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CHAPTER 11

The Activation of Trans-Acting Factors in Response to Hypo- and Hyper-Osmotic Stress in Mammalian Cells

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1. Introduction

Osmoregulatory processes are crucial to all living organisms since the maintenance of intracellular osmotic pressure (or chemical potential of metabolites) is of fundamental importance for cell survival. Osmoregulation also plays an important role in enhancing solute transport in cells during growth stimulation. Mammalian cells respond to either hypo- or hyper-osmotic stress by changing the cell volume like an osmometer. The physiology and subsequent metabolic changes associated with volume have been extensively reviewed (e.g. Lang et al., 1998). Change in osmotic pressure represents a change in Gibbs free energy, ΔG, in a closed system. How living cells sense and transmit the signals generated from ΔG change and how those signals eventually lead to various biological responses, including gene expression, remains unclear.

The heat shock response represents another important biological defense to physical stress. The original observation of puff formation on the *Drosophila* polytene chromosome upon heat treatment (Ritossa, 1962), has led to the identification of a large family of heat shock proteins (HSPs) in almost all living organisms. HSPs are highly conserved polypeptides whose biosynthesis is prominently stimulated by heat treatment (e.g. Nover and Sharf, 1991). In addition to heat, more than 100 chemicals, including amino acid analogs, transition metals, short-chain alcohols, oxidants and certain physiological and pathological conditions, could also lead to the induction of HSPs (reviewed in Nover, 1991). In eukaryotic cells, expression of HSP genes is controlled by heat shock transcription factor (HSF). Under heat stress or other stressed conditions, the inactive latent HSF is activated to form a homotrimer that recognizes specifically a heat shock response element (HSE) that is present in the promoter region of all HSP genes (Nover, 1991). Heat stress represents an increase of enthalpy (ΔH) of the system. How the living cells sense and transmit the signals generated from ΔH change and eventually lead to heat shock response is a subject under intense investigation.

Since a diverse array of inducers and stressors can elicit heat shock response, it is not surprising that HSPs have also been proposed to serve as molecular chaperons in cells during osmotic stress (e.g. Rauchman et al., 1997). Although several studies have shown that a delayed accumulation of HSP70 mRNA occurs in cells under hyper-osmotic stress (see table 11.1), the underlying mechanism is unclear. Since HSF activation is the prerequisite of HSP gene expression, we have investigated the effect of osmotic stress on the activation of HSF (Huang et al., 1995; Caruccio et al., 1997). In addition to HSF, another trans-acting factor, TonEBP (tonicity-responsive enhancer element binding protein), has also been shown to be responsive to osmotic stress. This review will discuss these two trans-acting factors in the context of the effect of osmotic stress on gene expression.

2. Effects of osmotic stress on gene expression

To maintain the intracellular homeostasis, cells will have to mount an effective defense strategy

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Table 11.1. Effect of osmotic stress on gene expression

Gene	mOsM[a]	-fold[b]	Time (min)[c]	Cell type	Reference
Genes induced by hypo–osmotic stress					
β-actin	225	~2×	120	hepatocyte	Schultz et al. (1991)
c-fos	200	~5×	30	myocyte	Sadoshima et al. (1996)
c-jun	205	~5×	60	hepatoma	Finkezeller et al. (1994)
Egr-1	160	~2×	360	mIMCD	Zhang and Cohen (1997)
ODC	200	7×	360	LLC-PK1	Lundgren (1992)
Tubulin	190	~2×	120	hepatocyte	Haussinger et al. (1994)
Genes induced by hyper–osmotic stress					
AR	600	18×	1200	MDCK	Garcia–Perez et al. (1989)
BGT1	515	12×	720–1440	MDCK	Miyakawa et al. (1998)
SMIT	500	10×	960–1440	MDCK	Yamauchi et al. (1993)
Taurine T	515	>5×	1440	MDCK	Uchida et al. (1992)
OSP94	515	>10×	720	mIMCD	Kojima et al. (1996)
HSP70	515	~5×	360	MDCK	Sheikh-Hamad et al. (1994)
c-fos	515	1.5×	360	MDCK	Cohen et al. (1991)
PEPCK	405	~3×	200	rat liver	Newsome et al. (1994)
Egr-1	515	~2×	120	MDCK	Cohen et al. (1991)

[a] The values are estimated based on literature information.
[b] The value refers to an increase in the maximal level of mRNA.
[c] The value refers to the approximate time when mRNA level is maximal. mIMCD, mouse inner medulla collecting duct cells; MDCK, Madin-Darby canine kidney cells; AR, aldose reductase; BGT1, betaine-GABA cotransporter; SMIT, sodium-dependent myo-inositol transporter; Taurine T, taurine transporter; PEPCK, phosphoenolpyruvate carboxykinase.

to cope with osmotic stress, either chronic or acute. One strategy will be to alter the expression of certain genes whose products may have critical regulatory or protective roles (reviewed in Burg et al., 1997; Lang et al., 1998). Table 11.1 lists some of the genes whose mRNA levels have been shown to be increased in response to osmotic stress. In general, these genes can be divided into three groups: (1) osmoregulatory genes whose gene products are responsible for the accumulation of compatible organic osmolytes in the cell; (2) heat shock family stress genes; and (3) genes whose roles in osmotic stress are not immediately clear. Since medium composition, osmolarity, cell types and the growth state of cell culture can all affect the level of gene expression, it is important to define these variables in order to make meaningful comparison of the literature data. Thus, the data in table 11.1 are by no means conclusive. For example, Sadoshima et al (1996) showed that hypo-osmotic stress (200 mOsM) induces c-fos within 30 min in cardiac myocytes, and Cohen et al (1991) showed a moderate increase in c-fos mRNA 6 h after hyper-osmotic stress (515 mOsM) in Madin-Darby canine kidney (MDCK) cells. It will be more informative, if the expression of c-fos is investigated over the complete range of medium osmolarity in these two different cell types.

2.1. *Hypo-osmotic stress on gene expression*

None of the hypo-osmolarity-induced genes listed in table 11.1 belong to either the group of osmoregulatory genes or to the stress gene family. The induction of c-fos (Sadoshima et al., 1996) and c-jun (Finkenzeller et al., 1994) is interesting because their gene products are components of heterodimeric AP1 transcription factor, and the AP1 site is commonly present in many gene promoters. However, the protein amount and the AP1 binding activity were not examined in these two studies. It is also unclear whether, or not, c-fos expression can be induced

by hypo-osmolarity in other cells (e.g. MDCK cells). Ornithine decarboxylase (ODC) is the key enzyme for polyamine biosynthesis. An increase in ODC activity in response to hypo-osmolarity and a subsequent accumulation of putrescine have been observed not only in mammalian cells but also in bacteria (Munro et al., 1972, 1975). Hypo-osmotic stress induces ODC mRNA in LLC-PK1 cells (Lundgren, 1992), but not in a variant, DFMO-resistant L1210 cells (Poulin and Pegg, 1990). Instead, hypo-osmolarity causes a large increase in the synthesis and stabilization of ODC protein in the variant L1210 cells (Poulin and Pegg, 1990). Thus, the effect of osmotic stress on gene expression is not limited to transcription, but can be extended to translational and posttranslational level. This example also illustrates the importance of polyamines in discussing the osmotic stress response. Polyamines are organic cations and have the potential to modulate the intracellular ionic strength during hypo-osmotic stress. The physiological role of polyamines in osmoregulation is an interesting topic that should be further explored.

2.2. *Hyper-osmotic stress and gene expression*

Because of their physiological role in osmosis, renal cells have been frequently used to study the effect of hyper-osmotic stress on gene expression (table 11.1). Hyper-osmolarity prominently induces the expression of osmoregulatory genes that encode enzymes or transporters for organic osmolytes. Thus, aldose reductase (AR), betaine/γ-aminobutyric acid transporter (BGT1), sodium-coupled myoinositol transporter (SMIT) and taurine transporter are responsible for the accumulation of sorbitol, betaine, inositol and taurine, respectively, in the cells under hyper-osmotic stress. When the SMIT transporter activity is inhibited, cells undergo necrosis under hypertonic conditions (Kitamura et al., 1998); indicating the importance of osmoregulatory genes to cell survival under hypertonic conditions. The expression of AR, BGT1, and SMIT genes has been shown to be regulated at a transcriptional level, but with very slow kinetics. In general, the mRNA appears 6–10 h after hyper-osmotic stress and reaches maximal value about 20 h later (table 11.1). Several reports showed that hyper-osmolarity can induce stress genes such as HSP70 and Osp94 (table 11.1); the mechanism of induction, however, has not been investigated. Other genes such as c-fos, egr-1 and PEPCK only show moderate increase in mRNA and the physiological significance of these effects remains to be investigated.

2.3. *Promoter organization of the osmoregulatory BGT1, AR and SMIT genes*

The sequences of promoter regions of BGT1, AR and SMIT genes are available, making it possible to identify *cis*-elements and trans-acting factors that may be directly responsive to medium osmolarity. The cDNA for betaine transporter (BGT1) encodes a single protein of 614 amino acids with 12 putative membrane-spanning regions (Yamauchi et al., 1992). A *cis*-element, termed tonicity-responsive enhancer element (TonE, TACTTGGTGGAAAGTCCAG), has been found to be sensitive to medium osmolarity in both reporter gene assay and gel mobility shift assay (Takenaka et al., 1994). Sequence comparison and mutational analysis of the several BGT1 promoter sequences obtained from different species have revealed the consensus sequence of TonE as YGGAAAnnYnY where Y represents C or T (Miyakawa et al., 1998). Using similar approaches, Ferraris et al (1996) have identified a *cis*-element in the rabbit AR gene promoter, termed the osmotic response element (ORE, 5′-TGGAAAGTCCA-3′). A comparison of the sequences for TonE and the ORE from different species reveals a high degree of similarity, indicating that they represent the same *cis*-element with a refined consensus sequence TGGAAAnnYnY. SMIT cDNA encodes a single protein of 718 amino acids, with 12 putative membrane-spanning regions (Kwon et al., 1992). TonE is also present in the promoter region of the SMIT gene. Figure 11.1 illustrates the organization of the TonE *cis*-element in the promoter

region of human AR, bovine SMIT and canine BGT1 gene. All three genes contain perfect TonE sequences in their promoter region. However, the copy number, the location and the neighboring *cis*-elements of TonE in these promoters are all different. For example, the two TonEs in BGT1 gene are only 300 bp upstream from the ATG codon whereas the first TonE in the AR gene is almost 1 kb upstream from ATG. With a 1.5 kb region upstream from ATG, five TonEs can be found in human AR gene, but only two are present in the promoter region of either bovine SMIT or canine BGT1 genes.

With more refined promoter analysis techniques available, it is also possible that additional *cis*-elements capable of responding to osmolarity may be found in these and other osmoregulatory genes. For example, Iwata et al (1997) recently reported a new *cis*-element, termed aldosse reductase enhancer element (AEE, 5′-GGGTGTTGGAAGAGTGCCAAATTT-3′), which is also involved in the osmotic response activity of the rat AR promoter. In some osmoregulatory genes, other *cis*-elements such as NF-Y and AP1 are also present near the TonE sites (fig. 11.1). Clearly, future work will be directed toward understanding how the promoter organization of these *cis*-elements can affect the cross-talk among different DNA binding proteins and thus lead to controlled gene expression during osmotic stress.

2.4. *Trans-acting factors*

The DNA binding protein that recognizes TonE has not been fully characterized yet. Miyakawa et al (1998) found that a 200 kDa polypeptide can be UV cross-linked specifically to a TonE containing oligonucleotide, suggesting that this polypeptide may be either a component of TonE binding protein (TonEBP) or the TonEBP itself. Further identification of TonEBP will have to await its purification and cloning. Ultimate proof will come from in vitro reconstitution experiment using purified protein components. Without detailed knowledge of the sequence and structure of TonEBP, it is difficult at this moment to speculate how TonEBP senses the hyper-osmotic signal and how it is activated. It has been suggested that the intracellular ionic strength could be the cause that leads to the induction of osmoregulatory genes (e.g. Burg et al., 1997). Whether TonEBP can be activated by high ionic strength is testable once recombinant TonEBP is available. The cloning of TonEBP will also enable us to investigate how this trans-acting factor interacts with other transcription factors (NF-Y, AP1 etc.) during hyper-osmotic stress.

2.5. *MAPK signal transduction pathways*

Elucidation of the signal transduction pathway will be essential for us to understand the mystery of the process that converts changes in ΔG into biological signals that elicit osmotic stress response. Research in yeast has shed some light on the possible involvement of protein phosphorylation in osmotic response. Using complementation approach, several genes, termed HOG (high osmolarity glycerol response genes), have been cloned from yeast (Brewster et al., 1993). Further study revealed that a two-component osmosensing system (Sln1p/Ssk1p), similar to that in prokaryotic cells, is operative in yeast. In this system, the histidine kinase sensor, Sln1p and the response regulator, Ssk1p, work in tandem to regulate the osmosensing MAP kinase cascade (Maeda et al., 1994). In view of the simplicity and elegance of this model, it is certainly attractive to speculate that similar pathway may also be present in mammalian cells. If so, one can expect that alterations in the phosphorylation of trans-acting factors such as TonEBP may be the cause for enhanced DNA binding activity. Figure 11.2 illustrates schematically the three MAPK signal transduction pathways present in mammalian cells. These pathways, termed ERK, JNK and p38 pathways, are grouped according to the signature phosphorylation motif at the MAPK proteins. Each pathway contains a serine/threonine kinase (MAPKKK) which phosphorylates and activates a dual-specificity threonine-tyrosine kinase (MAPKK). MAPKK then phosphorylates MAPK on two phosphoryla-

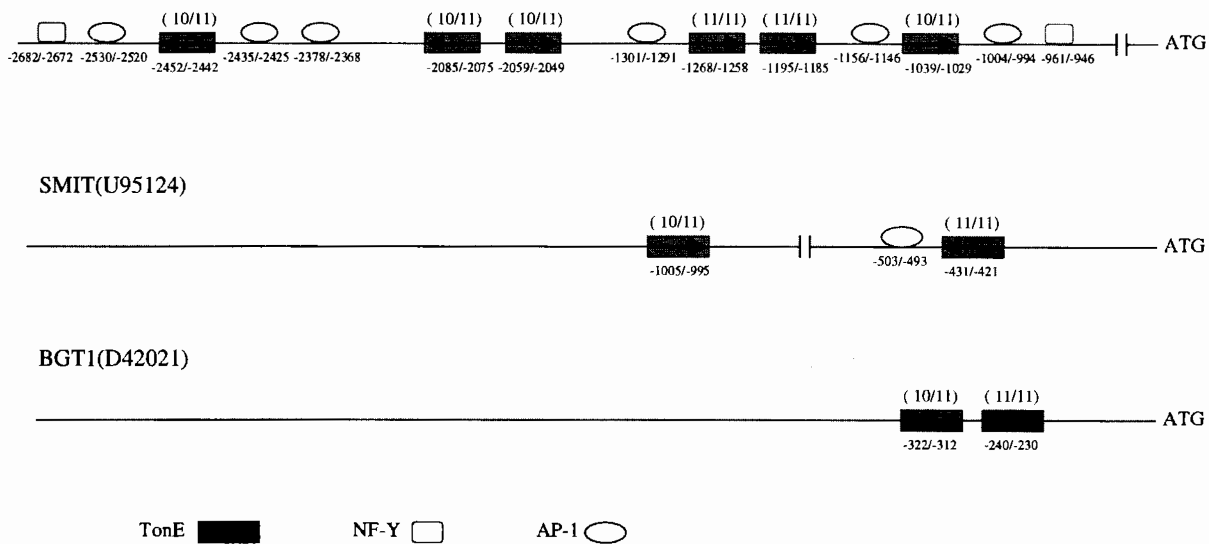

Fig. 11.1. Promoter organization of human AR gene, bovine sodium dependent myo-inositol transporter gene and canine betaine/γ-amino-n-butyric acid transporter gene. The accession number of each gene is indicated in the parenthesis. The numbers above the TonE box indicates degree of sequence match. The number underneath each *cis*-element indicates the position relative to the ATG initiation codon, since the transcription initiation site is not known for all the genes. Other *cis*-elements near TonE were identified by using GCG program, FINDPATTERN.

tion sites within a TXY motif (X: glutamic acid, proline or glycine) that is adjacent to the catalytic cleft of the kinase (Hanks et al., 1988). The three different TXY motifs are TEY, TPY and TGY, representing the signature motif for ERK, JNK and p38, respectively. Once activated, MAPKs will phosphorylate their substrates on the PX(T/S)P consensus motif where X can be any amino acid residue (Songyang et al., 1996).

Although it has been reported in many studies that osmolarity change can activate kinases of MAPK pathways, literature results appear to be conflicting with each other in some instances. For example, some studies showed that hyperosmolarity activates ERK kinases in cells (Matsuda et al., 1995; Itoh et al., 1994, Kwon et al., 1995), but other studies reported that it has no effect on ERK (Warskulat et al , 1998). Similarly, one study showed that hyper-osmolarity activates the p38 pathway (Han et al., 1994), but another study showed a lack of effect of hyperosmolarity on p38 (Sadoshima et al., 1996). At this stage it is unclear whether any of the MAPK pathway components is directly linked to the expression of the genes listed in table 11.1. Using specific kinase inhibitors, it has been shown that the induction of SMIT and BGT1 mRNA may not need ERK (Kwon et al., 1995) and that the induction of AR may not require p38 or JNK (Kultz et al., 1997). Notwithstanding the conflicting results, it is still likely that MAPKs may have an important role in osmotic stress response in mammalian cells. However, since phosphorylation-dephosphorylation is a dynamic and transient event, a more systematic approach is needed to tackle its role in osmotic stress response. For example, parameters such as cell type, time course, different osmolarities (e.g. 500 vs. 600 mOsM), and the method of generating hypo- or hyper-osmotic medium (NaCl, sorbitol, water etc) should be assessed for their effects on MAPK activation. Future studies should also be directed toward the identification of the upstream players (e.g. MAPKKKK and receptors) and downstream targets during osmotic stress.

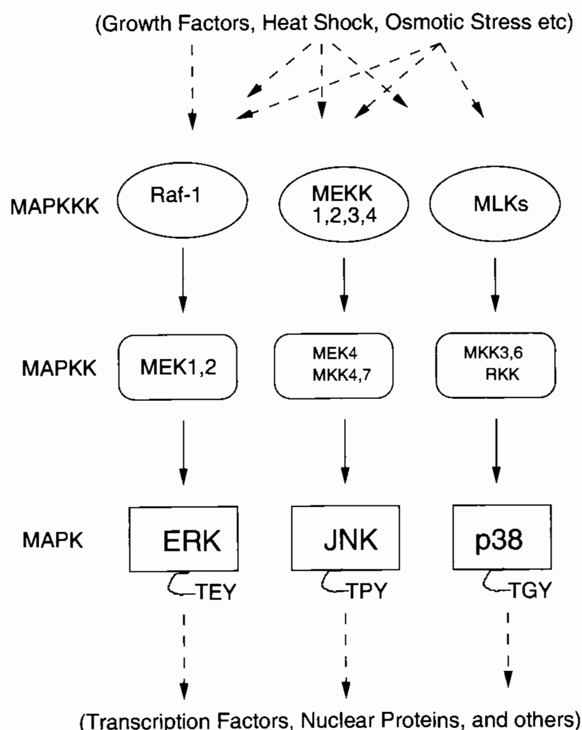

Fig. 11.2. Schematic diagram of three MAPK signal transduction pathways in mammalian cells. The environmental signal (growth factors, heat shock or osmotic stress) activates membrane bound receptors or kinases which initiates the cascades (dotted arrows) that lead to the activation of three MAPK signal transduction pathways: ERK, JNK and p38 pathways. The TXY sequence in the MAPK kinase domain is subject to dual phosphorylation at T and Y by dual specificity MAPKK. The substrate proteins of MAPK include transcription factors and other nuclear proteins. MAPK, mitogen activated protein kinase (also termed proline-directed serine/threonine kinase); MAPKK, mitogen activated protein kinase kinase (a dual specificity kinase); MAPKKK, mitogen activated protein kinase kinase kinase; MEK, MAP/ERK kinase; MEKK, MEK kinase; ERK, extracellular regulated kinase; JNK, Jun N-terminal kinase; p38, reactivating kinase; MKK, MAP kinase kinase; MLK, multilineage kinase; Raf-1, Ras-activated factor 1.

3. Osmotic stress and heat shock response

It has been reported that hyper-osmolarity could lead to a moderate increase in HSP70 mRNA (Cohen et al., 1991). The magnitude of induction, however, differs from cell-type to cell-type (Petronini et al., 1993; Sheikh-Hamad et al., 1994). It is unclear whether the induction is controlled at the transcriptional level. In addition, a concomitant increase in HSP70 protein has not been demonstrated in these studies. The induction kinetics of osmotic stress-induced increase in HSP70 mRNA are much slower than that in heat shock response. The prolonged increase in HSP70 mRNA, up to 24 h (Sheikh-Hamad et al., 1994) and relative high level of constitutive HSP70 mRNA in MDCK cells (Cohen et al., 1991) make it difficult to rule out contributions from posttranscriptional events such as mRNA stabilization. In contrast to these reports, we and others did not observe any HSP70 mRNA accumulation in cells within 3 h after either hypo- or hyper-osmotic stress (Caruccio et al., 1997; Hatayama et al., 1997; Alfieri et al., 1996). Since HSF activation occurs rapidly within minutes after both hypo- and hyper-osmotic stress and the activated HSF has a short half-life (Caruccio et al., 1997), it seems unlikely that HSF activation will be directly responsible for the delayed HSP70 mRNA accumulation during hyper-osmotic stress. Hyper-osmotic stress has also been shown to selectively induce the expression of the osmotic stress protein 94 (Osp94), a member of the HSP110/SSE stress protein subfamily (Kojima et al., 1996). Osp94 mRNA can be induced by either heat shock or hyper-osmotic stress. The levels of induction in both cases are comparable, but the kinetics of induction differ; heat-induced Osp94 mRNA peaks within 3 h, whereas, hyper-osmolarity-induced Osp94 mRNA peaks 12–24 h later. It is unclear whether HSF activation is involved in the hyper-osmolarity-induced increase in Osp94 mRNA. Thus, the hyper-osmolarity-induced stress genes have similar induction kinetics as those of osmoregulatory genes, but much slower than those observed in heat shock response. In this regard, it is interesting to note that the TonE sequence (YGGAAAnnYnY) can be identified in the promoter region of a number of heat shock genes, including HSP70, with high sequence match (10/11 or 11/11). Since the expression of TonE-containing osmoregulatory genes such as BGT1, SMIT and AR exhibits delayed induction kinetics, one wonders whether TonE or TonE-like sequences that are present in certain HSP gene promoters may also contribute to the delayed induction of

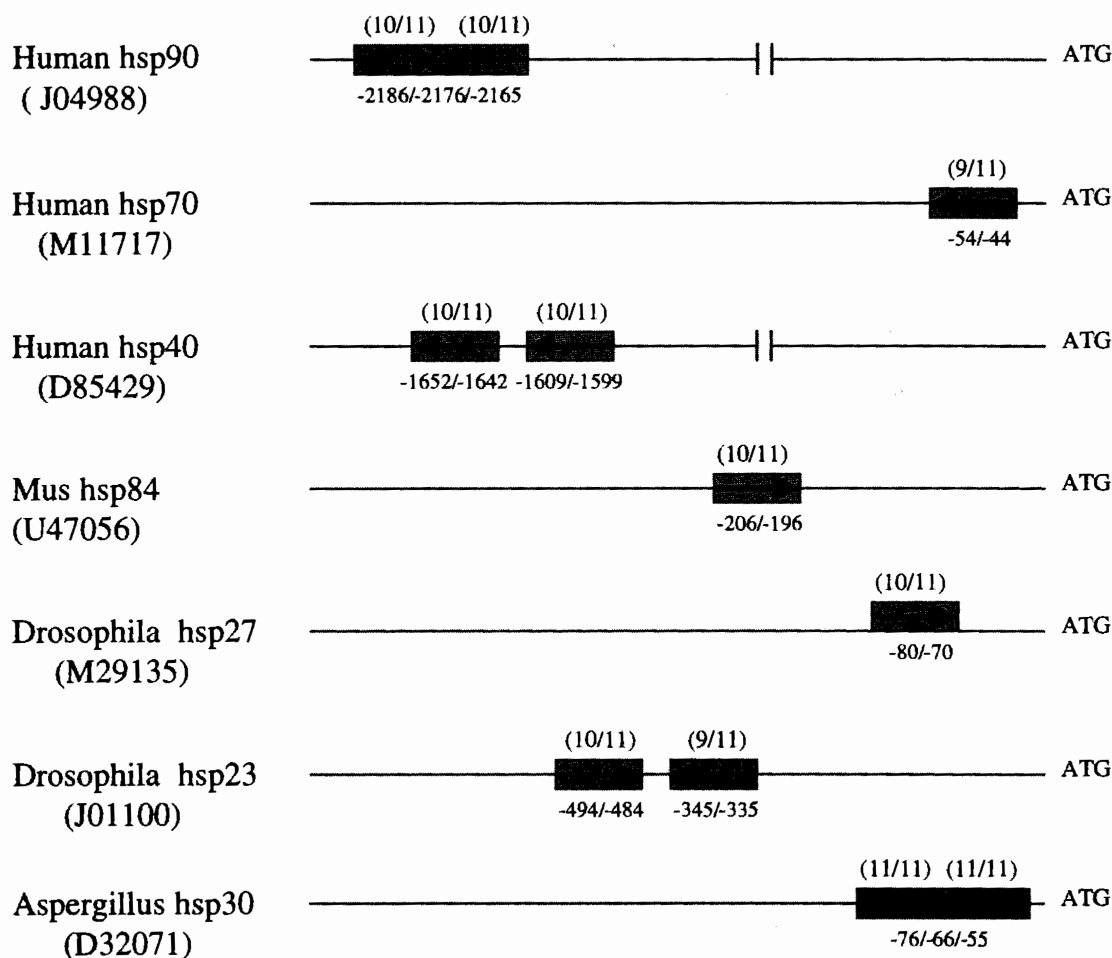

Fig. 11.3. TonE sequence in the promoter region of heat shock genes. The promoter region of several heat shock genes were scanned using BLAST analysis with the consensus TonE sequence, 5′-TGGAAAnnYnY-3′. Accession number of each gene is indicated in parenthesis. The number above the TonE box indicates the degree of sequence match and the number underneath the box indicates the position relative to the ATG initiation codon.

these genes under conditions of hyper-osmotic stress.

4. Heat shock transcriptional factors and stress response

4.1. *Heat shock element*

The induction of heat shock genes in response to heat shock is mediated by the activation of latent heat shock transcriptional activator (HSF) and the binding of HSF to a short and highly conserved upstream response element, termed the heat shock element (HSE). HSE was first identified in *Drosophila* heat shock gene promoters (Holmgren et al., 1981), and the consensus sequence was defined as 5′-CTnGAAnnTTCnAG-3′ (Pelham, 1982). Since it has been shown that an isolated monomeric HSF DNA binding domain can bind to a single nGAAn unit (Kim et al., 1994), the 5-bp nGAAn can be considered as the minimal basic unit of HSE. However, it is unclear whether HSF can bind to the 5-bp basic unit in vivo. A complete HSE consists of contiguous, alternating repeats of the 5-bp unit nGAAn, now refined to AGAAn (n stands for less conserved nuc-

leotides), arranged in either head-to-head or tail-to-tail orientation (Sorger and Nelson, 1989; Sorger, 1991).

The HSE is present in the promoter region of all heat shock family proteins. Figure 11.4 shows the promoter organization of *cis*-elements in several heat shock genes. Although perfectly matched HSE is the prevalent signature of these genes, it can be noted that there is no definitive pattern in terms of the copy number, location or orientation of HSE within the promoter region of HSP genes. The arrangement and the nature of other *cis*-elements close to HSE also shows great variation among HSP genes.

4.2. Trimerization of heat shock transcription factor (HSF)

The HSF is a sequence-specific DNA binding protein that binds specifically to HSE with high affinity. The activation of HSF and its subsequent binding to HSE are key steps in regulating the expression of almost all heat shock genes. Depending on the species studied, HSF can either be a unique gene (e.g. *Drosophila*) or a family (up to 5) of related genes (e.g. human, mouse, tomato). In both human and mouse cells, HSF1 appears to be the one most responsive to heat and other heat shock-like stresses. Other members, HSF2 and HSF3, may respond differently to various forms of stresses or may have functions other than heat shock response (e.g. Tanabe et al., 1997). HSF proteins from various species differ significantly in size, but they all share conserved core domains for DNA binding and for trimerization. Figure 11.5 shows the position of these two domains in *Drosophila* HSF protein and the ribbon structure of the DNA binding domain which spans about 100 amino acid residues. Three α-helices and four β-strands can be identified within the DNA binding domain, and α-helix 3 (aa 54–63) seems to be the region for HSE recognition as indicated from genetic analysis (Vuister et al., 1994; Hubl et al., 1994).

Co-crystallization of the DNA binding domain with HSE oligonucleotides should yield more definitive information. The trimerization domain is located immediately downstream of the DNA binding domain, spanning over aa 147–241 in *Drosophila* HSF. This region is characterized by extensive hydrophobic heptad repeats forming helical coiled-coil structure, typical of multileucine zipper proteins (Rabindran et al., 1993). However, the HSF homotrimer is unique since almost all other leucine-zipper proteins exist as homodimers or heterodimers. Under normal physiological conditions, mammalian HSF is latent and present in monomeric form without DNA binding activity. In response to heat or other stresses, the monomeric HSF is converted into a trimer which exhibits high affinity binding activity to HSE (Sorger, 1991; Morimoto, 1993; Rabindran et al., 1993). It is intriguing, however, that HSF in yeast exists as a trimer with DNA binding activity under normal conditions (Nieto-Sotelo et al., 1990).

4.3. Mechanism of activation

Activation of HSF1 by heat stress is a multistep process that includes trimerization of the HSF monomer, nuclear localization, DNA binding and trans-activation. The process does not involve new protein synthesis and, thus, appears to be controlled posttranslationally. The binding of HSF trimers to HSE is necessary but insufficient for trans-activation; in several cases, DNA binding can be uncoupled from transcriptional activation (e.g. Jurivich et al., 1995). The question of the subcellular localization of HSF monomers under normal, unstressed conditions is not fully resolved. Some studies indicate that HSF1 is a nuclear protein prior to exposure to stress (e.g. Mercier et al., 1997). However, a nuclear localization signal domain has been located in *Drosophila* HSF1. Mutation in this region prevents nuclear localization but is without effect on the heat-induced trimerization (Orosz et al., 1996; Zandi et al., 1997). Furthermore, Zandi et al. (1997) showed that the nuclear entry is a heat stress-dependent process. Taken together, it seems that monomeric HSF1 in most cells exists in the cytosol under unstressed conditions. Trimerization of HSF1 is the key step for HSF

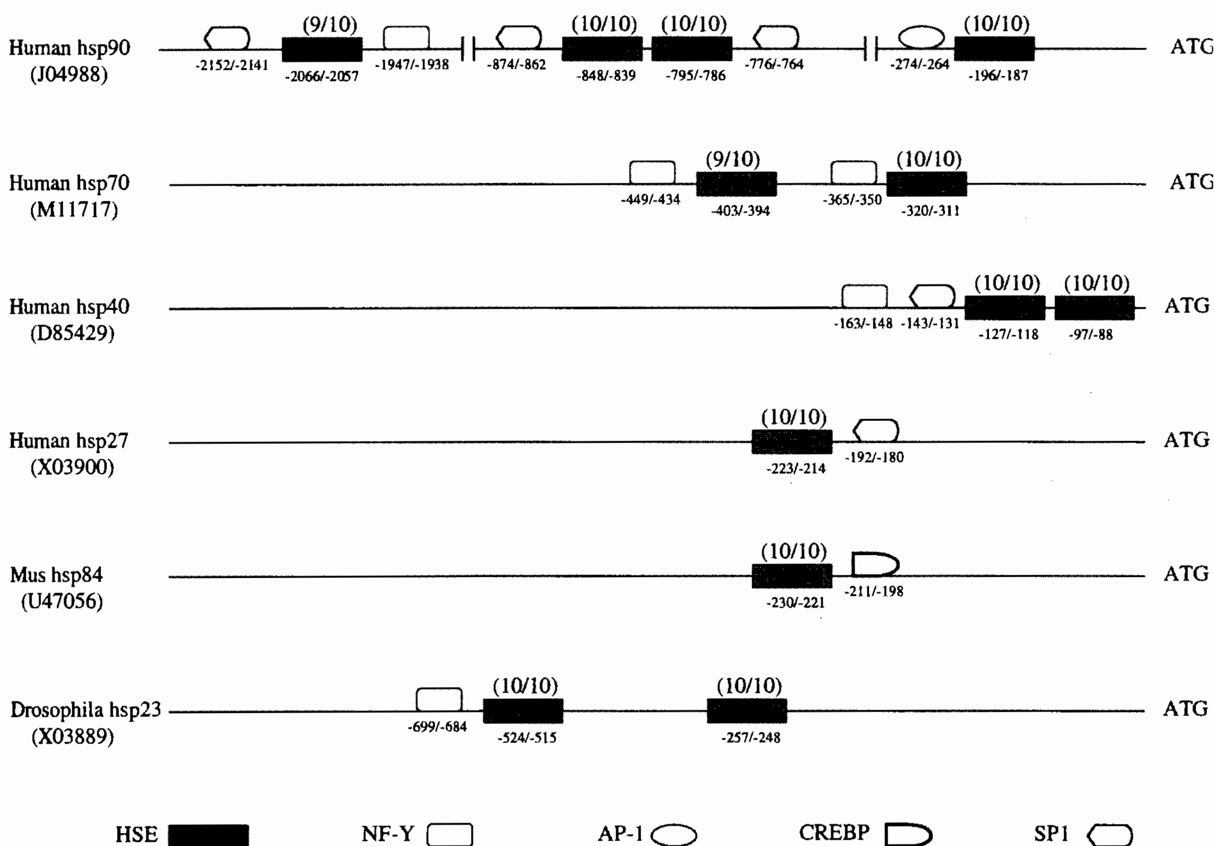

Fig. 11.4. Promoter organization of HSP genes. The arrangement of HSE and other *cis*-elements within the promoter region of several heat shock genes was determined by using GCG program, FINDPATTERN. The number above the HSE box indicates the degree of sequence match with consensus HSE. SP1 and NF-Y sequences are most commonly seen near HSE. The position of each *cis*-element is indicated relative to the ATG initiation codon.

activation. With regard to the monomer-trimer interconversion (i.e. activation and repression), one or several of the following mechanisms could, directly or indirectly, be responsible for this conversion: (a) Intramolecular repression and activation. It has been shown that temperature elevation, pH change, and hydrogen peroxide treatment can induce trimerization of HSF monomers in vitro (Goodson and Sarge, 1995; Farkas et al., 1998; Zhong et al., 1998), suggesting that no external regulator is needed. (b) MAPK signal transduction pathways. HSF1 can also serve as an in vitro substrate for kinases of all three MAP kinase families (Kim et al., 1997). Heat stress causes hyperphosphorylation of HSF (Larson et al., 1988), and specific kinase inhibitors for p38, ERK and JNK have been used to demonstrate the involvement of phosphorylation in HSF activation (e.g. Hung et al., 1998). Hyperphosphorylation may be important in both activation and deactivation , including the stability of the trimer state (Xia and Voellmy, 1997). However, there is also evidence suggesting that phosphorylation is not required for trimerization, but may be important in transactivation (Wu, 1995). (c) Autoregulatory control. Both HSP90 and HSP70 have been shown to bind to HSF and lead to the inactivation of HSF (Zou et al., 1998a). Thus, HSP70 or HSP90 may form an inactive complex with HSF monomers (repression) and the dissociation of the complex during heat shock leads to HSF trimerization (activation) (Morimoto, 1993). (d) Ubiquitin-proteasome pathway. Proteasome inhibitors have been used

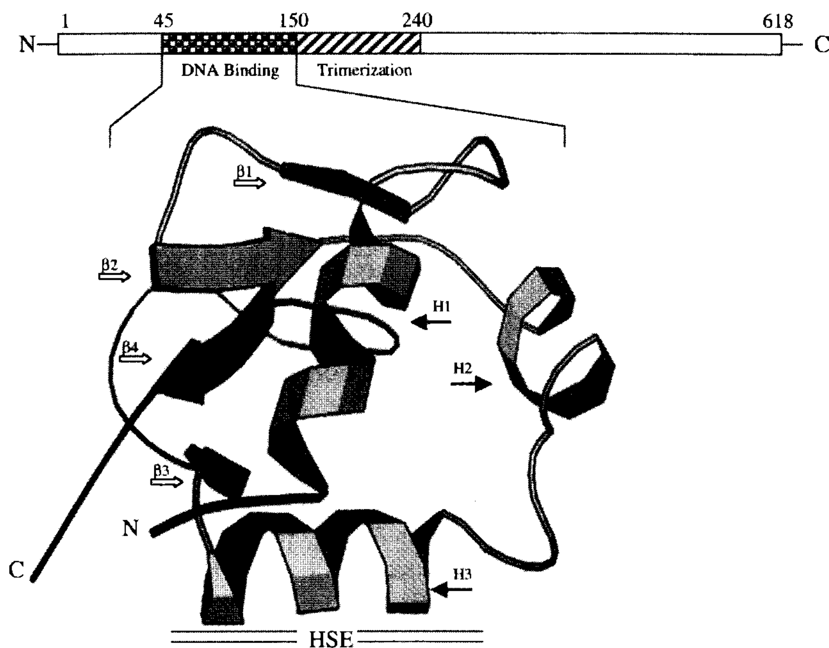

Fig. 11.5. The motif structure of *Drosophila* HSF1 protein and the three dimensional ribbon structure of DNA binding domain in *Drosophila* HSF1 (amino acid residue 45–150). $\beta1$ to $\beta4$ indicates the different β-strand regions and H1 to H3 indicates different α-helix regions. A segment of HSE is included to indicate the interaction with H3 (aa 54–63) in the DNA binding domain of HSF. The ribbon structure was generated with RasMac v2.6 program.

to show that the protein-degradative machinery is involved in HSF activation (Kawazoe et al., 1998, Mathew et al., 1998). (e) Redox regulation. Glutathione oxidation correlates closely with HSF activation, suggesting that the redox state of the system may influence HSF trimerization (e.g. Zou et al., 1998b; Zong et al., 1998).

5. Heat shock transcription factor and osmotic stress

Like heat stress, osmotic stress represents a major physical stress that living organisms may encounter. In view of the key role of HSF in the heat shock response, it is certainly of interest to know whether HSF activation may be also involved in osmotic stress response. A detailed dose-dependent study of the effect of medium osmolarity, from 100 mOsM to 900 mOsM, on HSF activation is shown in fig. 11.6.

HSF activation, as measured by gel mobility shift assay, is prominently induced in mammalian cells under conditions of both hypo-osmolarity (100–250 mOsM) (Huang et al., 1995) and hyper-osmolarity (500-900 mOsM) (Caruccio et al., 1997). Unlike any other types of osmotic shock response, the two opposing physical forces, hypo- and hyper-osmotic stress, produce an almost identical biological response.

5.1. *Characteristics of osmotic stress-induced HSF activation*

The osmotic stress-elicited HSF1 DNA binding activity exhibits a sharp biphasic nature in that HSF activation is prominently induced in cells when the medium osmolarity deviates from iso-osmolarity in either direction (fig. 11.6). HSF activation induced by either hyper- or hypo-osmotic stress shares many similarities (Caruccio et al., 1997; Huang et al., 1995), including the following: (i) both give rapid induction kinetics, detectable within 5 min after stress; (ii) both are independent of protein synthesis; (iii) trimerization and nuclear entry are involved in activation;

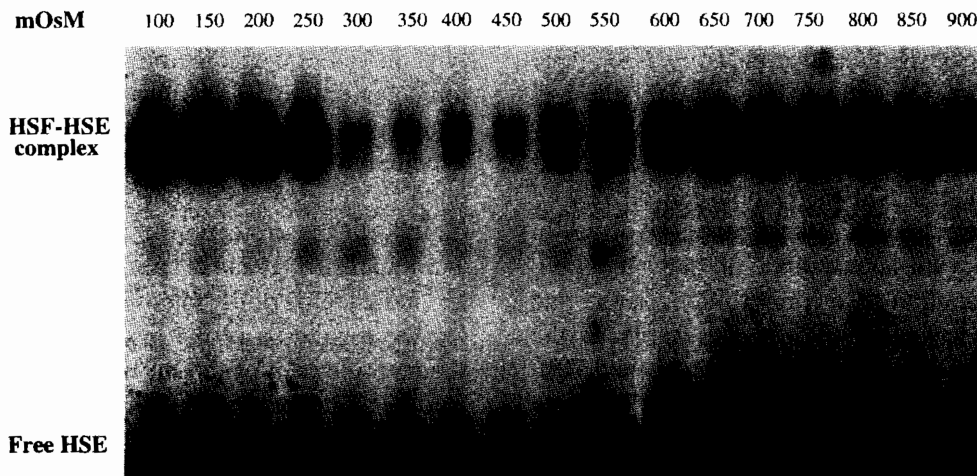

Fig. 11.6. Effect of osmolarity on the activation of HSF1 DNA binding activity. Confluent cultures of HeLa cells were washed with prewarmed isotonic sorbitol solution and then incubated in sorbitol solution with the indicated osmolarity for 20 min. Cells were then harvested for DNA binding activity using gel mobility shift assay (Caruccio et al., 1995).

(iv) the magnitude of induced HSF-DNA binding activity is comparable to that induced by heat shock; and (v) both appear to be uncoupled from HSP gene trans-activation.

5.2. *Mechanism of activation*

The characteristics of HSF1 activation induced by osmotic stress are also shared by heat shock-induced HSF activation. In addition, similar to heat shock, HSF hyperphosphorylation also occurs during osmotic stress as indicated by the slower mobility of the monomeric form on SDS-PAGE (Caruccio, 1995). Since the activation of MAPKs by heat or by osmotic stress occurs rapidly with a time course similar to that of HSF activation (i.e. within minutes), it is tempting to speculate that HSF activation could be mediated by the activation of MAPK pathways. At present, however, there is no direct evidence to support this notion. If indeed some MAPK pathway is involved in osmotic stress-induced HSF activation, this pathway must be activated equally well by both positive change (hyper-osmotic stress) and negative change (hypo-osmotic stress) in ΔG.

The half-life of HSF DNA binding activity induced by either hypo- or hyper-osmotic stress is short, with $T_{1/2}$ estimated to be less than 25 min (Caruccio, 1995). The deactivation of HSF is sensitive to inhibition by cycloheximide, suggesting an involvement of new protein synthesis. The mechanism of HSF deactivation has not been carefully studied. Future study of the deactivation mechanism may shed more light on the mechanism of HSF monomer-trimer interconversion.

5.3. *Possible physiological significance of HSF activation during osmotic stress*

Hypo- and hyper-osmotic stress represent two opposing physical forces applied to a living organism, hence one would expect that they will elicit different or opposite physiological responses. For example, in the yeast two-component osmosensing system, hypo-osmotic stress activates the Sln histidine kinase whereas hyper-osmotic stress inactivates it (Brewer et al., 1993; Maeda et al., 1994). Thus, among many known osmotic stress responses, HSF activation is unique. However, the physiological role of HSF activation during osmotic stress is unclear. During heat stress, HSF activation is directly responsible for transcriptional activation of heat shock family genes and synthesis of HSPs. The physiological heat shock response (e.g. themotolerance, protein folding, etc.) is mediated by various HSPs (Nover and Scharf, 1991). This does not appear to be the case for osmotic stress-

induced HSF activation because of the apparent uncoupling of HSF activation from HSP gene transcription (Caruccio et al., 1997; Alfieri et al., 1996). Since HSE exists almost exclusively in the promoters of HSP genes, it is difficult to envision that HSF activation can be related to the expression of other genes involved in the osmotic stress response. If activated HSF does not function as a transcription factor, one wonders what function it would serve in the osmotic stressed cells. Could the activated HSF trimer, being present in the nucleus, be involved in chromatin stabilization during both hypo- and hyper-osmotic stress? Could it be possible that the binding of HSF to DNA at strategic positions may protect chromosomal DNA from nuclease attack? Recently, HSF, but not HSPs, was found to be required in *Drosophila* under normal growth conditions for oogenesis and early larval development (Jedlicka et al., 1997). Therefore, it is not impossible that HSF in osmotically stressed cells may serve physiological functions independent of HSP gene expression.

6. Conclusions

The osmotic stress-induced HSF activation is unique in that: (i) it is induced within minutes after osmotic stress; (ii) it is inducible by both hypo- and hyper-osmolarity; and (iii) it is uncoupled from transcriptional activation. Except for TonEBP, which has yet to be fully identified, HSF is the only known DNA binding protein that is responsive to osmotic stress. Unlike TonEBP, which responds only to hyper-osmotic stress, HSF activation is sensitive to both hypo- and hyper-osmotic stress. In view of its sensitivity to both temperature and osmolarity changes, HSF can be consider not only as a molecular thermostat, but also a molecular osmometer. HSF activation (trimerization and DNA binding) occurs rapidly within minutes under both heat stress and osmotic stress. In contrast, TonEBP activation, as measured by TonE binding activity, occurs more slowly, usually 6–10 h after hyperosmotic stress. The TonE-containing genes are responsible for the slow accumulation of osmolytes in cells for adaptation to prolonged hypertonic conditions. The function of HSF during both hypo- and hyper-osmotic stress is unclear. Whether HSF activation by osmotic stress is related to delayed HSP gene expression or more directly to the protection of chromosomal DNA from nuclease attack at specific sites remains to be studied. One of the major challenges in studying the regulation of the heat shock response is the identification of the intracellular signals that lead to the activation of HSF by a diverse array of inducers, including osmotic stress. If a common signal exists, this signal must be sensitive not only to enthalpy change but also to changes of osmotic pressure in either direction. In this regard, osmotic stress-induced HSF activation also provides a nice model system to study the mechanism of HSF activation.

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