mM $MgCl_2$, 5mM DTT, 50ug/ml bovine serum albumin) and incubated. Transformation of the ligated DNA into competent E·coli strain JM109 (Yanisch-perron et al., 1985) was carried out as follows: 50ul JM109 calcium chloride competent cells was mixed with 5ul reaction product above and incubated on ice for 30 minutes. Then the mixture was transferred to a 42°C water bath for 90 seconds before placing it on ice again for 2 minutes. 1ml LB media without ampicillin was added to the reaction and placed in 37°C water bath to warm up for short time. The cells were grown in this media at 37°C for 45 minutes with vigorous shaking (200rpm). Then 50-100ul of the culture was transfered to LB/Amp, X-gal, IPTG plates (100ug/ml Ampicillin, 40ug/ml X-gal, 40ug/ml IPTG) and spread. The plates were placed in 37°C incubator overnight. The white colonies were picked up, grown in 5ml LB/Amp media at 37°C overnight with vigorous shaking. Then the culture was subjected to a mini-prep.

3.7 PLASMID MINI-PREP

The culture was centrifuged, and the medium was removed by aspiration, leaving the bacteria pellet as dry as possible. The pellet was resuspended by vortexing in 100ul of an ice-cold solution containing: 50mM glucose, 10mM EDTA, 25mM Tris-HCL pH8.0 and stored at room temperature for 5 minutes. Then 200ul of a freshly prepared solution containing 0.2N NaOH, 1% SDS was added to the resuspension, mixed by inversion and incubated for 5 minutes on ice.

Then another 150ul of ice-cold potassium acetate (pH4.8) (The solution is 3M with respect to potassium and 5M with respect to acetate.) was added, mixed by inversion for 10 seconds and incubated on ice for 5 minutes. The incubation was stopped by centrifugation for 5 minutes and the supernatant was transfered to a fresh tube, avoiding the white precipitate. This step was repeated once. RNase A was added to the supernatant to a final concentration of 20ug/ml, and was placed at 37°C to incubate for 20 minutes. Then an equal volume of phenol/chloroform was added, mixed, and centrifuged. The aqueous phase was transfered to a fresh tube and precipitated with 2.5 volume absolute ethanol at -80°C for 30 minutes. The DNA precipitate was obtained by centrifugation, rinsed once with 70% ethanol, and dissolved in 16.8ul of deionized water. Plasmid DNA was precipitated again by adding 3.2 ul 5M NaCl, 20ul 13% PEG (polyethylene glycol; MW 8,000) on ice for 30 minutes. Then the tube was centrifuged and the pellet was washed with 70% ethanol, dried and dissoved in 10ul of sterile deionized water.

3.8 DOUBLE STRANDED DNA SEQUENCING

Double stranded DNA sequencing (Chen, E.Y.and P. H. Seeburg 1985) is favored because of its simplicity, and convenience. However, some factors had limited the popularity of this method until the modified T7DNA polymerase (Sequenase™) replaced the Klenow polymerase I (Tabor, S. and C.C. Richardson. 1987. 1989). Since then it has become very common for sequencing. The primary procedure is according to Sequenase™(United States Biochemical Corporation, Step-By-Step Protocol For DNA Sequencing with Sequenase™

Manual). The template was prepared by adding NaOH to 0.2M and EDTA to 0.2mM to 3ug purified plasmid DNA in a 20ul volume and incubating at room temperature for 5 minutes. Then the solution was neutralized by adding 2ul of 2M NH_4Ac (pH4.6), mixing well on ice. The DNA was precipitated at 4°C, washed with 70% ethanol and dried in a Speedvac (Zhang et al. 1988.). The DNA pellet was dissolved in 7ul deonized H_2O. 1ul reverse sequencingprimer and 2ul reaction buffer (200mM Tris·HCl pH7.5, 100mM $MgCl_2$, 250mM NaCl. USB) were added to the DNA. The solution was mixed well, heated at 65°C for 2 minutes and then gradually cooled down to 30°C. Once below 30°C, the solution was placed on ice. To this template-primer solution, 1ul of 1:20 diluted labelling mix (0.375uM dGTP, 0.375uMdCTP, 0.375uMdTTP. USB), 0.5ul 10uci/ul (alpha-^{35}S)dATP, 2ul of 1:10 diluted sequencing version 2.0 enzyme(enzyme dilution buffer: 10mM Tris·HCl pH7.5, 5mM DTT, 0.5mg/ml BSA. USB) were added, mixed and incubated 1 minute at room temperature. The reaction was terminated by adding 3.5ul of the reaction to each prewarmed 2.5ul of ddGTP, ddATP, ddTTP and ddCTP(USB) aliquot and incubated at 37°C for 5 minutes. Then 4ul stop solution I (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each tube, mixed thoroughly and heated up at 80°C for 2 minutes. Afterwards 3ul of this solution was loaded on each lane of prerun 6% acrylamide gel (19:1 acrylamide:bis). Gel was transferred to Whatman 3MM filter paper, dried, and exposed to X-ray film (Kodak XAR-5) at room temperature for several days. Four expected sequences were read out in Fig 4 and were named 15mer,30-rev,30mer and 45mer.

3.9 PROBE PREPARATION

Probes for these experiments were prepared as follows: pUC118-15-mer, pUC118-30-mer, and pUC118-45-mer cut with Hind III (for top strand labelled probes) or pUC118-30-rev cut with EcoR I (for bottom strand labelled probes) were dephosphorylated with calf intestinal alkaline phosphatase, purified, and stored until further use. Labelling at the 5'end was conducted as follows: A 2-4 ug aliquot of linearized dephosphorylated plasmid, 150-200 uci of (r-^{32}P)ATP(6000ci/ml), and 10 units of T4 polynucleotide kinase in kinase buffer I (50mM Tris·HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA) in a 10ul volume. After incubation at 37°C for 1 hour following heat inactivation at 65°C, DNA was cut with a second enzyme (EcoRI for pUC118-15-mer,30-mer,45-mer; Pst I for pUC118-30-rev). Labelled probe was separated from labelled vector fragment by preparative 8% polyacrylamide gel electrophoresis followed by elution with TE buffer (10mM Tris, 1mM EDTA) and ethanol precipitation. The resulting series of probes denoted 15-mer, 30-rev, 30-mer and 45-mer were of size of 66, 69, 81 and 96 base pairs respectively.

3.10 BAND SHIFT ASSAY ANALYSIS

Band shift assays (Fried and Crothers, 1981; Garner and Revzin, 1981) were used as the primary assay for the detection of HSE binding activity of the purified cloned HSF1. The assay was performed as previously described with the use of the alternating copolymer poly(dI·dC)·poly(dI·dC) as a non-specific competitor DNA.

Typically, reactions were performed in a 15ul volume containing 5000-10,000cpm of 5'end ^{32}P labelled 30-mer (Hind III*-EcoR I) probe, 1ul; 10A_{260}U/ml poly(dI.dC)·poly(dI.dC),1ul; and 1ul of purified and concentrated HSF in H(7.0)G5ED NaCl100 buffer (20mM Hepes pH7.0, 5% glycerol, 0.1mM EDTA, 100mM NaCl, 1mM DTT). Incubations were performed on ice for 30 minutes. The reactions were loaded on a 4% polyacrylamide (79:1 acrylamide:bis in 10mM Tris-acetate pH7.5, 1mM EDTA, 0.06% wt./vol.ammonium persulfate, 0.06%vol./vol. TEMED). Buffer circulation was performed during the run which was conducted at 10V/cm for 1.5 hours at room temperature. Gel was transferred to Whatman 3MM filter paper, dried, and exposed to X-ray film (Kodak XAR-5) with an intensifying screen (Dupont Cronex Lighting Plus or Quanta III) at -80°C overnight.

3.11 MULTICOMPONENT BAND SHIFT ASSAY ANALYSIS

Multicomponent band shift assay were performed as previous described by Cunniff et al (Cunniff et al., 1991). Four labeled restriction fragments of different size (approximately 10^5 cpm of each) were incubated in H (7.0)G5ED NaCl100 buffer with 1.5ul of 10A_{260}U/ml poly(dI-dC)·poly(dI-dC) and 1ul of purified HSF at 0°C, 25°C, 37°C and 42°C respectively for 3 hours, and then electrophoresed on a 4% polyacrylamide (79:1 acrylamide:bis) as typically used for band shift assays. Circulation of gel buffer (10mM Tris-acetate pH7.5, 1mM EDTA) was performed during the run which was conducted at 10V/cm for 1.5 hours. The wet gel was exposed overnight at 4°C after which the autoradiogram was used to locate

and excise protein-bound and free probe bands. DNA was eluted by casting gel slice in 0.75% agarose in 1x TBE buffer (89mM Tris.base, 89mM boric acid, 2mM EDTA) followed by electroelution onto NA-45 membrane (Schleicher and Shuell) (Gilman et al., 1986). DNA was recovered by incubation of membrane strips in 150ul high salt buffer (20mM Tris-HCl pH8.0, 1mM EDTA, and 1.0M NaCl) at 65°C for 45 minutes. This eluate was removed and the procedure was repeated with fresh buffer(150ul). Cerenkov counting was performed on the combined eluates and each sample was appropriately diluted so as to contain equivalent counts per equal volume (300ul). To each combined eluate, 10ug of carrier yeast RNA was added followed by phenol/ chloroform extraction, chloroform extraction and precipitation with 1ml 100% ethanol. DNA was resuspended in 0.3M sodium acetate pH5.0 and precipitated with 3 volumes of 100% ethanol followed by a 70% ethanol wash. DNA was dried and resupended in 1 volume 0.1M NaOH, and 2 volumes of loading buffer containing formamide (90mM Tris-borate pH8.3, 2mM EDTA, 90% vol./vol. formamide, 0.1% wt./vol. xylene cyanol FF, 0.1wt./vol. bromophenol blue), heat denatured at 90°C for 3 minutes then applied to and electrophoresed on a 8% sequencing gel. Gel was transferred to Whatman 3MM filter paper, dried, and exposed to X-ray film (Kodak XAR-5) with an intensifying screen (Dupont Cronex Lightning Plus or Quanta III) at -80°C for sevral days.

3.12 DISSOCIATION RATE ANALYSIS

Dissociation determination was performed as previously described (Li et al. 1989). It was carried out by incubation of labeled

DNA fragment (~8000cpm) carrying different number of 5bp units with 6ul of puried HSF (0.12ug/ul) along with 6 microlitre of poly(dI·dC)·poly(dI·dC) $10A_{260}$U/ml in H(7.0)G5ED NaCl100 buffer in a 60ul volume at 0°C, 25°C, 37°C and 42°C respectively for 30 minutes. For time zero, a 10ul of aliquot was removed and loaded directly onto a prerun, 4% native polyacrylamide gel (79:1 acrylamide:bis) as typically used for band shift assays. Circulation of gel buffer (10mM Tris-acetate pH7.5, 1mM EDTA) was performed during the run which was conducted at 5V/ml. Afterwards, a 500 fold molar excess of competitor 60-mer (111bp of Hind III and EcoR I fragment containing 12 5bp units) was added to the reaction and 12ul of aliquots were removed and loaded on the gel at subsequent time points. Because it is the electric field in the gel that separates the competitor DNA from the bound DNA and thus marks the time point, the gel was left running as the samples for each time point were loaded. When all the samples were finished loading, the power condition was adjusted to 10V/cm for 1.5 hours at room temperature. Gel was transferred to Whatman 3MM filter paper, dried, and exposed to X-ray film with an intensifying screen (Dupont Cronex Lightning Plus or Quanta III) at -80°C overnight or for several days.

3.13 BINDING TITRATION ANALYSIS

5'end labeled DNA probe (2.85fmol) was incubated with 10ul, 7.5ul, 5ul, 4ul, 3ul, 2.5ul, 2.0ul, 1.5ul, 1.0ul and 0.5ul of HSF (0.12μg/ul) in a 50ul of volume. The incubations were carried out at 0°C, 25°C, 37°C and 42°C respectively for 3 hours. Then the samples

were loaded on 4% native acrylamide gel (79:1 acrylamide:bis),dried and exposed to X-ray film as typically used for band shift assays.

3.14 PORE EXCLUSION LIMIT ELECTROPHORESIS ANALYSIS

To estimate the size of the purified HSF protein molecules, "Gradipore electrophoresis" in polyacrylamide gels of graded porosity (Arcus, 1970. Andersson et al., 1972) was performed. The principle of the separation is that the proteins migrate through progressively smaller pores, the sizes of which are regulated by the gel concentration, and finally tend to stop and concentrate where the pore size is too smalll to allow further migration. The assay was carried out as follows: ten microliters (1.2ug), twenty microliters (2.4ug) and thirty microliters (3.6ug) of purified HSF and high molecular weight marker proteins (Sigma) were electrophoresed on a 4% polyacrylamide (37:1 acrylamide:bis, 0.5xTBE, 3% glycerol)-20% polyacrylamide (37:1 acrylamide:bis, 0.5xTBE, 15% glycerol) gradient gel in 0.5xTBE. Electrophoresis was continued for at least 24 hours at 4°C (10V/cm). The long duration of electrophoresis was necessary for proteins to migrate to their exclusion limit (Andersson et al., 1972). The gel was stained with 0.1% coomassie blue R-250 (Pierce) in fixative (40% methanol, 10% HoAC) for 1 hour, destained with 40% methanol/10%HoAC to remove background for 3 hours, equilibrated in water, then dried between cellophane at 45°C for 3-4 hours.

3.15 DENSITOMETRIC ANALYSIS

Autoradiograms from 1. multicomponent band shift assay, 2. dissociation rate determination, 3. binding titration analysis and gel

from pore exclusion limit electrophoresis were quantified by laser densitometry on an LKB 2222-010 Ultroscan XL densitometer and densitometric data was analysed later.

3.16 DNASE I FOOTPRINTING ANALYSIS

DNAse I footprinting analysis (Galas and Schmitz, 1978) was performed essentially as previously described (Dynan, 1987; Briggs et al., 1986; Jones et al., 1985). The following components were assembled in microtubes on ice: 10ul of 10%wt./vol. polyvinyl alcohol, 0.5 fmol of 5'end labelled probe 15-mer (or 30-mer, 30-mer-y, 45-mer), 0.5ul poly(dI-dC)·poly(dI-dC) 10A260U/ml, 2.5ul of purified cloned HSF-1 (0.12ug/ul)in H(7.0)G5ED Nacl100 buffer, and water to a final volume of 50ul. Reactions were incubated for 20 min on ice. Fifty microlitres of a solution containing 5mM CaCl2, 10mM MgCl2 was added and incubated for 1 min at room temperature then 1.0ul of 10 ug/ml DNAse I (Boehringer Mannheim) was added and incubated for 1 minute at room temperature. Reactions were terminated with the addition of 90ul of a stop solution consisting of 0.2M NaCl, 20mM EDTA, 1% wt./vol. SDS, and 250ug/ml carrier yeast RNA. Reactions were then extracted with phenol/chloroform, chloroform and ethanol precipitated followed by a 70% ethanol wash. Dried pellets were redissolved in 1 volume 0.1M NaOH, and 2 volumes of loading buffer containing formamide, heat denatured, applied to and electrophoresed on a 8% sequencing gel. Gel was exposed to X-ray film (Kodak XAR-5) with an intensifying screen (Dupont Cronex Lightning Plus or Quanta III) at -80°C for several days.

4. RESULTS

4.1. HUMAN HSF1 EXPRESSED IN E. COLI CELLS IS ACTIVE AS A DNA BINDING FACTOR

The 529-amino acid ORF of hu-HSF1 was expressed in E.coli by using the GST-fusion expression system. Human HSF1 cDNA was inserted into pGEX-KG vector and specifically expressed as glutathion-S-transferase (GST)-HSF fusion protein in E.coli strain JM109. The expression was very successfully carried out under the induction of IPTG. To increase the production, prolonged culture was performed, and no bacteria concentration decline was observed on overnight culture. The fusion protein (M_r: 96kDa, estimated by SDS-PAGE) was soluble in aqueous solutions and was purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione. The human HSF1 protein was then cleaved from the GST carrier by digestion with site-specific protease thrombin which recognizes the amino acid sequence between the GST carrier and the human HSF. The whole procedure was carried out at low temperature except the thrombin cleavage step, and the fusion protein and cleaved human HSF1 were stable during the whole process. The purified HSF protein production was found to be at a relatively high level with yields of total protein concentration up to 2ug per ml media. The apparent molecular weight of the recombinant protein as measured by SDS-PAGE is 70kDa, which is concordant with the previous report (Rabindran et al, 1991), but lower than the apparent 83-kDa mass of the purified

protein from human cells (probably due to the absence of post-translational modification) (Figure 2).

Band shift assay analysis of purified extract was initially used to create the criteria for the identification of HSF binding activity. Human HSF was detected by its ability to retard the migration of a ^{32}P-HSE containing oligonucleotide probe upon native polyacrylamide gel electrophoresis (band shift assay). Using HSE24 (a 24bp synthetic double stranded oligonucleotide with the HSP70.1 promoter HSE sequence: AACCCCTGGAATATTCCCGACCTG) and 30-mer as radio labelled probes, extracts prepared from E. coli expressing the human HSF1 showed specific binding to the HSE in the absence of heat shock (Figure 3). The uppermost complex is the HSF specific complex which was verified by using competition band shift assay in previous work (Cunniff et al, 1991). This recombinant hu-HSF1 protein capable of functioning as a transcription factor suggests that, unlike cell extracts from unshocked animal cells where some treatments including exposing cell extracts to elevated temperature or to reagents that favor the dissociation and denaturation of protein complex are required to induce HSF, cloned HSF protein synthesized in bacteria has an intrinsic affinity for DNA. This observation is in agreement with previous reports (Rabindran et al.1991)

4.2. COOPERATIVE BINDING OF HU-HSF1

The cooperative binding of HSF appears to be important since the natural heat shock regulatory regions often contain multiple short arrays or a single long array of 5bp units. In mammals these

appear to display typical enhancer properties with more sites required when located at a greater distance from the TATA box (Bienz and Pelham, 1986). The HSP70 HSE by in vivo genomic footprinting shows strong interactions in the centre of the element (Abravaya et al.,1991) which is in good agreement with the primary high affinity in vitro binding site (Cunniff et al., 1991), and weaker interactions at more sites flanking this region. This may result from assembly of more than one HSF oligomer. It is not known how much the weaker interactions are involved in transcription activation. Cooperativity of binding has been studied on the natural HSP70 HSE and extended HSE mutants. The sequence differences between individual 5bp motifs complicates this analysis. For this reason we prepared synthetic HSEs consisting of multiple repeats of the same 5bp element.

The chosen 5bp consensus sequence was based on genetic analysis in our lab (Cunniff and Morgan., 1993). This work demonstrated that outer position 1 and 5 of the 5 base pair motif NGAAN, in addition to the most conserved triplet in the center can affect the activity dramatically, and the optimal activity occurred with the sequence AGAAC, thus, a HSF binding site can form a complex of lower free energy and, therefore, greater stability (Cunniff and Morgan., 1993). According to these results, we prepared 4 constructs, named 15mer, 30-rev, 30mer and 45mer containing various numbers of genetically idealized 5bp sequence AGAAC (see figure 4). There is a hypothesis which proposes that HSF subunits interact tightly to form a homotrimer and each subunit of HSF binds

to one 5bp unit (Perisic et al., 1989; Peteranderl and Nelson, 1992). Therefore the 15mer construct contains one trimeric binding site, 30-rev and 30mer contain two trimeric binding sites, 45mer contains three trimeric binding sites.

The specific binding of HSF in vitro to these constructs was first examined directly by DNase I footprinting (Figure 5). DNase I protection allows the identification of DNA binding sites by exclusion of a cutting agent (DNase I) from a DNA region protected by a bound protein. As expected, the protected region in 15mer construct lies about 20-bp, 30mer and 45mer templates show extended protection region with the longer constructs. 30-rev gives an apparently different footprint because the probe labelling was at a distinct site on the bottom strand. These results demonstrated that the length of the footprint is proportional to the number of 5bp repeats in the arrays. Figure 5B is the summary of protection results with all four constructs. The protection patterns were consistent with prevous reports and with a model in which an HSF trimer interacts with 15bp regions (Perisic et al., 1989).

To further understand the significance of these arrays of the 5bp units, I tested the properties of these DNA fragments binding to the purified hu-HSF-1 protein at different temperature including heat shock temperature 42°C using the multicomponent band shift assay.

The procedure of this method consists of two steps. The first step is incubation of HSF with the end-labeled set of constructed DNA

fragments that contain various arrangements of the 5bp unit in a single binding reaction. The second step is resolution of the complexes from the unbound DNA by native, low-percentage polyacrylamide gel electrophoresis after binding for a time sufficient to achieve equilibrium. This is followed by excision, elution, and application of the resolved bands to a denaturing sequencing polyacrylamide gel. From the ratio of the bound to the unbound DNA, relative binding constants of the DNA fragments that bind to HSF can be determined.

After the incubation, the HSF-DNA complexes were separated with good resolution by gel electrophoresis, as illustrated in Figure 6A. Figure 6B shows a 24hr autoradiographic exposure of a sequencing ladder of the bound and unbound DNA. Quantifying each of the DNA species allowed the determination of relative binding constants. By comparing the distribution of the four probes between their free and complexed states, the equilibrium binding constant of each probe was determined relative to that of the 15-mer(The binding constant was first determined relative to the wild type HSP70.1 sequence on a 24bp oligonucleotide probe (HSE24). But further experiments demonstrated that the affinity of all of the four idealized HSE constructs was much higher than wild type HSE. For example, 15mer consensus element possesses 57-fold higher affinity than the HSE24 (Cunniff and Morgan, 1993). The low signal from the HSE24 oligonucleotide made it inconvenient for use as a standard. Therefore, we chose 15mer as a standard instead of wild type

HSE24). We express the results as the ratio of the binding constant Kn to the value K_{15}:

$$K_n/K_{15} = (C_n/D_n)/(C_{15}/D_{15})$$ (Liu-Johnson et al., 1986)

Where Cn is the concentration of probe n in the bound complex, and Dn is the concentration of free probe n. Table 1 presents the data of such an experiment. The results were plotted in Figure 6C. As can be seen, 30-mer shows a 3-fold higher affinity for HSF than a site containing three properly positioned 5bp units-15mer, at 0°C. When the temperature increases, the affinities increase too, they show 6-fold, 21-fold and 88-fold higher affinities at 25°C, 37°C and 42°C respectively. The temperature plays a same role on the 45-mer in a more apparent way: the affinity of 45-mer for HSF goes from 4-fold higher at 0°C to 132-fold higher at 42°C. These results are consistent with the idea that the HSF binds to 30-mer and 45-mer in a cooperative manner, and it seems that the cooperativity is strongly temperature dependent. The temperature dependency is the most apparent at 42°C-the heat shock temperature. Therefore, it suggests that the highly cooperative binding may play a critical role during heat shock.

Since the 30mer and 30-rev possess the same six 5bp units , the same higher binding affinity might be expected for both of them. However, 30-rev seems insensitive to the temperature. Its affinity for HSF is slightly higher than 15mer, but it does not increase significantly along with the temperature. This can be explained from the structure of the 30-rev. From the definition of HSE, we know that

the HSEs are composed of varying numbers of 5bp unit organized in continuous arrays where each unit is inverted relative to the immediately flanking units. In Figure 4, the fourth 5bp unit of 30-rev is not inverted relatively to the third one, it is possible that two HSF trimers bind to this structure in more independent manner or with a very low level of cooperation.

4.3. DISSOCIATION KINETICS

The kinetics of protein-DNA interactions were analysed by using gel electrophoresis retardation assays. In this experiment, the labeled DNA fragments were incubated with HSF at four temperatures from 0°C to 42°C. For time zero, an aliquot was removed and loaded directly onto a prerun, nondenaturing polyacrylamide gel. Afterwards a 500-fold excess of specific competitor DNA (containing a 60 bp HSE composed of 5bp units) was added to the reaction, and aliquots were removed and loaded on the running gel at subsequent time points. By quantitation of complex band with densitometry, the bound complex signal is expressed as percent compared to the original bound complex at time zero. Figure 7A presents an example of such an experiment performed on 30mer and 15mer at 0°C and 37°C. The data are plotted on the graph 7B as the percentage of cpm measured over cpm at time zero versus minutes after the addition of the competitor DNA. First order dissociation rate constants (k) were determined by non-linear least square analysis:

$$\text{Fraction Bound} = \text{Initial} \times \text{Exp}(-k \times t) + \text{offset}$$

The results are shown in Table 2. Generally, as it demonstrates, the length of the constructs influence the dissociation kinetics: the dissociation rate decreases as the number of 5bp repeats increases. The rate of dissociation as a function of temperature can also be seen in Table 2: it increases with increasing temperature. The half-lives of the HSF-DNA complex are given in this table too. The half-lives were determined from the value of k by the equations:

$$A = A_0 e^{-kt}$$

$$A/A_0 = e^{-kt}$$

at $t = 1/2$, $A/A_0 = 1/2$

then $$1/2 = e^{-kt_{1/2}}$$

$$\ln(1/2) = -kt_{1/2}$$

$$0.693/k = t_{1/2}$$

where A is the percent of complex signal compared to the complex signal at time zero.

Like the range of binding dissociation rate constants (k), the range of HSF-HSE half-lives, from 73min (45mer, 0°C) to 0.9min (15mer,42°C) also showed strong relationship with length of HSEs and the temperature. In general, the longer HSEs at low temperature have longer half-lives. This observation excluded the possibility that the temperature-dependent increase in affinity in multiple probe band shift assays, described above, for the 30mer and 45mer

constructs, might be due to greater kinetic stability at high temperature.

4.4 TITRATION ANALYSIS

To characterize the interaction of hu-HSF1 with HSE constructs, titration experiments were carried out. In this experiment, a constant amount of DNA fragment was mixed with various amount of purified HSF1 protein and incubated for 3 hours at different temperatures in order to reach equilibrium. The protein-DNA complexes were resolved by polyacrylamide gel electrophoresis (as typically used in band shift assay). Low ionic strength conditions and the gel matrix itself stabilize the complex against dissociation. Gel autoradiograms and densitometry of gels allow quantitation of the protein-DNA complex and free probes. Figure 8A shows two examples of the experiment with the 30mer construct at 37°C and 42°C. These represent total fraction of bound complex I (the minor band, migrating slightly faster) and complex II (the major band, migrating more slowly) which are consistent with two bound species, containing a single HSF1 trimer, or a pair of trimers (or hexamer). The resultes are plotted in Figure 8B. The shapes of the curves are sigmoidal, qualitatively demonstrating the presence of cooperative binding interactions at indicated temperatures.

To quantitatively analyze the cooperativity in this nucleic acid binding protein, we employed an model previously described by Draper and Von Hippel (1978). This model is appropriate for studying the cooperativity of binding of a protein to a nucleic acid

lattice by measuring the binding of two proteins to DNA fragment of appropriate length. The binding isotherm which describes the cooperative interaction of two binding proteins with a DNA construct contains four variables: the intrinsic binding constant, K_{int}, which represents the direct interaction between the protein and the oligonucleotide; the cooperativity parameter, y, a unitless quantity representing the ratio of the equilibrium constant for binding two ligands contiguously to that for binding the two ligands separately on a DNA construct (if binding is cooperative $y>1$; for non-cooperative binding, $y=1$, and for anti-cooperative binding, $y<1$); two statistical factors, S1 and S2, which are the number of ways of arranging one and two protein molecules, respectively, on the oligonucleotide lattice.

S_1 and S_2 are defined as follows: $S_1 = (l-m+1)$ where l is oligonucleotide length, and m is ligand binding site size (the length of oligomer actually interacting with the protein); S_2 is the number of sites available for potentially cooperative binding of a second ligand, when first site is already occupied. Then, two binding constants must be defined: K_1 for binding a protein to a free oligomer lattice, and K_2 for binding a protein to a lattice already occupied by one protein. The model can be adapted to the present case of HSF-HSE interactions in the following manner: the length l is the number of correctly oriented AGAAC or GTTCT tandem repeats, and the site size for HSF (considered as trimer) is three. In the case shown for the 30mer probe ($l=6$), $S_1 = 4$, and $S_2 = 2$. Then, K_1 is the product of intrinsic

affinity of the protein(K_{int}) and the number of ways the protein can bind to the oligonucleotide(S_1) for the oligomer:

$$K_1 = S_1 K_{int}$$

The association constant K_2 for binding of the second ligand is expressed as:

$$K_2 = (S_2/S_1) \text{ y } K_{int}$$

the extent of binding of the labeled restriction fragment probe (D) can be represented by a binding polynomial:

$$(D)_{bound} = K_1 (P) (D)_{free} + K_1 K_2 (P)^2 (D)_{free}$$

where P is the concentration of free protein. Since the method of experiment used gives the fraction of probe bound (containing one or more protein molecules), rather than the binding density, the treatment diverges from the original. The total concentration of restriction fragment probe must be:

$$(D)_{total} = (D) + K_1 (P) (D) + K_1 K_2 (D) (P)^2$$

Then, the fraction of probe bound, designated r, is described by:

$$r=(D)_{bound}/(D)_{total}=(4K_{int}(P)+2yK_{int}^2(P)^2)/(1+4K_{int}(P)+2yK_{int}^2(P)^2)$$

A titration of 30mer with HSF1 is shown in Figure 8A , plotted in Figure 8B and fitted to this equation by non-linear least-square analysis (UltraFit, Biosoft). Reasonable fits could be obtained with a range of values, by simultaneously adjusting the two parameter K_{int} and y. Therefore, a constant value of K_{int} was set, and the

corresponding value of y was estimated by curve fitting. The nonlinear least-squares best fit to the 30mer titration data gives values of y by a given value of $K_{int}=10^6$. Table 3 presents these data at three temperatures. It is also shows the temperature dependent pattern. The binding is weaker at 0°C than it is at other temperatures, and the parameter y gives the similar higher value at 37°C and 42°C (the curves at these two temperatures are almost identical).

The titration experiment was also carried out to analyze the hu-HSF1 binding to 15mer and 30-rev (Figure 9A). The results were collected and applied to empirical (Hill) equation for cooperation binding:

$$\text{Bound} = K_{app} \times P^n / (1 + K_{app} \times P^n)$$

where n=3 (decided by the best fitting); K_{app} represents the association constant; P is the protein concentration .

By comparing the parameters (Table 4), we found that 15mer probe had lowest affinity at 0°C and highest at 37°C. Very surprisingly, the binding affinity of the 15mer probe decreased moderately when temperature increased from 37°C to 42°C. The 30mer-rev probe also showed a small decrease in binding at 42°C compared to 37°C. The analysis of the data showed here made us rethink the results obtained from multiple-probe band shift assay described above. It appears, from the data showed here, that the higher relative affinity for the (cooperatively binding) 30mer probe compared to 15mer and 30mer-rev probe at 42°C, compared to 37°C,

results from a decrease in binding to the latter two probes (between 37°C and 42°C) while the 30mer probe affinity is relatively constant in this range.

From the dataof Figure 9B, we also noticed that the curves of 15mer binding to hu-HSF are clearly sigmoidal at all temperatures examined. These sigmoidal curves are contrary to expectation that binding of a single HSF1 trimer to a 15bp site might follow a simple hyperbolic relation. Furthermore, two distinguishable complexes, migrating at the same positions as those with the 30mer probe, were visible (Figure 9A), presumably trimeric and hexameric forms of HSF1. This is consistent with the idea that more than one protein trimer binds to 15mer and that the binding is cooperative. Thus, formation of complexes containing more than 1 trimer might also occur with probes containing a single 15bp site, presumably by binding either one or two probe molecules to an HSF1 hexamer. This fact indicates that there may be cooperative binding interactions even when two HSE elements are located on different DNA molecules.

4.5 OLIGOMERIC STATE OF HU-HSF1 IN SOLUTION

The oligomeric state of HSF may occur as a trimer as suggested in yeasts and Drosophila (Perisic et al., 1989. Sorger et al., 1989) or even hexamer and larger forms (Clos et al 1990). but the oligomeric form of HSF has not been established for the human protein. It was proposed that the apparent molecular weight of the polypeptide, from UV-cross-linking, and its migration on gel filtration chromatography and nondenaturing gel electrophoresis are

consistent with a large oligomeric form (Wagner and Morgan., unpublished data). It was observed in our lab that the hydroxy radical protection assays presenting three strong contact regions between the protein and three perfect 5bp motifs (Cunniff et al., 1991) appear to be consistent with results of functional binding studies of Drosophila HSF and with the trimeric (or hexameric) model of HSF structure. A similar observation was also obtained that binding of a pair trimers required six nGAAn motifs (Cunniff et al., 1991, Perisic et al, 1989). The exact nature of oligomeric form of HSF is still under investigation.

In this part, I present the results of the recombinant hu-HSF1 size measured by SDS-PAGE and pore exclusion limit analysis.

The apparent molecular mass of cloned hu-HSF1, purified from E. coli extracts, was determined to be about 70kDa by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2), significantly lower than the 83kDa and 110kDa size of purified protein reported elsewhere (Goldenberg et al., 1988; Zimarino et al., 1990, Wagner and Morgan., unpublished data.), but higher than that predicted from the cDNA sequence(57,000). The apparent difference in mass between the natural and recombinant human HSFs may be due to posttranslational modification of the natural protein (Larson et al., 1988).

The native size of cloned HSF was measured by pore exclusion limit analysis. In this procedure, proteins are electrophoresed for extended periods (24hr) on nondenaturing polyacrylamide gradient

gels, each protein migrates until it reaches the pore exclusion limit, which is dependent on the size of the protein. There are two species of recombinant hu-HSF1 separated on the native gel, one migrates with an estimated size of 440kDa, and the other with 220kDa (Figure 10). Therefore, we propose that the states of HSF are composed of a hexamer and a trimer of the 70kDa subunit in the solution. The two species bands were quantified by densitometry. The ratio of the two species varies according to the loading protein concentration. When the loading protein quantities are 6ug, 12ug and 18ug in 50ul solution, 220kDa species /440kDa species are 40%/60%, 30%/70%, 18%/82% respectively. It seems that the concentration of protein could affect the oligomeric status of hu-HSF1 free in solution.

It has previously reported that HSF polypeptide have the ability to associate to form homotrimers, and it was suggested that this association of HSF subunits is via a three-stranded coiled coil. More recently, the mobility analysis of Drosophila HSF on native gel electrophoresis was consistent with that of a hexameric protein or even higher oligomers at nonshock temperatures, suggesting that the HSF polypeptide has an intrinsic ability to form aggregates in the absence of DNA (Clos et al., 1991). Yeast HSF trimers also aggregate into higher forms at >= 100mM NaCl (Peteranderl and Nelson, 1992) Our results are consistant with a similar property for human HSF1, and it may contribute to the cooperative binding I presented above.

Figure 1. Preparation Scheme of Hu-HSF1

Human HSF1 was prepared and purified according to this scheme. Inserting and transformation were described in Materials and Methods. Preparation of crude extract was performed as previously described (Waterman et al., 1991). The HSF-fusion protein was then chromatographed through a glutathione-sepharose column and cleaved with thrombin. Concentrated HSF was then used as the source of HSF for subsequent protein/DNA interaction studies.

FIGURE1. PREPARATION SCHEME OF HU-HSF1

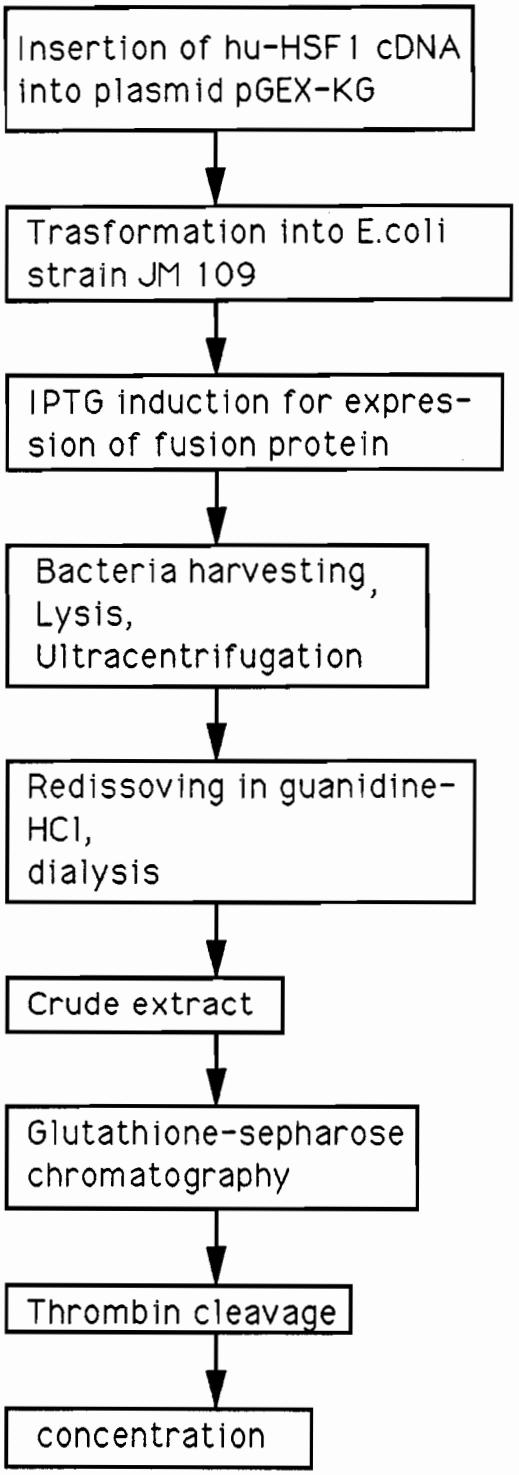


Figure 2. SDS-PAGE of Recombinant Hu-HSF1

Four microliters of hu-HSF1 and ten microliters of HSF-fusion protein were separated on a 7.5% polyacrylamide gel and visualized by Coomassie blue staining. Middle lane is molecular mass standards (kDa), they are marked on the left. Denatured sizes (kDa) of fusion-HSF1 and hu-HSF1 are indicated on the right.

FIGURE 2. SDS-PAGE OF FUSION-HSF AND HU-HSF1

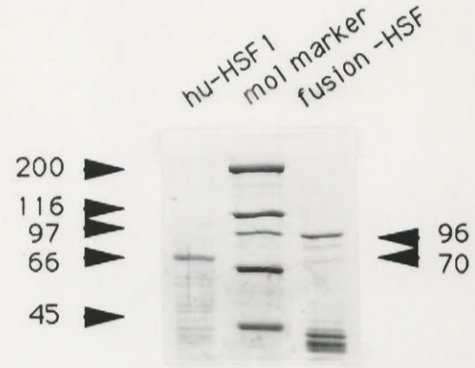


Figure 3. Band shift Assay Analysis of Recombinant Hu-HSF1

The binding activity of HSF-fusion protein and hu-HSF1 was detected using a band shift assay with human HSP70.1 HSE24 probe (AACCCCTGGAATATTCCCGACCTG) and HSE construct 30mer. Complex: specific HSF/HSE complex. Free: unbound ^{32}P labelled probe. Lane 1: fusion GST-HSF1 binding with probe HSE24. Lane 2: cleaved hu-HSF1 binding with probe HSE24. Lane 3: cleaved hu-HSF1 binding with HSE construct 30mer.

Figure 3. Band Shift Assay of Fusion-HSF and Hu-HSF

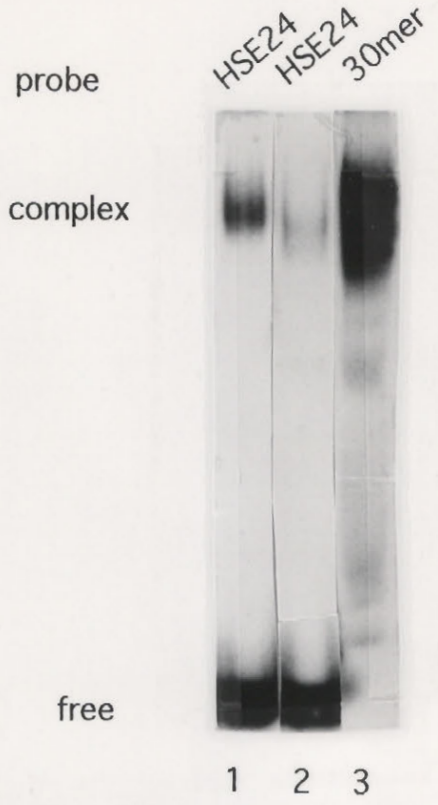


Figure 4. Idealized Heat Shock Elements Constructs

Four constructs containing various number of genetically idealized 5bp sequence AGAAC were named 15mer (three 5bp motifs), 30mer (six 5bp motifs), 30mer-rev (six 5bp motifs in reversed orientation) and 45mer (nine 5bp motifs). Arrow indicates the direction of each unit. They were created by self-ligation of a double-stranded 15bp idealized HSE construct oligonucleotide (AGAACGTTCTAGAAC), followed by blunt-end ligation into a site in the pUC118 polylinker. These constructs were subsequently used as probes to test the property of HSE binding to recombinant hu-HSF1 by cleavage with restriction enzymes at sites within the polylinker.

FIGURE 4. IDEALIZED HEAT SHOCK ELEMENTS

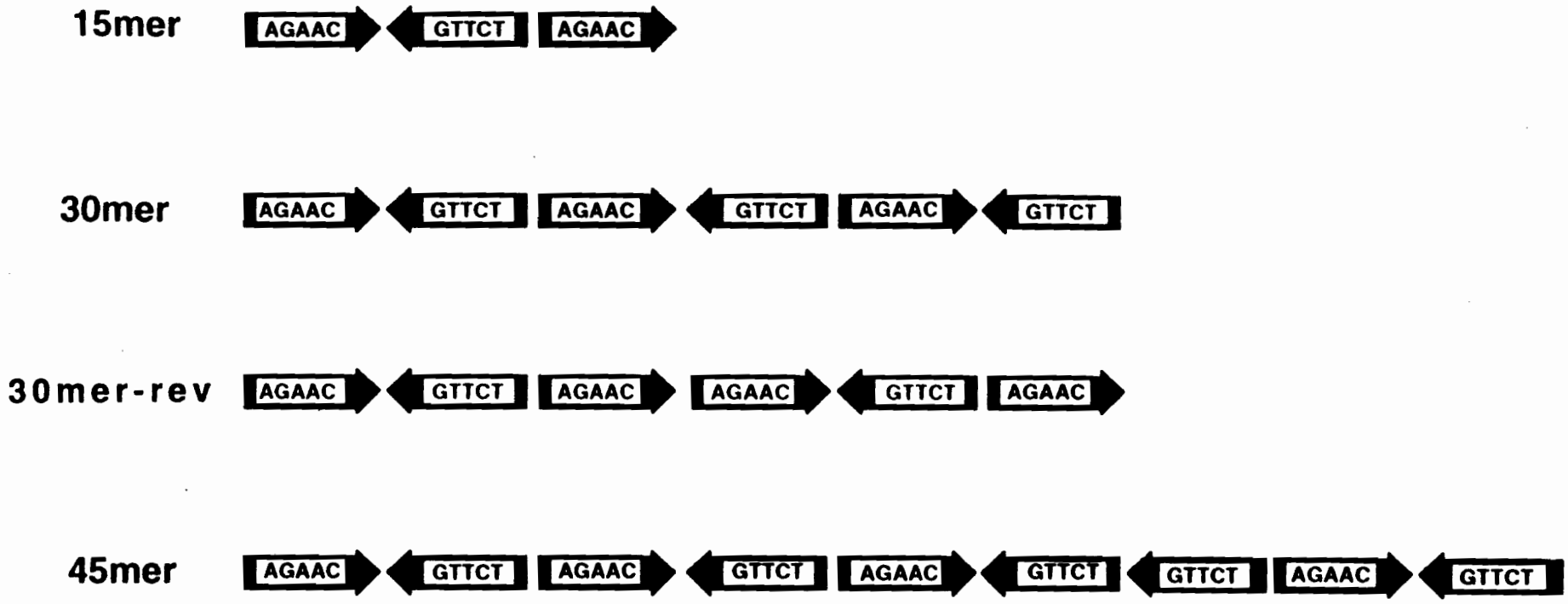


Figure 5. DNase I Protection by Human HSF1

A. Autoradiogram displays the DNase I protection pattern of the HSE constructs by hu-HSF1. The four constructs were labeled at top strands (15mer, 30mer, and 45mer) or at the bottom strand (30-rev). Recombinant hu-HSF1 (1ul); or no HSF, control was incubated with the labeled fragment and subject to DNase I digestion. Fragments were purified and analyzed on an 8% sequencing gel. (+) indicates reactions in the presence of the HSF. (-) indicates reactions without protein. Rectangle shows the actual length of 5bp motifs.

B. DNase I protection of human HSF on four HSE constructs summarized here is based on the examination of figure 5A. The approximate boundaries are between the ends of thick line segments and the arrowheads. The footprintings of 15mer, 30mer, 45mer indicate for the top strands; the 30-rev footprinting shows for the bottom strand.

FIGURE 5A. DNASE I FOOTPRINTING OF FOUR CONSTRUCTS

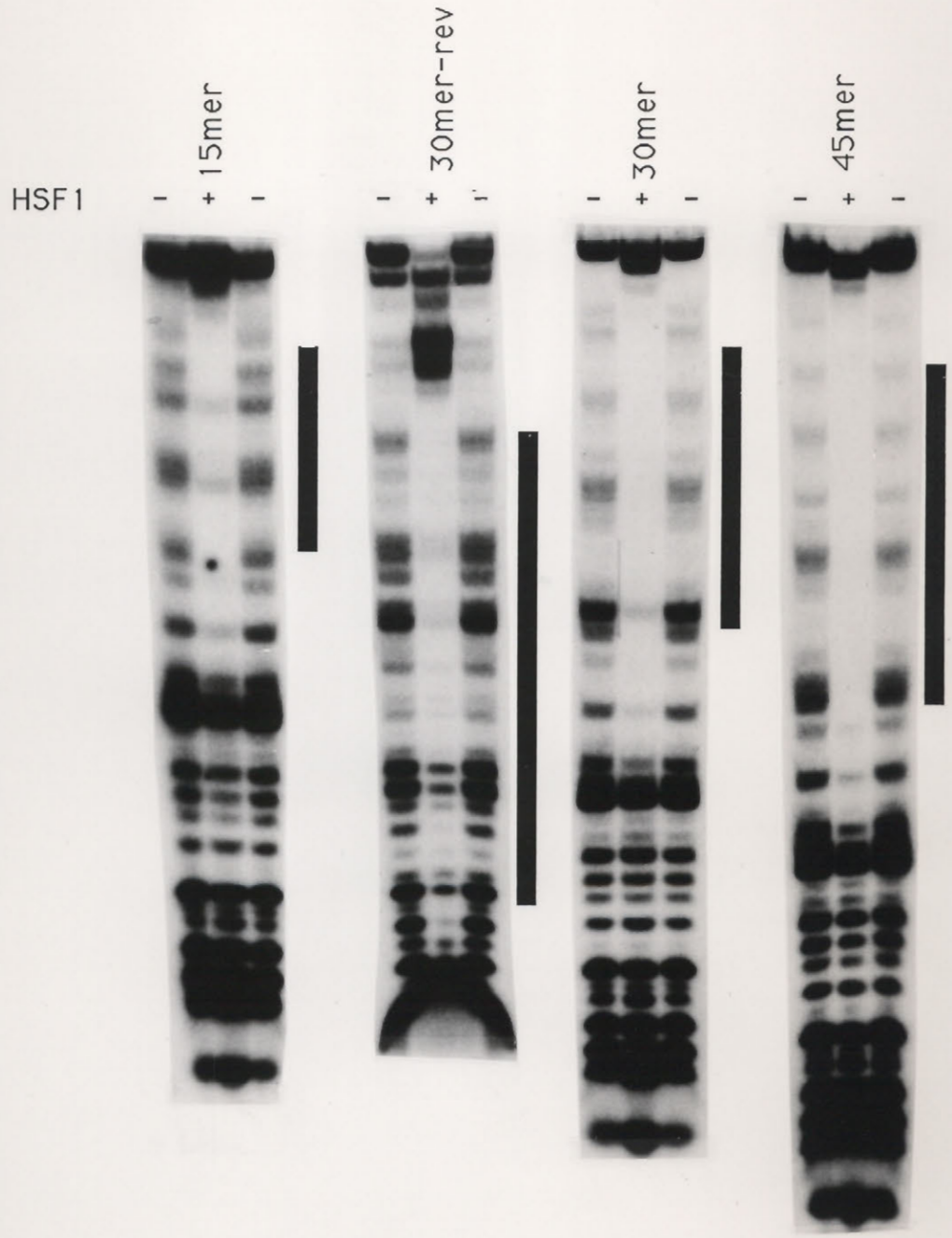


FIGURE 5B. SUMMARY OF DNASE I FOOTPRINTING OF FOUR CONSTRUCTS

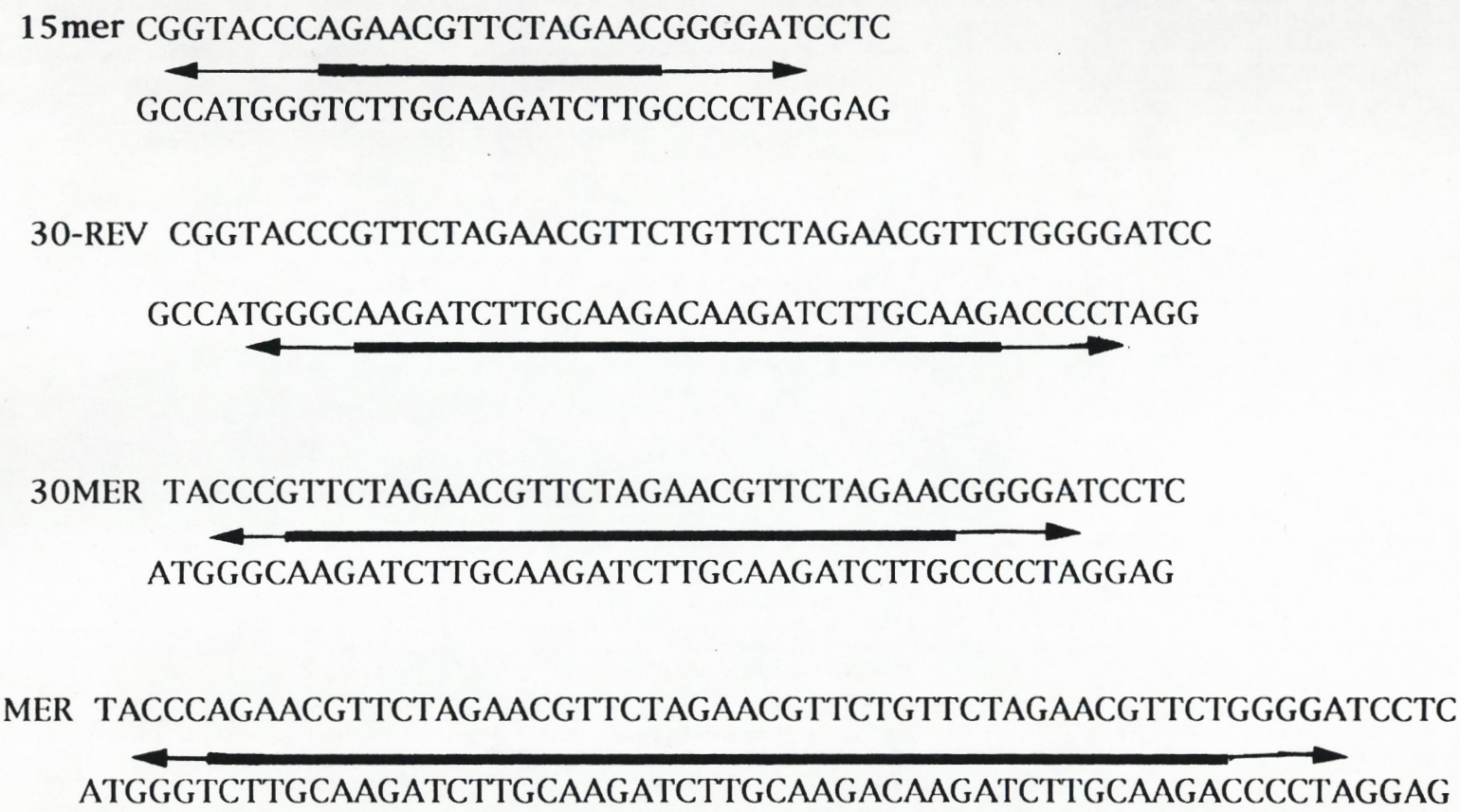


Figure 6. Multicomponent Band Shift Assay

A. A sample band shift assay with recombinant hu-HSF1 is shown here. The upper complex, labeled complex, consists of a mixture of the four probes bound to the HSF protein. Free indicates the unbound four fragment probes. These bands were eluted and electrophoresed on denaturing sequencing gel.

B. The bound complexes and free probes containing four probes were electroeluted, separated on denaturing polyacrylamide gels. Lane F: free probe bands; Lane B: protein-bound complex. The position of 4 probes on the gel are marked on the left. The results were quantified by densitometry.

C. The data quantified from B are plotted here.

FIGURE 6A. A SAMPLE BAND SHIFT ASSAY

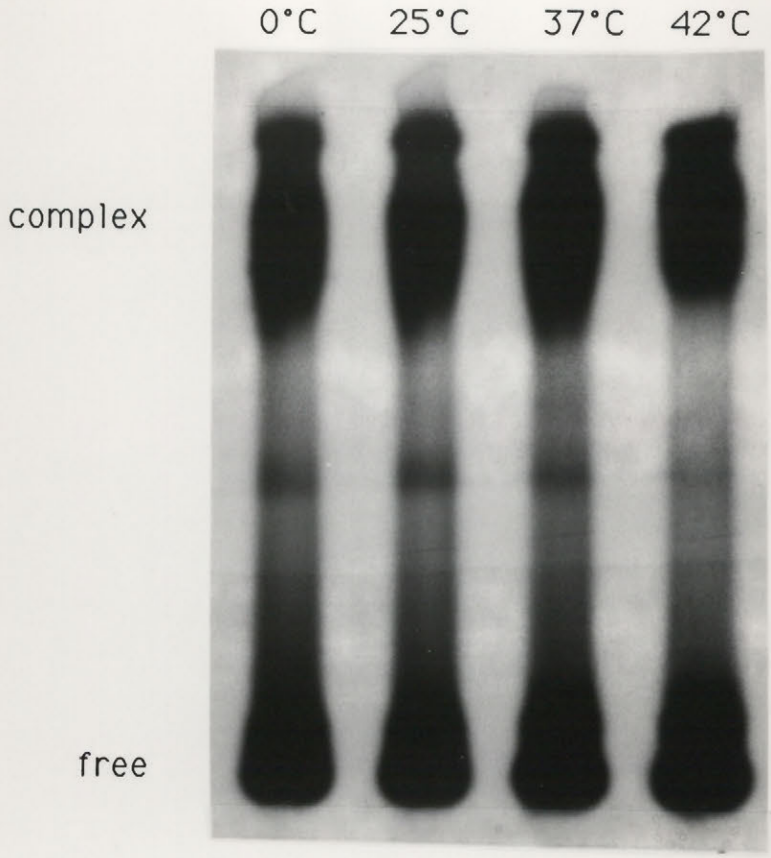


FIGURE 6B MULTICOMPONENT BAND SHIFT ASSAY

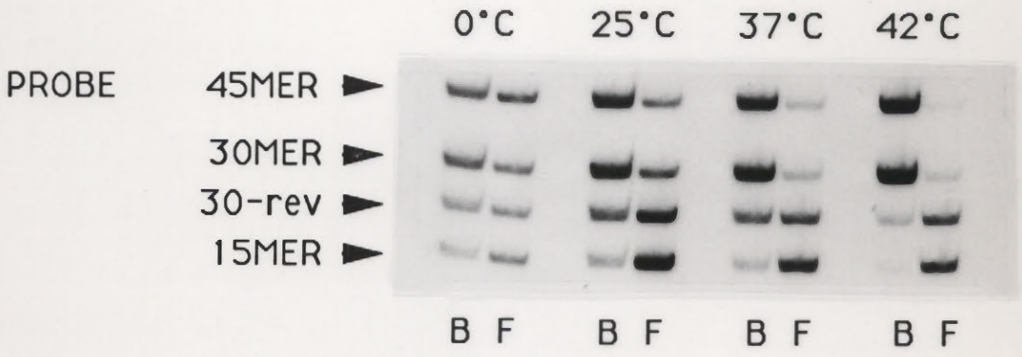


FIGURE 6C. SUMMARY OF MULTI COMPONENT BAND SHIFT ASSAY

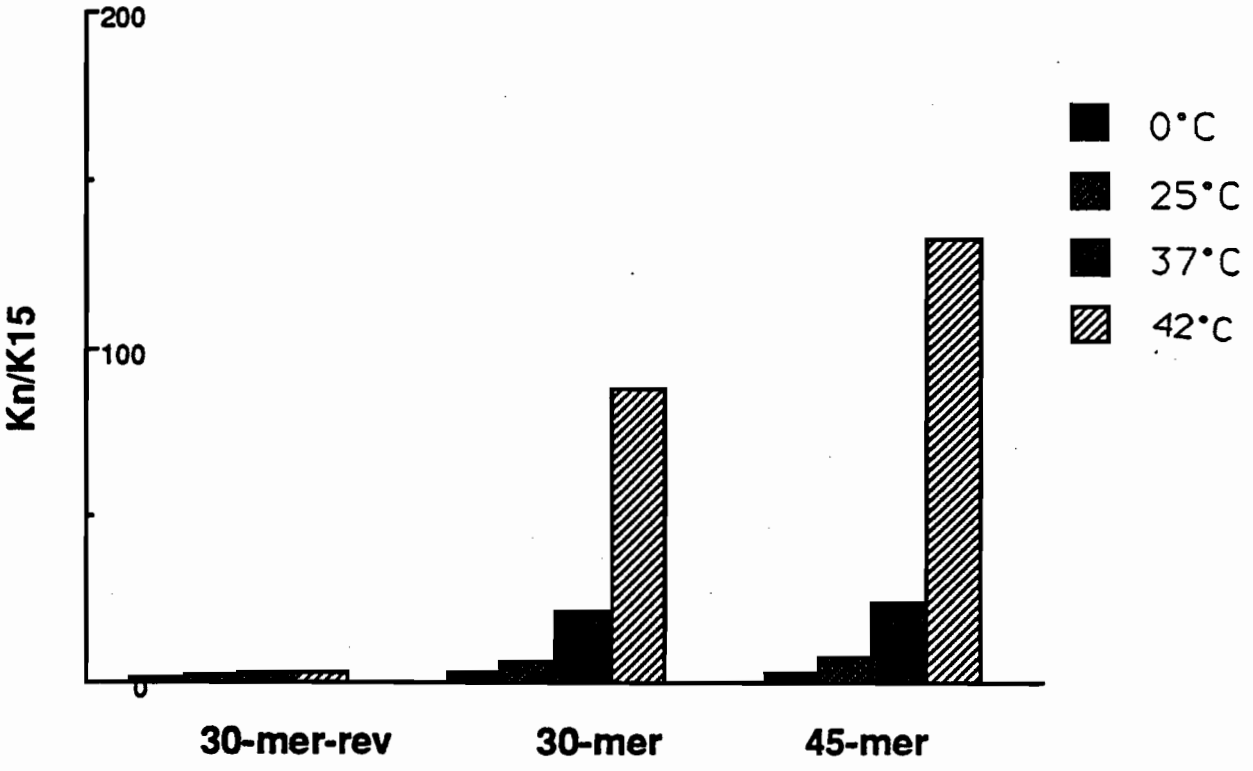


Table 1. Summary of multiple probe band shift assay

The data from multiple-probe band shift assays are summarized here. Values indicate the relative binding affinity of 30mer, 30-rev, and 45mer probes relative to that for the 15mer probe, calculated as described previously.

TABLE 1.SUMMARY OF MULTICOMPONENT BAND SHIFT ASSAY

	Kn/K15			
	0°C	25°C	37°C	42°C
30-mer-rev	1.7	2.3	3.1	3.1
30-mer	3.2	6.2	21.3	87.9
45-mer	3.4	7.9	23.5	132.0

Figure 7. Dissociation Kinetics

A. Labeled DNA fragment bearing various numbers of 5bp unit were incubated with protein as described in Materials and Methods. At time zero, an aliquot was removed and loaded directly onto a prerun gel, then excess of competitor 60mer (bearing twelve 5bp units) was added to the reaction and aliquots were loaded onto a running gel at selected time points. Here are some examples of the experiment. The probes, temperatures and times are indicated above the lanes. Bound complexes and free probe bands from gels were quantified by densitometry. Only the bound complexes are shown.

B. Data from 15mer and 30mer at four temperatures are plotted on the graph. Shifted cpm at time x over shifted cpm at time zero are plotted versus time in minutes.

FIGURE 7A DISSOCIATION KINETICS

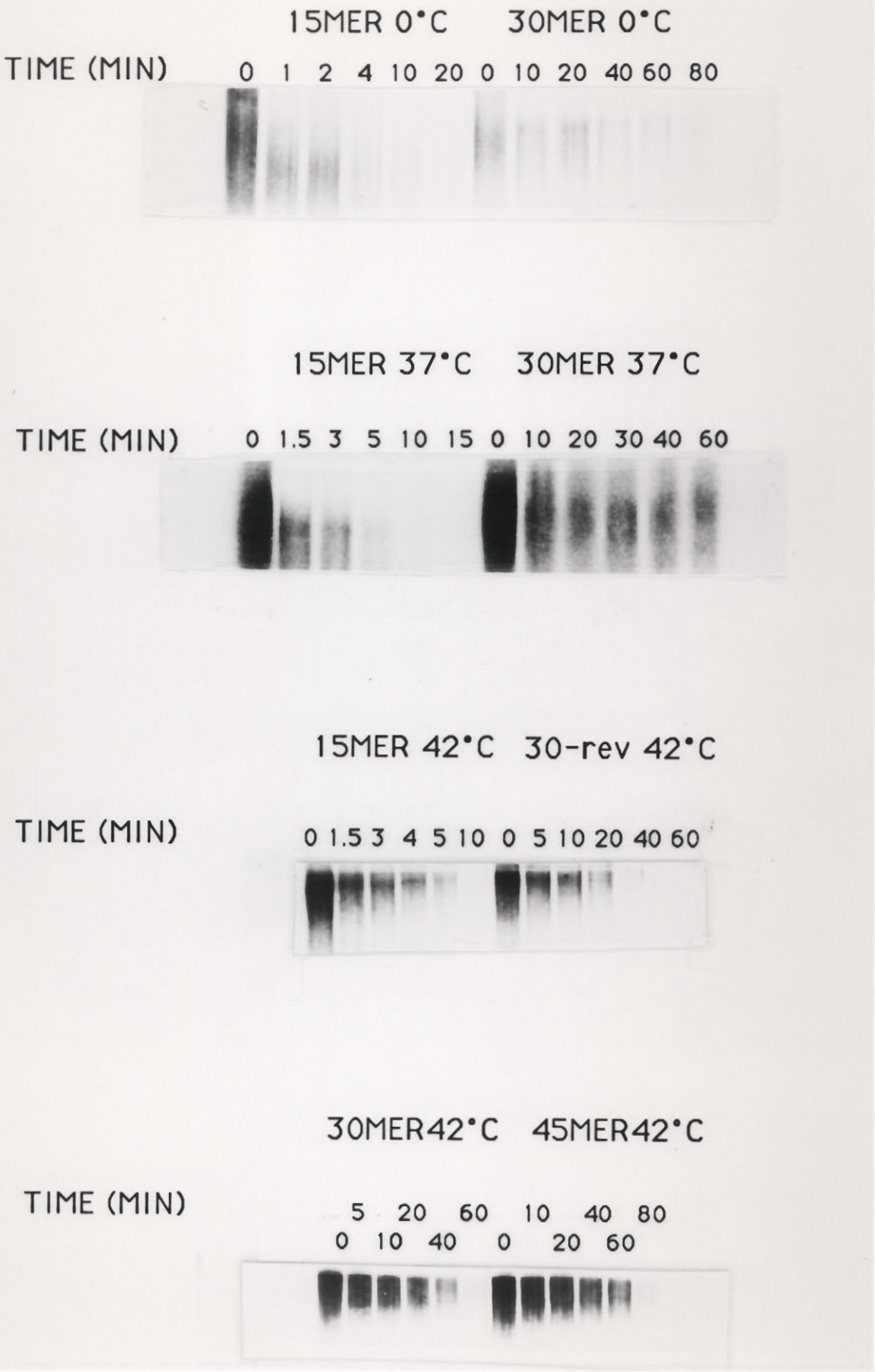
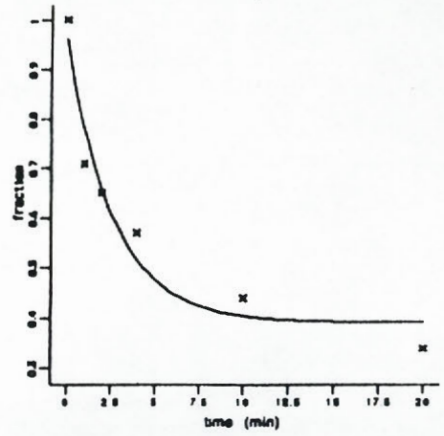
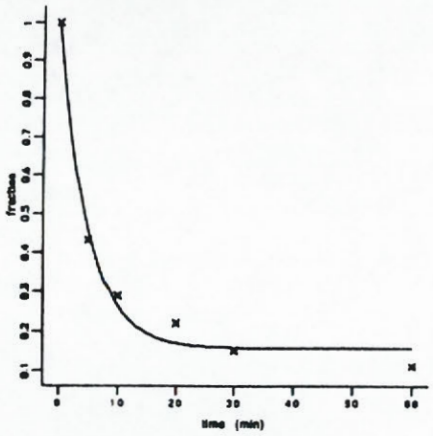


FIGURE 7B. DISSOCIATION KINETICS

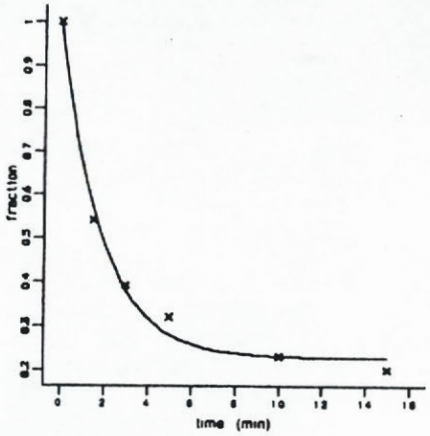
15mer, 0°C



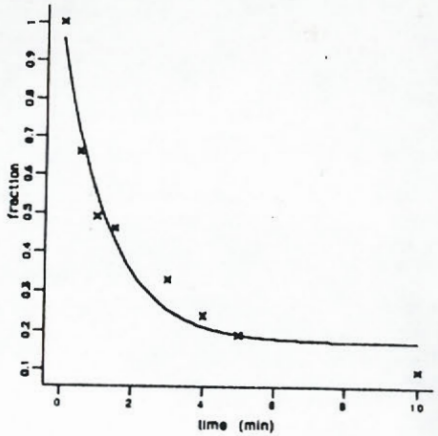
15mer, 25°C



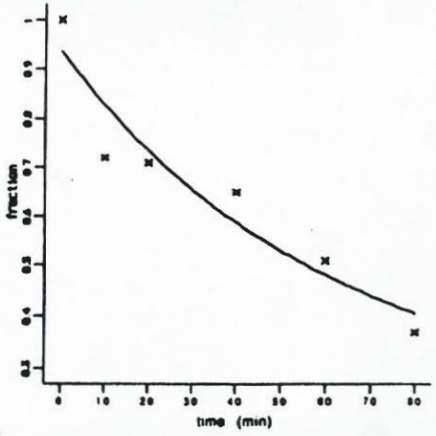
15mer, 37°C



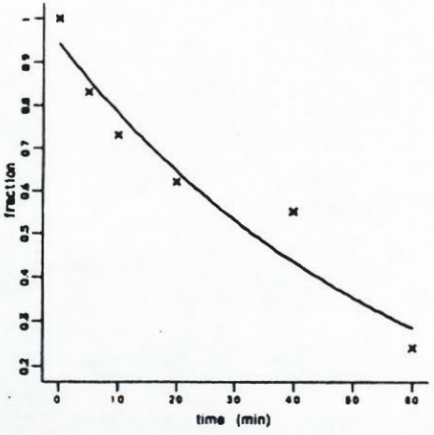
15mer,42°C



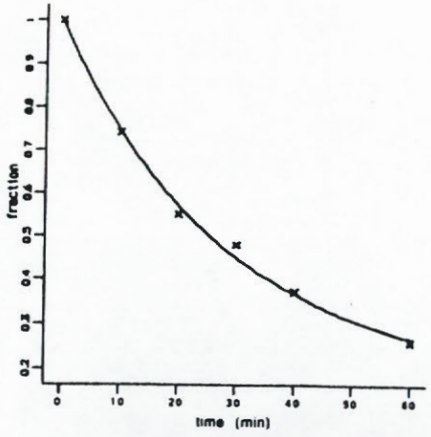
30mer, 0°C



30mer, 25°C



30mer, 37°C



30mer, 42°C

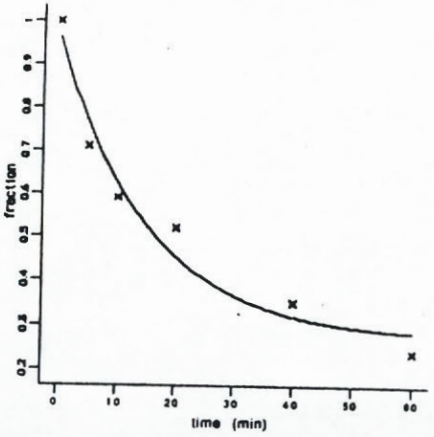


Table 2. Summary of dissociation kinetics

Summary of data from all four constructs at four temperatures. k (dissociation constant) and t (half lives) were obtained from equations as described in Results.

TABLE 2. SUMMARY OF DISSOCIATION KINETICS

	15mer		30mer-rev		30mer		45mer	
	$k(x10^{-3}s^{-1})$	$t_{1/2}$	$k(x10^{-3}s^{-1})$	$t_{1/2}$	$k(x10^{-3}s^{-1})$	$t_{1/2}$	$k(x10^{-3}s^{-1})$	$t_{1/2}$
0°C	o.915	12.6	0.887	13.0	0.270	42.7	0.158	73.0
25°C	3.400	3.4	1.230	9.4	0.283	40.8	0.265	43.5
37°C	8.960	1.3	1.390	8.3	0.612	18.9	0.667	17.3
42°C	12.500	0.9	2.830	4.1	1.130	10.2	0.993	11.6

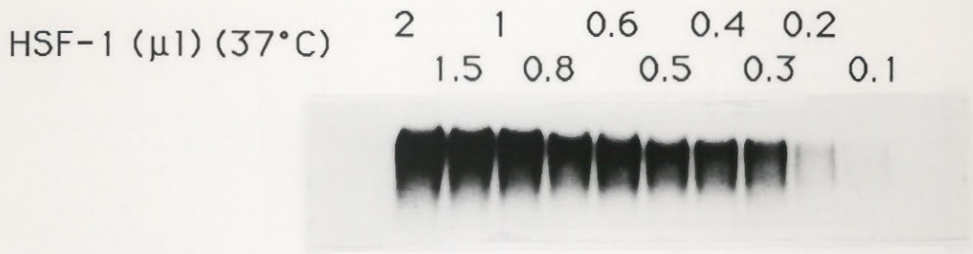
k: dissociation constant
$t_{1/2}$: half life (min)

Figure 8. 30mer Probe Titration

A. Titration of the 30mer with hu-HSF1 at three temperatures. Amount of probe was constant (2.85 fmol) for each lane, the volume of hu-HSF1 are marked on the above lane. the concentration of hu-HSF1 here is 0.12ug/ul.

B. The percent bound probe at each temperature was quantified by densitometry of autoradiogram. Data are plotted on this graph. the curves were drawn by nonlinear least-squares analysis with the equation described above. Fraction in Y axis presents the fraction of probe bound, protein concentration in X axis is molar $x10^{-7}$ and is given in terms of concentration of trimers.

FIGURE 8A 30MER PROBE TITRATION



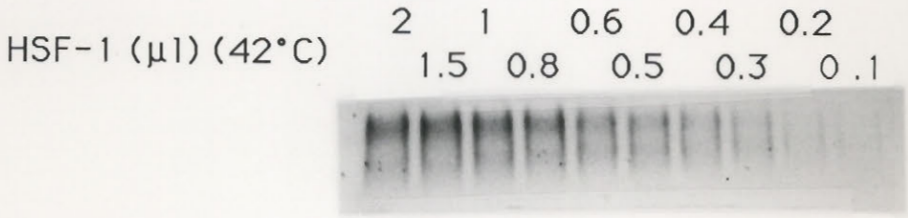
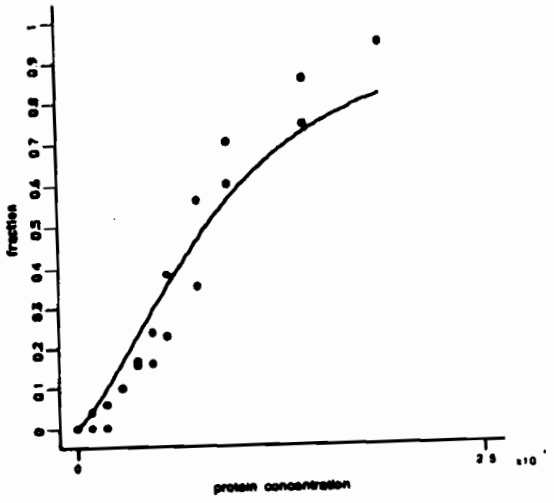
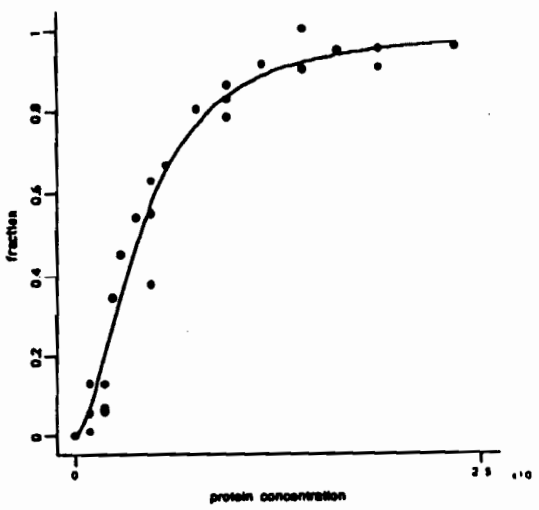


FIGURE 8B 30MER PROBE TITRATION

30mer 0°C



30mer 37°C



30mer 42°C

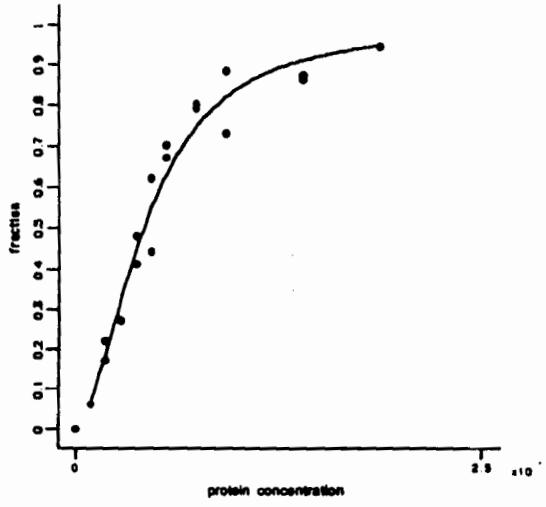


Table 3. Summary of 30mer titration

Summary of parameter y obtained by non-linear least-square treatment of data from A. K_{int} was given a value 10^6. the equation is indicated at the bottom of the table.

TABLE 3 SUMMARY OF 30MER PROBE TITRATION

	y		
	0°C	37°C	42°C
30mer	52	247	237

*1. Equation: Bound=(4*Kint*P+2*y*Kint^2*P^2)
/(1+4*Kint*P+2*Kint^2*P^2)

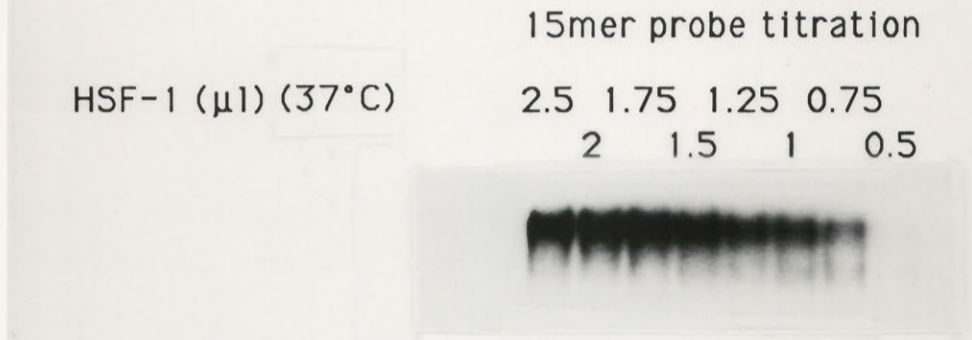
*2. Kint=1E6

Figure 9. 15mer and 30-rev titration

A. Examples of 15mer and 30-rev titration at 37°C and 42°C respectively. The binding condition is described in Material and Methods. The volume of hu-HSF1 are shown on the top. The concentration of hu-HSF1 was 0.12ug/ul.

B. Titration data of 15mer and 30-rev are plotted on the graph. Y axis represents the fraction of probe bound, X axis indicates the protein concentration on molar $x10^{-7}$, and is given in terms of concentration of trimer.

FIGURE 9A 15MER AND 30-rev TITRATION



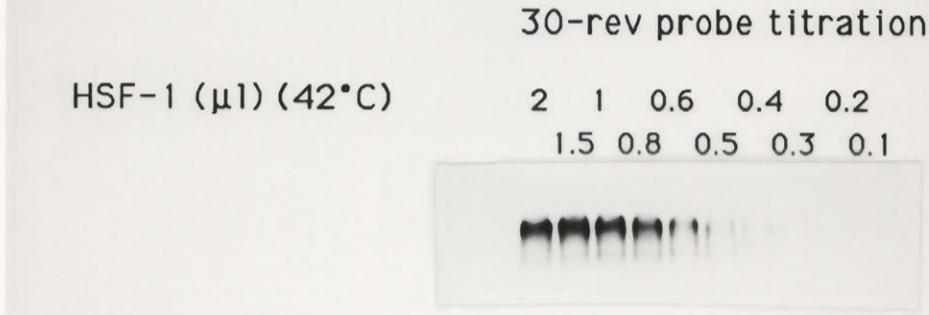
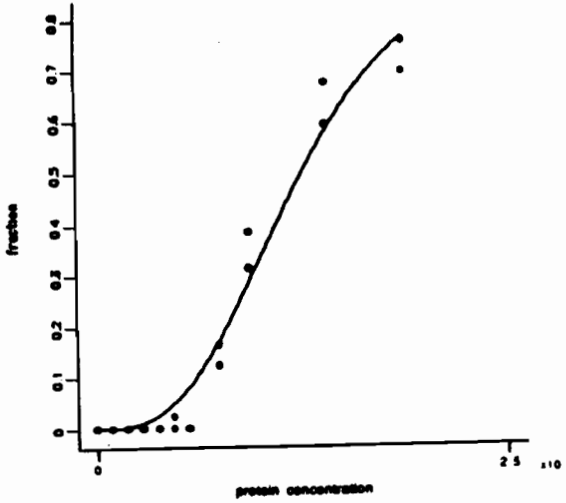
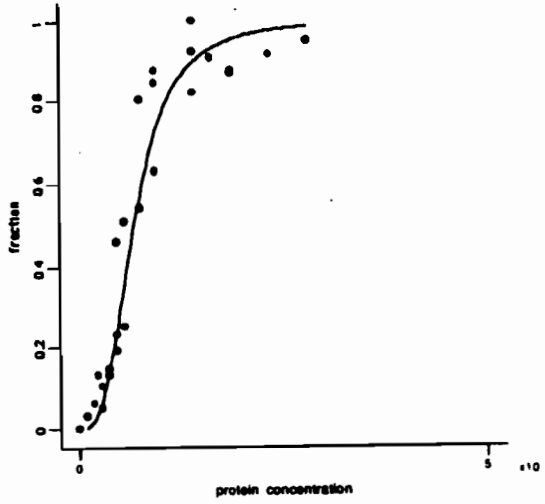


FIGURE 9B 15MER AND 30-rev PROBE TITRATION

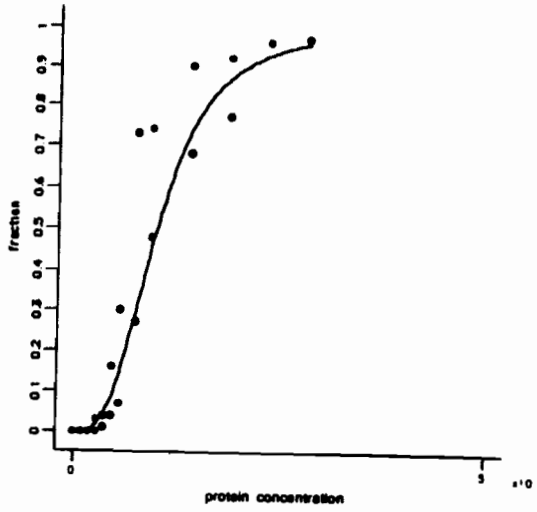
15mer 0°C



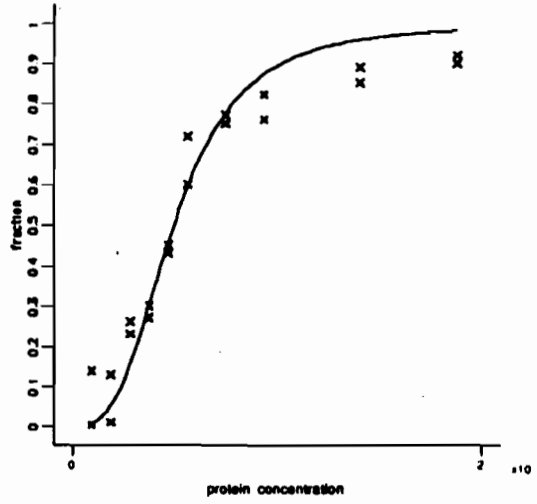
15mer 37°C



15mer 42°C



30-rev 37°C



30-rev 42°C

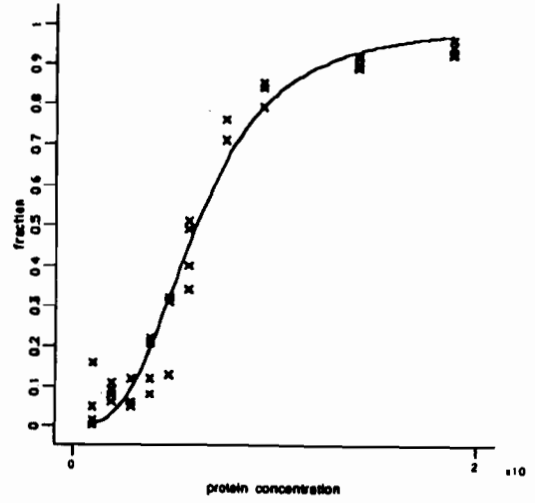


Table 4. Summary of 15mer and 30mer-rev

Data from A was applied to equation: Bound = K_{app} x P^n / (1 + K_{app} x P^n), where n was given of 3, K_{app} was obtained from this treatment and summarized here.

TABLE 4. SUMMARY OF 15MER AND 30-REV TITRATION

	K_{app}(association)	
	15mer ($x10^{21}$)	30-rev ($x10^{21}$)
0°C	0.5	
37°C	3.9	8.3
42°C	1.6	4.3

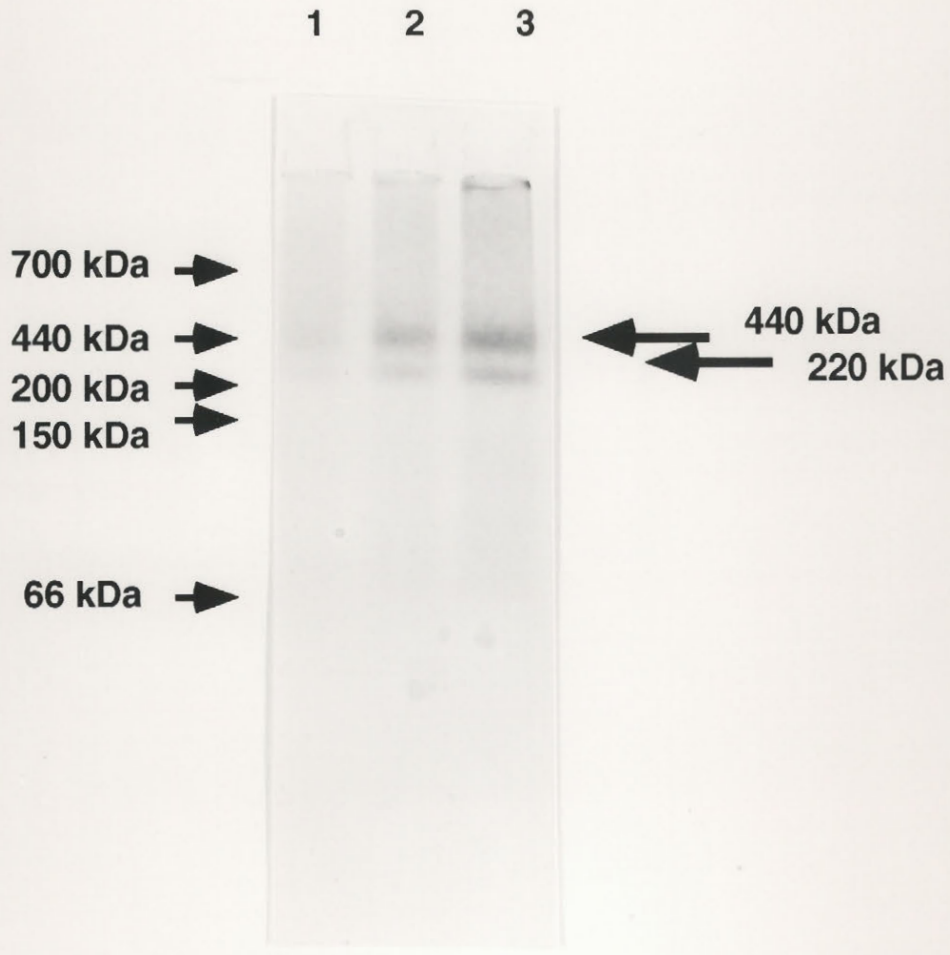
*1. Equation: Bound = $K_{app} \times P^n / (1 + K_{app} \times P^n)$

*2. n = 3

Figure 10. Pore Exclusion Limit Electrophoresis

Different amounts of purified hu-HSF1 were loaded on 4%-20% polyacrylamide gradient gel and electrophoresed for extended periods. Protein bands were detected by Coomassie blue staining. Nondenaturing gel markers (Sigma) were: thyroglobulin, 690kDa; apoferritin, 440kDa; beta-amylase, 200kDa; alcohol dehydrogenase, 150kDa; bovine serum albumin, 66kDa. They are marked on the left. The molecular mass of hu-HSF1 complexes expressed in E.coli was marked on the right.

PORE EXCLUSION LIMIT GEL ELECTROPHORESIS



6. DISCUSSION

From our analysis, we showed that recombinant hu-HSF1 purified from E. coli binds to four idealized HSE constructs with high intrinsic affinity and that the binding to tandem HSE arrays in correct alternating orientation is in a cooperative manner. There was a significant relation between cooperativity and temperature.

The strong temperature dependence of the cooperative binding to the constructs with tandem sites is clearly demonstrated in multiple probe band shift assays: the arrays containing six 5bp units (30mer) and nine 5bp units (45mer) showed 21-fold and 23-fold higher affinity at 37°C, and 90-fold and 130-fold higher affinity at 42°C than the arrays containing three 5bp (15mer) at same temperatures (Figure 6C and Table 1). This may mean that during a temperature increase from 37°C to 42°C, the distribution of hu-HSF1 may be affected by increasing the preference for tandem rather than isolated sites.

Further analysis of thermodynamic parameters for binding affinity and cooperativity from direct titrations gave us quantitative data and indicated a more clear pattern of cooperativity: formation of complexes containing more than one HSF trimer can occur with probes containing not only multiple 15bp sites, but probably also a single 15bp site; that is cooperative binding exists with 15mer too, presumably by binding either one or two probe molecules to an hu-HSF1 hexamer. The increase in HSF binding to 15mer reached the highest point at 37°C instead of 42°C while the affinity of hu-HSF1 to

30mer remained relatively constant between 37°C and 42°C. The apparent increase in cooperative binding of hu-HSF1 to 30mer and 45mer at 42°C results from the decreased binding of hu-HSF1 to 15mer at higher temperature. This may account for the HSF preference for long arrays instead of short arrays of HSE at high temperature. Our quantitative analysis of thermodynamic parameters provided very interesting insights into the behavior of hu-HSF1 in DNA-protein interactions. The evidence we present here is consistent with the qualitative previous report that Drosophila heat shock factor binding to HSE is highly cooperative and the cooperativity is particularly important at 37°C (heat shock temperature for Drosophila) (Xiao et al, 1991).

The mixture of trimer and hexamer oligomeric state of hu-HSF1 might provide the evidence to explain the cooperativity nature of protein-DNA binding: by binding to standard trimeric sites, the larger multimers provide the potential for interactions with additional 5bp units and a means of assembling stable HSF-DNA complexes even over separated HSEs. The sigmoidal curve of 15mer binding to hu-HSF1 gives us an interesting example here: presumably by binding one or two probe molecules to an HSF1 hexamer, the cooperative binding interactions could occur between two HSEs located on different DNA molecules that could be brought together by a looping mechanism.

Cooperative binding of heat shock factor to heat shock element is not restricted to the heat shock genes. It is well documented that synergistic activation can occur in the hormone response enhancer

element (Tsai et al., 1989) and can be achieved by duplication of specific motifs or by assembly of combinations of different motifs within the SV40 enhancer (Herr and Clarke., 1986; Jones et al., 1988) and the adenovirus E4 promoter (Horikoshi et al.,1988). More and more data have been accumulated for the synergically regulating gene expression. The study presented here should also be contributive to explaining in vivo occupancy of HSE sites as a function of the concentration of free activated HSF and provide very useful data required for the further analysis of heat shock gene regulation.

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