Regu tion of Heat Shock Response in east and Mammalian Cells

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Nothing shocks me. I'm a scientist.

Harrison Ford (1942-), as Indiana Jones

SUMMARY

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Cells of both unicellular and multicellular organisms experience conditions threatening the integrity of their proteome. Circumstances affecting protein folding include environmental stress, chemical stress, or pathophysiological states of multicellular metazoans. This study characterizes the regulation of components of the heat shock response, a cellular stress defense mechanism present in all eukaryotes. The hallmark of the heat shock response is an increase in heat shock protein expression, which results from transcriptional activation accomplished by heat shock transcription factors.

The human K562 erythroleukemia cell line can be induced to differentiate along either the erythroid or the megakaryocytic lineage. In the present study, differentiation lineage-specific expression patterns of human heat shock transcription factor 2 (HSF2) were detected. During hemin-induced differentiation of K562 cells, HSF2 is upregulated, accompanied by activation of DNA-binding. This upshift was detected at the protein and at the transcriptional level, and was shown to be due to mRNA stabilization in addition to transcriptional induction. In contrast, megakaryocytic differentiation of K562 cells induced with 12-O-tetradecanoyl-phorbol-13-acetate led to downregulation of HSF2 expression and DNA-binding. This downregulation occurred *via* the HSF2 promoter.

Using *Saccharomyces cerevisiae*, a novel regulatory mechanism of yeast chaperones was identified. When yeast cells, grown at 24°C, were preconditioned at 37°C, exposed to a brief thermal insult at 50°C and thereafter returned to 24°C to recover, chaperone expression was induced several hours after the thermal insult, although the cells were maintained at 24°C. This novel regulatory mechanism was designated Delayed Upregulation (DUR). The heat shock proteins Hsp104 (cytosol), BiP/Kar2p and Lhs1p (endoplasmic reticulum), as well as Hsp78 (mitochondria), were subject to DUR, showing that it occurs in various compartments of the yeast cell. For both Hsp104 and BiP/Kar2p, the heat shock promoter element (HSE) was necessary and sufficient for DUR. In the case of Hsp104, the MAP kinase Hog1p and the transcription factors Msn2/4p were required for DUR. Hog1p was also necessary for removing cytosolic heat-aggregated proteins during recovery. The biological functions of BiP/Kar2p, translocation of polypeptides into the endoplasmic reticulum and their folding to secretion-competent forms, were abolished by the thermal insult. However, translocation recovered concomitantly with DUR of BiP/Kar2p, followed by resumption of exit from the endoplasmic reticulum and secretion.

ABBREVIATIONS

ARE	messenger-RNA adenylate- and uridylate-rich (AU-rich) element
BiP	immunoglobulin heavy chain binding protein
cDNA	complementary DNA
DUR	delayed upregulation of chaperones after thermal insult
EM	electron microscopy
ER	endoplasmic reticulum
HSE	heat shock element
HSF	heat shock transcription factor
HSP	heat shock protein
IRES	internal ribosome-entry sequence
kDa	kilodalton
MAPK	mitogen-activated protein kinase
miRNA	micro RNA
mRNA	messenger ribonucleic acid; messenger-RNA
MSF	mitochondrial stimulating factor
NAC	nascent chain-associated complex
NMD	nonsense-mediated decay pathway of messenger-RNA
PABP	poly(A)-binding protein
qPCR	real-time quantitative PCR
RNAP	RNA polymerase
Rubisco	1,5-bisphosphate carboxylase/oxygenase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electroforesis
snRNP	small ribonuclear protein complex
STRE	stress response element
TPA	12-O-tetradecanoylphorbol-13-acetate
uORF	upstream open reading frame
UPR	unfolded protein response
UPRE	unfolded protein response element
UTR	untranslated region in mRNA
wt	wild type

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals, and on unpublished results presented in the text.

I Pirkkala, L., Alastalo, T.-P., Nykänen, P., **Seppä, L.,** and Sistonen, L. 1999. Differentiation lineage-specific expression of human heat shock transcription factor 2. *FASEB J.* 13: 1089-1098.

II **Seppä, L.,** Hänninen, A. L., and Makarow, M. 2004. Upregulation of the Hsp104 chaperone at physiological temperature during recovery from thermal insult. *Mol. Microbiol.* 52: 217-225.

III **Seppä, L.** and Makarow, M. 2005. Regulation and recovery of functions of the yeast chaperone BiP/Kar2p after thermal insult. Manuscript.

REVIEW OF THE LITERATURE

1 Regulation of eukaryotic gene expression

All cells in a multicellular organism contain the same set of genes. Yet the structure and function of different cell types vary greatly. This is because only a fraction of the genes is expressed within any given cell at a given time. The pattern of gene expression is what distinguishes a skin cell from a liver cell, and a cancerous cell from a normal cell. The term gene expression refers to all processes that are needed to convert the genetic information contained in a gene to produce a functional protein (Fig. 1).

Regulation of gene expression in an eukaryotic cell is a series of complex control mechanism, starting with the activation and nuclear transport of the transcription factors, transcription in the nucleus, followed by modification and splicing of the transcript, export of the mRNA from the nucleus to the cytoplasm, cytoplasmic distribution of the mRNA, and finally, translation to protein (Fig. 1). Subsequently the activity, multimerization and degradation of the functional protein are likewise tightly regulated. The following sections review the steps that are subject to regulation for controlling expression of a protein.

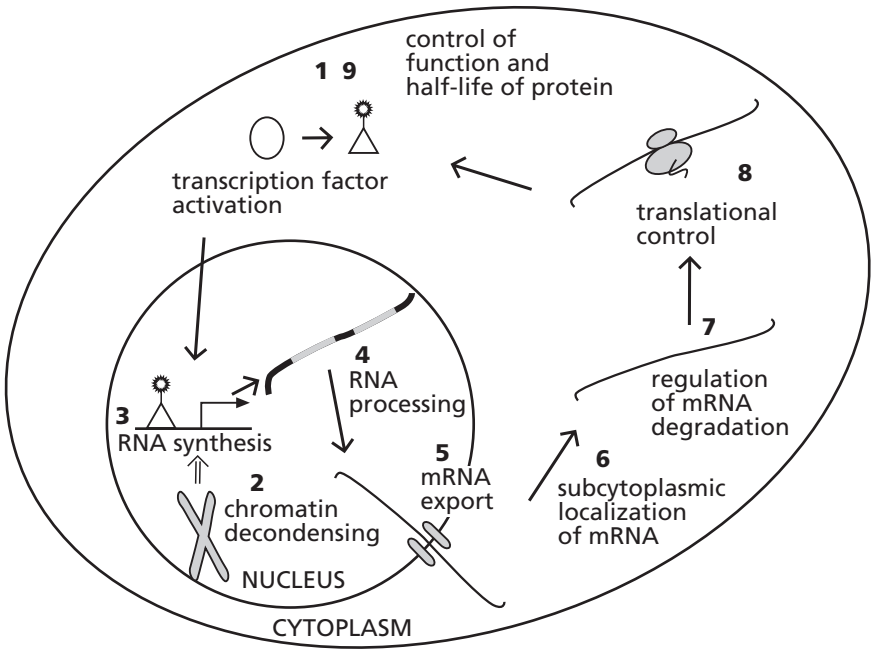

Figure 1. Steps at which eukaryotic gene expression can be controlled. Gene expression is a multi-step process in which the information contained in genomic DNA is converted *via* mRNA to protein. The key steps of gene expression are transcription (3) and translation (8), followed by folding, post-translational modification and targeting (9) of the gene product. The pattern of proteins that a cell expresses depends on the tissue, the developmental stage of the organism and the metabolic or physiologic state of the cell.

1.1 Transcriptional regulation

In transcription, a single-stranded RNA molecule is produced, using genomic DNA as a template, in a reaction catalyzed by RNA polymerases (RNAP). In eukaryotes, there are three types of RNA polymerases, RNAPI to III, each of which transcribes its own subset of RNAs. This chapter focuses on expression of genes transcribed by RNAPII, which is responsible for the expression of protein-coding genes.

Transcription is accomplished by a protein complex called the general transcription machinery, which, in addition to RNAPII complex, consists of a series of additional factors called general transcription factors. They recognize specific promoter sequences and perform the steps required for transcription initiation. The general transcription machinery binds to a specific region upstream of a gene, referred to as the core promoter, located typically 40 to +50 relative to the initiation site (+1). DNA-binding is followed by RNA synthesis performed in three steps, transcription initiation, transcript elongation and termination.

Before the general transcription machinery can act, however, it must be activated by *trans*-acting regulatory proteins called transcription factors (Fig. 1, steps 1 and 3). They bind to DNA regulatory sequences upstream of the core promoter and modulate the activity of the general transcription machinery. Transcription factors play a key role in regulation of gene expression; the fact that 5% of human genes encode transcription factors reflects their importance (Tupler *et al*., 2001). Transcription factors allow tight regulation of transcription in response to nutritional, environmental or hormonal stimuli, and in a manner specific for tissue type and developmental stage.

The transcription-regulating, *cis*-acting sequences fall into two different classes. The first class, promoters, are usually located within 100-400 basepairs upstream from the transcriptional start site. They are composed of various combinations of short, conserved DNA sequences called promoter elements specifically recognized by transcription factors. Examples of promoter elements are heat shock elements (HSEs), which are present in the promoters of numerous stress-induced genes, e.g. *HSP104* and *KAR2* in the yeast *Saccharomyces cerevisiae,* and which are recognized by the yeast heat shock transcription factor 1 (Hsf1p).

The second class of *cis*-acting regulatory sequences, enhancers, consists likewise of modular elements recognized by transcription factors. However, unlike promoters, enhancers can be located kilobases away from the promoter and they are orientation- and position-independent. Enhancers may exist upstream, downstream, or within a gene.

Promoters of most eukaryotic genes contain binding sites for several transcription factors, allowing the gene to be activated in response to a range of stimuli. For example, the above-mentioned *KAR2* gene promoter contains HSEs, binding sites for Hsf1p, allowing the gene to be induced upon heat stress. Unfolded protein response elements, UPREs, which are binding sites for the transcription factor Hac1p, allow the *KAR2* gene to be induced upon accumulation of unfolded proteins inside the endoplasmic reticulum (ER; Kohno *et al*., 1993). On the other hand, one transcription factor frequently activates several genes, allowing concerted expression of all genes needed in response to a specific stimulus. Such is the case for Hsf1p and Hac1p, which both activate numerous genes.

Most transcription factors contain at least two modular domains, a DNA-binding

domain and a regulatory domain. When bound to their target promoter *via* the DNA-binding domain, transcription factors induce or increase transcription by attracting, positioning and modifying the general transcription factors, chromatin modifying proteins and RNAPII at the promoter so that transcription can begin. Examples of the specific mechanism of transcription factor action are described in chapter 3.2.3 concerning heat shock transcription factors 1 and 2.

For transcription to occur, the transcription factors must be present in the nucleus and activated, but in addition, the appropriate chromosome segments must be made accessible. Regulation of chromatin structure (Fig. 1, step 2) plays an important role in gene expression by altering DNA packaging and nuclear localization of the chromosomal DNA, and hence DNA accessibility. Untranscribed regions of the genome occur in a highly-condensed state called "heterochromatin", whereas actively transcribed segments are packaged into "euchromatin". Each cell and tissue type has its own pattern of DNA packaging into heterochromatin and euchromatin, and this pattern is inherited in cell division. Regulation of chromatin structure is a complex and dynamic network of regulatory mechanisms. Chromatin decondensation frequently occurs jointly with binding of sequence-specific transcription factors to the gene promoter. In a widely accepted model chromatin remodeling proteins are recruited to the promoter by the transcription factors (reviewed in Narlikar *et al*., 2002).

1.2 Regulation of mRNA splicing

After and during transcription, the RNA transcript emerging from the transcription apparatus is subject to extensive modifications (Fig. 1, step 4), such as cleavage, adding of a polyadenine tail to the 3' end, adding of a cap structure to the 5' end, and splicing of introns. One of the most crucial steps in RNA processing is RNA splicing, where the non-coding introns are cut from the transcript and protein-coding exons are ligated to form a functional mRNA. RNA splicing takes place in the nucleus within the spliceosome, a large complex consisting of 145 different proteins and five small nuclear RNA molecules, which form small ribonuclear protein complexes (snRNP; reviewed in Jurica and Moore, 2003).

An average human gene is 28 000 nucleotides long, whereas an average mRNA is 960 nucleotides long (Lander *et al*., 2001). This means that the exons are small and exist within extensive intron sequences. mRNA splicing is typical for multicellular eukaryotes. *S. cerevisiae*, in contrast, has introns in only ~3% of its genes (Barrass and Beggs, 2003). Unlike the human introns, which are thousands of nucleotides long, the yeast introns are in average only 270 nucleotides long (Barrass and Beggs, 2003). Furthermore, usually the spliced yeast genes only contain one intron, whereas the average human gene contains 7-8 introns (Lander *et al*., 2001).

One major factor contributing to human proteome complexity is alternative splicing, which results in production of different forms of a protein from the same mRNA. 35-65% of human genes are assumed to be alternatively spliced, which explains how approximately 30 000 human protein-coding genes can give rise to more than 90 000 different proteins (Mironov *et al*., 1999; Modrek and Lee, 2002). The most usual type of alternative splicing is performed by skipping an exon, resulting in a shorter and a longer isoform of the same protein (reviewed in Ast, 2004). This is the case for human and mouse heat shock transcription factors 1 and 2, which are both expressed as a longer α isoform and a shorter

β isoform (Goodson and Sarge, 1995; Goodson *et al*., 1995). Alternative splicing is regulated in a cell type- and developmental stage-specific manner, which results in specific expression of the isoforms in different tissues.

Transcription efficiency affects splicing efficiency, and surprisingly, the same is true in the opposite direction. Splicing occurs frequently cotranscriptionally. For instance, the human gene for dystrophin is 2 400 000 nucleotides long. It would take 16 h to transcribe this gene, and it is obvious that the splicing of the dozens of dystrophin introns would be mechanically difficult from the huge pre-mRNA. Dystrophin is, indeed, spliced cotranscriptionally (Tennyson *et al*., 1995). Coupling of transcription and pre-mRNA processing is thought to depend on the ability of RNA polymerase II to recruit some of the pre-mRNA processing factors in a complex forming a "mRNA factory" (Du and Warren, 1997; McCracken *et al*., 1997a; McCracken *et al*., 1997b; Hirose and Manley, 1998). At least 30 out of the 145 spliceosomal proteins are anticipated to be involved in coupling between splicing and transcription (Zhou *et al*., 2002). However, it is not only that transcription affects splicing. Introducing experimentally an intron immediately downstream from a promoter enhances transcription both in mammalian and yeast genes (Furger *et al*., 2002). Furthermore, spliceosomal factors, by interacting with the human transcription elongation factor TAT-SF1, stimulate polymerase II elongation (Fong and Zhou, 2001), suggesting a strong connection between transcription and splicing.

1.3 mRNA half-life

After synthesis and processing, the newly-made mRNA is exported from the nucleus (Fig. 1, step 5) and distributed to its correct subcytoplasmic localization (step 6). The abundance of a given mRNA species is the sum of its production and decay, which in turn is determined by the stability of the message (Fig. 1, step 7). In many cases, the regulation of protein production is principally performed by controlling mRNA levels. The half-life of different eukaryotic mRNAs varies between some minutes and 24 hours (reviewed in Tourrière *et al*., 2002). Often mRNAs for constantly expressed, "housekeeping" proteins are very stable, like the β-globin mRNA in mammalian cells and the *PGK1* mRNA for 3-phosphoglycerate kinase in yeast cells (Muhlrad *et al*., 1995). The long-lasting message provides the cell a prolonged time window for translation. In contrast, mRNAs for proteins that are transiently expressed in response to a specific stimulus such as developmental, nutritional, hormonal, or environmental input, are often unstable, which enables the cell to downregulate translation rapidly. Examples of such unstable transcripts are mRNAs for growth factors, proto-oncogenes, cytokines, and lymphokines (Schiavi *et al*., 1994; Staton and Leedman, 1998). Apart from regulating the expression of an individual protein, mRNA turnover is a major control mechanism to synchronize gene expression. Recent studies suggest that mRNAs involved in similar mechanisms decay at similar rates (Wang *et al*., 2002; Yang *et al*., 2003), and that the cell achieves this synchronization of decay by concentrating mRNA degradation to discrete cytoplasmic locations (van Dijk *et al*., 2002; Ingelfinger *et al*., 2002; Sheth and Parker, 2003; Cougot *et al*., 2004).

Sequence elements regulating mRNA stability are present throughout the mRNA sequence. mRNA decay is interplay of these *cis*-acting elements with *trans*-acting factors that recognize and bind to them. In the untranslated regions of eukaryotic transcripts, the cap structure at the 5′ end,

the 5′ untranslated region (UTR), the polyadenylated (poly(A)) tail at the 3′ end and the 3′ UTR are determinants of RNA stability. In addition, sequences in the protein coding region of the mRNA have been suggested to act as instability elements, as well (Parker and Jacobson, 1990; Wisdom and Lee, 1991; Ito and Jacobs-Lorena, 2001). The 3′ poly(A) tail, which occurs on most eukaryotic mRNAs, stabilizes the message in which it is present. The highly expressed poly(A)-binding protein (PABP) binds to the poly(A) with high affinity and protects the mRNA from ribonuclease attack (Gorlach *et al*., 1994; for review, see Shim and Karin, 2002). In addition to protecting the poly(A) tail from deadenylases and 3′-5′ exonucleases, PABP also enhances the message by increasing the rate of translation (Otero *et al*., 1999). The 5' cap, in turn, likewise protects the new transcript from attack by nucleases, and serves as a binding site for proteins involved in export of the mature mRNA into the cytoplasm and its translation into protein.

Many unstable mRNAs contain destabilizing elements in their 3′ UTR region. These *cis*-acting elements can be quite variable in length and sequence, but some of them contain adenylate- and uridylate-rich (AU-rich) elements (AREs). AREs are typical for mRNAs that are upregulated during cell growth and differentiation (reviewed in Tourrière *et al*., 2002). The first step in ARE-mediated decay is thought to be deadenylation of the mRNA (Brewer and Ross, 1988; Wilson and Treisman, 1988; Shyu *et al*., 1991; Chen and Shyu, 1994). The presence of AREs is a very widespread and efficient determinant of RNA instability, and their effect is in part due to their potent stimulatory effect on decapping and deacetylation processes (Xu *et al*., 1997; Gao *et al*., 2001).

In addition to the pathways that stabilize or degrade mRNAs specifically, there is a pathway that functions independently of the individual nature of the mRNAs. This nonsense-mediated decay pathway (NMD) targets aberrant or truncated mRNAs for degradation (reviewed in Alonso, 2005). NMD targets incorrectly spliced transcripts and mRNAs with nonsense codons for rapid degradation to prevent accumulation of defective and non-functional proteins (He *et al*., 1993; Pulak and Anderson, 1993).

1.4 Translational control

Protein synthesis (Fig. 1, step 8) is one of the key points in regulation of gene expression. Translational control enables the cell to rapidly manipulate protein production without new RNA synthesis, processing and export. It is especially important, when transcription is silenced, like in oocytes of many species during meiotic maturation, or when a concentration gradient of a protein is created by local translation in a polarized cell.

Eukaryotic cells employ two general modes of translational control. In global control, translation of most cellular mRNAs is regulated as a whole. mRNA-specific control, in turn, is applied to modulate the translation of a defined group of mRNAs without affecting overall translation in the cell (reviewed in Gebauer and Hentze, 2004). Global control is performed by regulating general translation-initiation factors, whereas mRNA-specific control involves regulatory protein complexes that recognize particular elements present in the 5′ or 3′ untranslated regions (UTR) of the target mRNAs. Furthermore, mRNA-specific control can also be regulated by small micro RNAs (miRNAs) that hybridize to specific sequences found in the 3′ UTR of their target mRNAs (Ambros *et al*., 2003; Stark *et al*., 2003; Brennecke *et al*., 2005).

Recently, Iborra and co-workers (2001) reported that mRNA translation also takes place in the nucleus. Nuclear translation has been suggested to represent an additional mechanism of translational control and to be involved in nonsense-mediated RNA decay. Thus, the purpose of nuclear translation seems not to be the production of proteins, but scanning of aberrant mRNAs by nuclear ribosomes (Wilkinson and Shyu, 2002; Iborra *et al*., 2004).

In the mRNA molecule itself, there are several different structural features and regulatory sequences which control translation of the mRNA on which they exist. The cap structure at the 5′ end and the poly(A) tail in the 3′ end of the mRNA have a strong enhancing effect on translational initiation, whereas upstream open reading frames (uORFs) reduce translation from the main ORF. Secondary and tertiary structures found in the 5′ end of the mRNA, in turn, commonly block initiation of translation, while internal ribosome-entry sequences (IRESs) mediate cap-independent translation by bypassing the usual translation initiation process (for review, see Dever, 2002). All these regulatory motifs found in the mRNA UTRs base their activity on specific interactions with different RNA-binding proteins, in particular the translation initiation complex of ribosomes. Upstream open reading frames (uORFs) regulating translation are found both in eukaryotes and prokaryotes. Commonly, they impair translation from the downstream ORF, probably by "misleading" the ribosomal 43S preinitiation complex, which scans along the 5′ UTR until it reaches and identifies an initiation codon (for review, see Dever, 2002). One of the best-studied examples of regulatory uORFs is the *S. cerevisiae GCN4* transcript, which contains four uORFs (Mueller and Hinnebusch, 1986). These uORFs regulate *GCN4* translation by derepression in response to amino acid limitation. Gcn4p is a transcriptional activator that induces at least 40 genes encoding amino acid biosynthetic enzymes (reviewed in Hinnebusch, 1997).

1.5 Regulation of protein half-live and function

As the final steps in regulation of gene expression, the functionality of the proteins is controlled in a variety of ways (Fig. 1, step 9). Some proteins, such as hormones, are produced to function only for short times, whereas others, such as myosin chains in muscles are meant to persist. Protein half-life is tightly regulated by several different mechanisms. One of them is ubiquitin-mediated proteasomal degradation, which plays a central role in regulating destruction of both aberrant and normal proteins. Up to 30% of the newly-synthesized polypeptides in a cell are selected for rapid degradation in the proteasome because they do not pass the quality control system of the cell. In addition to degradation of abnormal proteins, the ubiquitin system plays an important role in a broad array of basic cellular processes. Among these are regulation of cell cycle, modulation of the immune and inflammatory responses, control of signal transduction pathways, development and differentiation (Hershko *et al*., 2000).

Proteins are functional only in interaction with appropriate partners and modifying factors, localized in the correct cellular compartment, and activated appropriately. Human heat shock factor 2 is an example of all these processes. When inactive, it is localized in the cytoplasm as dimers. Upon activation, it forms trimers, localizes to the nucleus and is additionally activated by sumoylation (Sistonen *et al*., 1994; Goodson *et al*., 2001).

2 Protein folding and functions of molecular chaperones

A living cell synthesizes approximately 100 000 different proteins. Virtually every cellular task, on which our lives depend, is performed by proteins. Every protein has its unique, native three-dimensional structure, which is essential for its function. Protein folding is the process, where the linear information in the amino acid sequence of a polypeptide chain is interpreted to a well-defined three-dimensional structure of a functional protein.

Molecular chaperones are proteins that assist the folding of other proteins. Molecular chaperones are found in every organism, every cell and in nearly every compartment of the cells. They are conserved in evolution and essential for all life (for review, see Hartl, 1996). The term molecular chaperone was coined by Laskey and co-workers (1978) when describing the histone-assembling function of nucleoplasmin. More recently, the definition of molecular chaperones has expanded to describe a variety of proteins. The common feature for all these proteins is that they prevent improper interactions between their immature clients and favor more appropriate contacts. Furthermore, molecular chaperones are not part of the final protein structures. Chaperones do not themselves contain the information for folding (Anfinsen, 1973) and do not covalently modify their target proteins, but help the self-assembly of proteins by preventing alternative folding pathways leading to nonfunctional products. At the same time, they prevent aggregation of exposed hydrophobic regions in the protein. The binding mechanisms of chaperones are rather unspecific, because they recognize such a wide variety of targets. In a study, where random synthetic peptides where presented to BiP/Kar2p, which resides in the ER, the chaperone was shown to preferentially bind to 4-7 amino-acid peptides with a high content of hydrophobic residues (Flynn *et al*., 1991). The *Escherichia coli* chaperone SecB preferentially binds to positively charged regions of polypeptide chains (Randall, 1992). The current view is that chaperones bind to reactive surfaces of polypeptides, and by doing so, stabilize an otherwise unstable conformation of their targets, and thereby promote their correct fate: folding, transport, assembly, or switching between active/inactive conformations. The roles of molecular chaperones in these processes are described in the following sections.

2.1 Folding of newly synthesized polypeptides in eukaryotic cells

To be functional, a protein has to assume its correct three-dimensional structure and to find its correct location in the cell. All the information required for proper protein folding is contained in the primary amino acid sequence of the polypeptide chain (Anfinsen, 1973). When a polypeptide emerges from the ribosome, it cannot fold correctly until an entire domain is completed. Translation takes seconds to minutes, whereas folding intermediates build in milliseconds (for review, see Hendrick and Hartl, 1993). Consequently, actively growing cells contain large numbers of aggregation-prone, unfolded polypeptides. A polypeptide emerging from the ribosome into the cytosol is first bound by a complex termed the nascent chain-associated complex (NAC), which protects the first 30 C-terminal residues (Wang *et al*., 1995). When disassociated from the ribosome in the cytoplasm, folding of the newly synthesized chain is assisted by Hsp70 (DnaK, Ssb), Hsp40 (DnaJ), and Hsp60 (CCT, GroEL) type of chaperones (Bukau *et al*., 2000). These

chaperones promote the proper conformation of the newly-synthesized polypeptide through repeated cycles of binding, folding and releasing the substrate.

2.2 Molecular chaperones and translocation into organelles

The majority of proteins functioning in different compartments of the cell is produced in the cytosol from nuclear genes. Thus, they have to be specifically recognized, transported to the surface of the organelle, and vectorially translocated into the organelle lumen. Molecular chaperones are involved in virtually all of these transport processes.

Cytosolic Hsp70 family chaperones bind to the target polypeptide and assist its transport to the appropriate organelle membrane. In the organelle lumen, Hsp70 family chaperones stimulate the import of proteins. This is also the case for ER (Chirico *et al*., 1988). Inside the ER lumen, the Hsp70 chaperone BiP/Kar2p, binds transiently to the J-domain of the ER transmembrane Hsp40 protein Sec63p (Misselwitz *et al*., 1999). Both BiP/Kar2p and Sec63p are required for both cotranslational and posttranslational translocation of polypeptides (Brodsky *et al*., 1995).

Although mitochondria contain their own genome, most mitochondrial proteins are synthesized in the cytosolic ribosomes. Import into mitochondria may be described in analogous terms as translocation into the ER. Cytosolic molecular chaperones including Hsp70s and the mitochondrial stimulating factor (MSF) target the precursor proteins to the mitochondrial membrane (Hachiya *et al*., 1994). MSF is a cytosolic chaperone, which hydrolyzes ATP when interacting with precursors of mitochondrial proteins (Hachiya *et al*., 1994). In the mitochondrial matrix, heat shock protein 70 (mtHsp70) cooperates with its four cochaperones Mge1, Tim44, Pam18, and Pam16 at the mitochondrial inner membrane (Frazier *et al*., 2004).

2.3 Chaperone function and changing of protein subunits

When protein subunits are rearranged, hydrophobic patches in the protein are transiently exposed. Therefore chaperones are needed to prevent aggregation and incorrect associations between these reactive surfaces. Some cytoskeletal components are highly dynamic, i.e. they are constantly rearranged. Cytoskeletal microtubules are hollow cylinders constructed of tubulin, which is a heterodimeric protein consisting of α- and β-tubulin (for review, see Liang and MacRae, 1997). There is evidence that the cytosolic chaperone Hsp70 regulates tubulin assembly/disassembly. Mammalian Hsp70 binds to polymerized tubulin and may prevent microtubule formation by inhibiting the binding of microtubule-associated proteins, which are able to promote tubulin assembly and stabilize microtubules (Sánchez *et al*., 1994).

Chaperone activity is crucial for the assembly of the chloroplast enzyme 1,5-bisphosphate carboxylase/oxygenase (Rubisco), as well. Rubisco, responsible for CO_2 fixation during photosynthesis, exists in vascular plants and green algae as a 500-kDa holoenzyme. Eight of its subunits are of chloroplast origin and eight are synthesized in the cytosol (for review, see Gutteridge and Gatenby, 1995). The chloroplast chaperone cpn60 binds to newly synthesized or imported subunits of Rubisco, which are very prone to aggregation. cpn60 is related to the bacterial chaperonin GroEL (Hemmingsen *et al*., 1988) and like GroEL, it functions by stabilizing folding intermediates and thereby preventing their aggregation. The release of Rubisco subunits from cpn60 requires the cochaperonin cpn10 (Schmidt *et al*., 1994).

3 Stress responses

Cells of both unicellular and multicellular organisms experience every day conditions threatening the integrity of their proteome (Fig. 2). Circumstances affecting protein folding include environmental stress, such as fluctuations in temperature, hydration and nutrient balance, chemical stress caused by oxygen-free radicals, transition heavy metals, or pathophysiological states of multicellular metazoans such as ischemia, viral or bacterial infections or tissue injury. To cope with these stress conditions, organisms have evolved a variety of strategies operating on very different levels, from molecular responses to physiological and behavioural adaptation. Two examples of stress response mechanisms at the molecular level, both aiming at protection of proper protein conformation, are the unfolded protein response (UPR) and the heat shock response.

3.1 Unfolded protein response

The unfolded protein response (UPR) is an intracellular signaling pathway that controls transcription of genes encoding ER-resident chaperones, proteins involved in phospholipid biosynthesis, secretory pathway and ER-associated degradation (Travers *et al*., 2000). UPR is induced in response to accumulation of unfolded and misfolded proteins inside the ER lumen, following exposure to ER stressors such as tunicamycin, ditiothreitol, glucose starvation, disturbance of Ca^{2+} ion stores or genetic mutations. Activation of UPR is accompanied

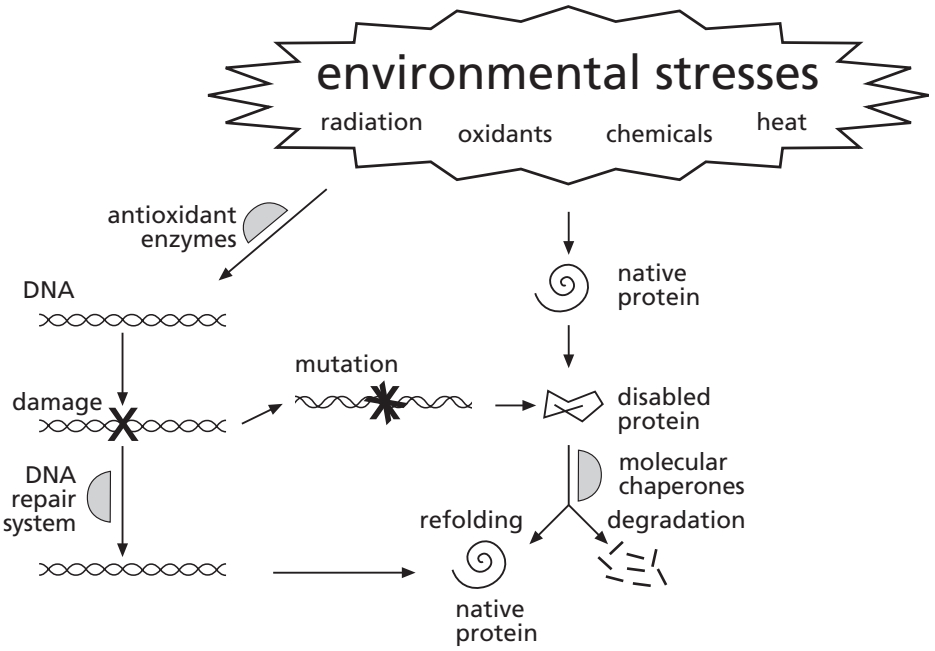

Figure 2. Cellular defense mechanisms against genotoxic and proteotoxic stresses. Misfolded and disabled proteins can occur as a result of alterations in the genomic DNA, direct damage to the cellular proteins by various stresses, or as a part of normal physiology. Antioxidant enzymes prevent oxidative damage of cellular components, DNA repair systems restore DNA integrity, and molecular chaperones refold damaged proteins or direct them to degradation. Modified from Ohtsuka and Hata, 2000.

with translational attenuation of protein synthesis, and leads to upregulation of hundreds of genes containing unfolded protein response elements (UPRE) in their promoters (for review, see Patil and Walter, 2001).

The UPR signal is transferred from the ER to the nucleus via rather unconventional mechanisms, first elucidated in yeast (Fig. 3). Accumulation of unfolded proteins inside the ER lumen activates the transmembrane kinase Ire1p by oligomerization and autophosphorylation by its cytosolic kinase domain (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). The kinase domain activates the carboxy-terminal domain of Ire1p, which is a site-specific endoribonuclease. The only known target of Ire1p endoribonuclease activity is the mRNA encoding the transcription factor Hac1p, which ultimately activates transcription of UPR target genes. The endonuclease Ire1p

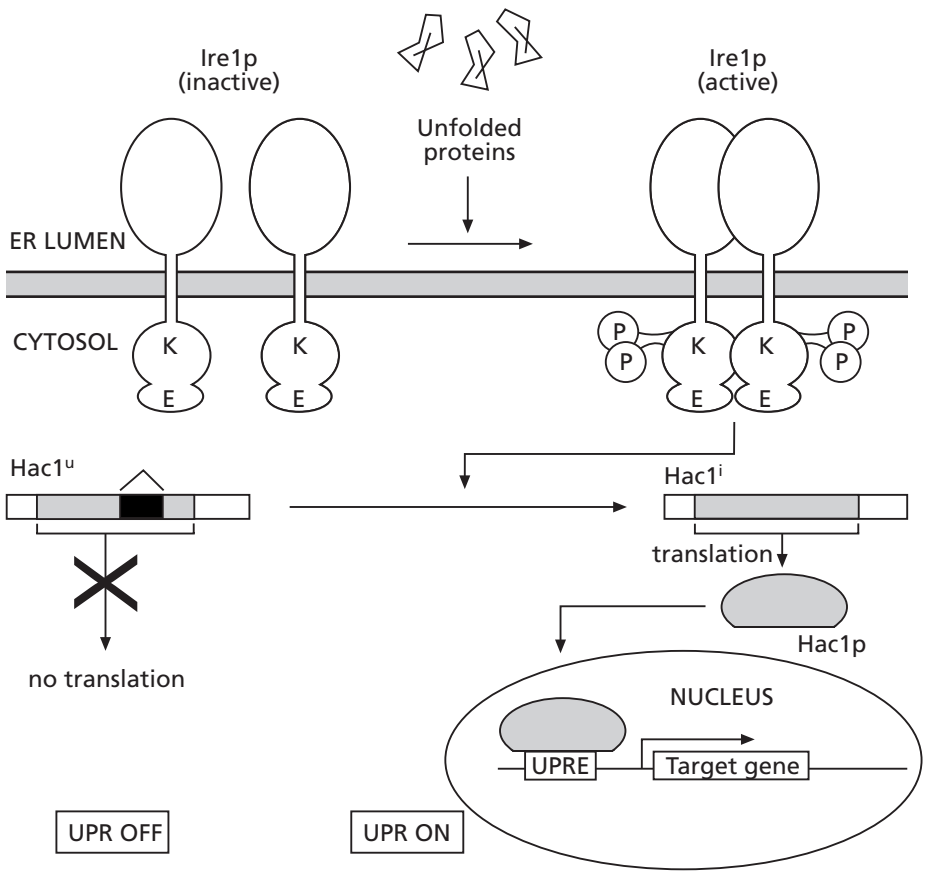

Figure 3. Schematic presentation of the unfolded protein response in yeast. Upon accumulation of unfolded proteins in the ER lumen, the transmembrane kinase-endonuclease Ire1p is activated by dimerization and autophosphorylation of its kinase domain (K). The activated endonuclease domain (E) removes a non-spliceosomal intron from the *HAC1*^{u} (uninduced) mRNA, resulting in *HAC1*^{i} (induced). *HAC1*^{u} mRNA is not translated, whereas *HAC1*^{i} produces the transcriptional activator protein Hac1p. Hac1p translocates to the nucleus and upregulates the expression of unfolded protein response (UPR) target genes by binding to the unfolded protein response element (UPRE) in the promoters of genes UPR target. Modified from Patil and Walter, 2001.

cuts *HAC1* mRNA at two sites, removing a non-spliceosomal intron (Sidrauski and Walter, 1997), producing *HAC1*^{i} mRNA (induced). *HAC1*^{u} mRNA (uninduced) is constitutively produced, but not translated into Hac1p (Chapman and Walter, 1997; Kawahara *et al*., 1997). The *HAC1*^{i} mRNA, free of the inhibitory intron, is efficiently translated to produce the transcription factor Hac1p, which then translocates to the nucleus and binds to UPR-specific upstream activating sequences, unfolded protein response elements (UPREs), on its target genes. Genes activated by UPR include the ER-resident chaperones *KAR2*, *PDI1* and *FKB2* (Mori *et al*., 1998), and the UPRE in their promoters is necessary and sufficient to activate transcription in response to UPR (Mori *et al*., 1992; Kohno *et al*., 1993).

UPR occurs in all eukaryotes, and most of the molecular mechanisms are conserved in yeast and mammals, among those Ire1p homologues, the function of their lumenal domains and dependence of UPR-induced BiP on Ire1 (for review, see Patil and Walter, 2001).

3.2 Heat shock response

One highly conserved stress defense mechanism in all eukaryotes is the heat shock response, whose hallmark is a strong increase in heat shock protein (hsp) expression. The induction of heat shock proteins by temperature shock was first reported in *Drosophila* salivary glands (Ritossa, 1962; Tissières *et al*., 1974). Later on, the expression was found to occur also in a wide variety of other stress conditions, such as exposure to cadmium sulfate, the amino acid analogue L-azetidine-2-carboxylic acid, puromycin, proteasome inhibitors, by mutations in the proteolytic pathways, by expressing mutant actin, or by simply injecting denatured proteins into living cells (reviewed in Georgopoulos and Welch, 1993). The common nominator for all these conditions is the accumulation of abnormally folded proteins. Furthermore, protein conformation-stabilizing agents such as glycerol reduce the heat shock response (Georgopoulos and Welch, 1993). Remarkable features of the heat shock response are the rapidity of its induction and the sensitivity of attenuation. This is achieved by tightly regulating the heat shock protein expression, described below.

Cells survive exposure to otherwise lethally high temperatures, if they are first preconditioned in a temperature moderately above their physiological temperature (Lindquist and Kim, 1996). This phenomenon called thermotolerance is due to production of heat shock proteins, among other cytoprotective agents such as trehalose in yeast cells (Iwahashi *et al*., 1997). Certain heat shock proteins play important roles in acquisition of thermotolerance, or tolerance to other forms of stress. The yeast chaperone Hsp104 is required for acquisition of thermotolerance (Sanchez and Lindquist, 1990). It also protects the cells against harmful effects of ethanol, sodium arsenite and long-term storage at low temperatures (Sanchez *et al*., 1992).

The phenomenon of cross-tolerance is likewise based on the increased production of heat shock proteins induced by exposure to one form of stress. The elevated amount of hsps in the cell thus protects the cell from damage by another form of stress.

3.2.1 Heat shock proteins

Heat shock proteins are needed both in stress conditions and in physiological conditions, where they accomplish similar tasks. Some heat shock proteins are inducible, some are constitutively expressed. Heat shock proteins comprise the largest class of molecular chaperones, but not all chaperones are heat shock proteins, or vice

versa, not all heat shock proteins are chaperones.

According to their apparent size and to their functions, hsps are classified into different families. The five main groups are Hsp100, Hsp90, Hsp70, Hsp60 and the small hsps. Below, these main groups are first shortly described, followed by a more detailed description of the proteins Hsp104, Hsp78, BiP/Kar2 and Lhs1p, that are the focus of this study.

The Hsp100/Clp family members have functions in protein disaggregation and thermotolerance. They form homo-oligomeric ring-shaped structures and modulate their substrates in an ATP-dependent manner. They cooperate with specific Hsp70 cochaperone systems. Some members of this family are the mammalian mitochondrial chaperone ClpP, the yeast cytoplasmic chaperone Hsp104 and mitochondrial Hsp78 in yeast, which are described below (Fig. 4).

Members of the Hsp90 family stabilize misfolded proteins and interact with regulatory signaling proteins. Examples of Hsp90 family proteins are the cytoplasmic chaperones Hsp90 in mammals and Hsp82 in yeast (Fig. 4).

The Hsp70 family is a very large family consisting of proteins with diverse functions. Many of them work together with cochaperones of the Hsp40/DnaJ family, which are thought to stimulate the ATPase activity of their Hsp70 partners (for review, see Ohtsuka and Hata, 2000). Hsp70 family members function in protein folding, damage protection and repair, and translocation of proteins. Examples of Hsp70 family chaperones are the ER chaperones BiP/Kar2p and Lhs1p, which are described below, and the cytoplasmic Hsp70/Ssa proteins (Fig. 4).

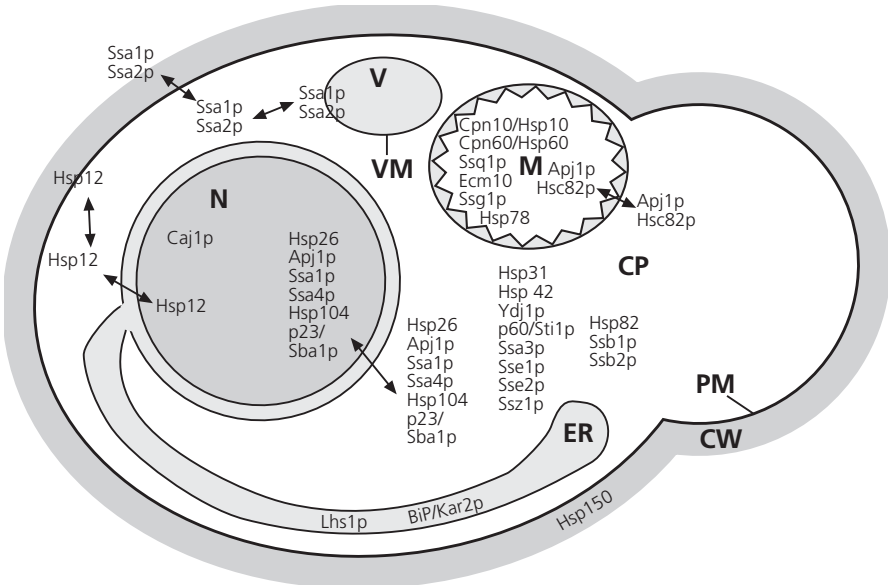

Figure 4. A. Heat shock proteins and their localization in yeast cells. CP, cytoplasm; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; PM, plasma membrane; V, vacuole; VM, vacuolar membrane.

Hsp60 family proteins are often called chaperonins. They share some homology with the bacterial chaperonin GroEL. Chaperonins function in assembly of multimeric protein complexes and folding of certain newly synthesized proteins. Members of the chaperonin family are e.g. the cytoplasmic chaperonin-containing t-complex polypeptide CCT, also called TriC, and mitochondrial Hsp60/Cpn60 (Fig. 4).

Hsp40 proteins are homologues of bacterial DnaJ proteins. They interact with Hsp70 family members and are as abundant as their partners: tens of Hsp40/DnaJ homologues have been identified both in yeast and in mammals (for review, see Ohtsuka and Hata, 2000).

The small hsps are the least conserved of the major hsp families and have divergent structures. They vary between 15 and 40 kDa in size and are targeted to the eukaryotic cytosol and are especially abundant in plants. Small hsps have been reported to function in stabilization of misfolded proteins and thermotolerance (for review, see Parsell and Lindquist, 1993). The most studied representatives of this family are the α/β-crystallins, which are structural proteins in the vertebrate eye lens, and additionally, function there in a chaperone-like manner (Fig. 4).

Hsp104

The cytoplasmic yeast chaperone Hsp104 is essential for acquired thermotolerance (Sanchez and Lindquist, 1990) and it functions in refolding of aggregated proteins in cooperation with the Hsp70/Hsp40 cochaperone system (Parsell *et al*., 1994). Prior to the reactivation of damaged proteins by the Hsp104/Hsp70/Hsp40 chaperone complex, Hsp26 sequesters the aggregated proteins and makes them accessible for refolding (Cashikar *et al*., 2005). Hsp104 is needed for refolding of heat-damaged proteins in the cytosol (Parsell *et al*., 1994) and unexpectedly, in the ER lumen, as well (Hänninen *et al*., 1999). The stress-protective

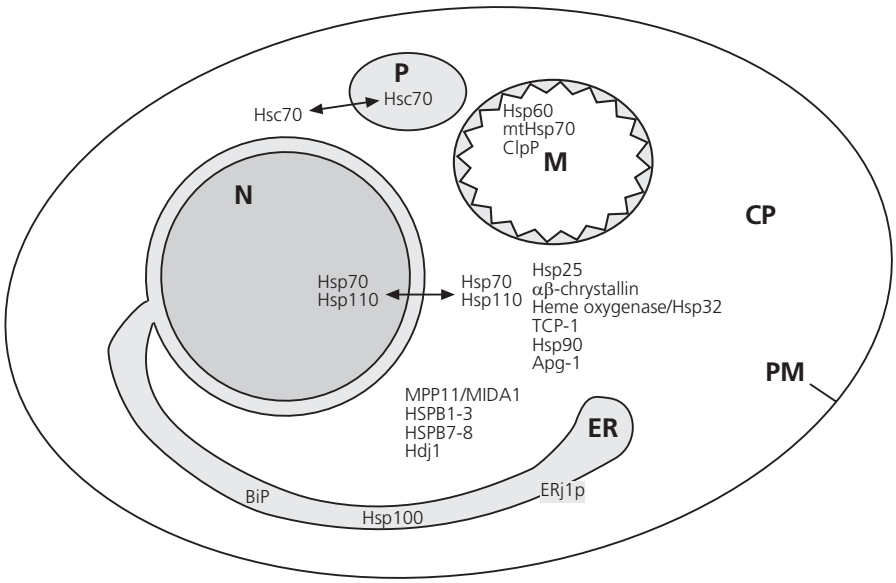

Figure 4. B. Heat shock proteins and their localization in mammalian cells. CP, cytoplasm; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; PM, plasma membrane; P, peroxisome.

yeast disaccharide trehalose assists Hsp104 in refolding of heat-damaged proteins in the cytosol (Singer and Lindquist, 1998) and inside the ER lumen (Simola *et al*., 2000).

Hsp78

In the yeast mitochondria, the Hsp100/ClB homologue Hsp78 is crucial for the maintenance of respiratory competence and resumption of mitochondrial protein synthesis after thermal insult at 50°C (Schmitt *et al*., 1996). Hsp78 functions as a component of the mitochondrial proteolysis system and is required for efficient degradation of substrate proteins in the matrix (Röttgers *et al*., 2002). Similar to Hsp104 in the cytosol, Hsp78 cooperates with the mitochondrial Hsp70 machinery in reactivation of denaturated mitochondrial proteins (Krzewska *et al*., 2001); however, Hsp78 does not affect acquisition of cellular thermotolerance (Schmitt *et al*., 1996).

BiP/Kar2p

BiP/Kar2p is a member of the 70-kDa heat shock protein (Hsp70) family, resident in the ER. Yeast BiP/Kar2p is an essential protein (Normington *et al*., 1989) with multiple roles. It functions in translocation of newly synthesized polypeptides through the Sec61 translocon into the ER (Corsi and Schekman, 1997; McClellan *et al*., 1998). BiP/Kar2p interacts with the lumenal J-domain of the co-chaperone Sec63p, a member of the Hsp40 family, and provides the driving force for translocation by hydrolyzing ATP (Brodsky and Schekman, 1993; Scidmore *et al*., 1993; Cyr *et al*., 1994). Like other Hsp70 proteins, BiP/Kar2p consists of two domains, an N-terminal domain harbouring an ATPase catalytic site and a C-terminal domain responsible for substrate binding (Flynn *et al*., 1989; McKay, 1993; Blond-Elguindi *et al*., 1993). Two models have been proposed for the function of BiP/Kar2p in protein translocation (for review, see Jensen and Johnson, 1999). In the ratchet or trapping model, BiP/Kar2p binds to the polypeptide emerging from the translocon preventing it from sliding backwards (Matlack *et al*., 1999). In the translocation motor model, BiP/Kar2p binds to the emerging polypeptide, and an ATP hydrolysis-dependent conformational change in BiP/Kar2p pulls the protein through the translocon (Glick, 1995). Moreover, BiP/Kar2p assists folding of polypeptides in the ER by shielding hydrophobic amino acid side chains from the aqueous environment of the ER lumen (Simons *et al*., 1995). BiP/Kar2p functions in quality control by binding to misfolded proteins and preventing their exit from the ER (Gething *et al*., 1986; Hurtley *et al*., 1989).

Lhs1p

Lhs1p is an Hsp70-related chaperone localized in the yeast ER lumen, member of the GRP170 subfamily of Hsp70 chaperones with homologues in mammals (Chen *et al*., 1996; Kuwabara *et al*., 1996). Unlike BiP/Kar2p, Lhs1p is not essential for viability, but cells carrying an *lhs1* null mutation exhibit a defect in post-translational translocation (Baxter *et al*., 1996; Craven *et al*., 1996; Hamilton and Flynn, 1996) and are constitutively induced in the unfolded protein response UPR (Baxter *et al*., 1996; Craven *et al*., 1996). Lhs1p is required for refolding of heat-damaged proteins inside the ER lumen and for acquisition of thermotolerance (Saris *et al*., 1997; Saris and Makarow, 1998). Recently, Lhs1p was found to interact with BiP/Kar2p. The ATPase cycles of Lhs1p and BiP/Kar2p are coupled: Lhs1p stimulates the nucleotide exchange phase of the BiP/Kar2p ATPase cycle, and reciprocally, BiP/Kar2p stimulates the hydrolysis phase of Lhs1p ATPase cycle (Steel *et al*., 2004).

3.2.2 Heat shock elements

Heat shock protein expression is controlled by specific sequences called heat shock elements (HSEs) located in the upstream regions of heat shock protein genes. Transcription of hsp genes is activated by binding of heat shock transcription factors (HSFs) to HSEs in their target genes. The HSE consensus sequence is defined by a repeating array of the 5-bp sequence nGAAn arranged in alternating orientations (Amin *et al*., 1988; Xiao and Lis, 1988). The number of nGAAn repeats in a functional HSE varies, but usually ranges from three to six. Furthermore, the number of HSEs and the distance between them in a heat shock gene promoter varies. The binding activity of HSF trimers increases as the number of nGAAn repeats in the promoter increases (Topol *et al*., 1985; Xiao *et al*., 1991). However, the sequence of HSE has been conserved in evolution among eukaryotes as diverse as yeasts, ciliated protozoa, insects, nematodes, amphibians, and mammals (for review, see Fernandes *et al*., 1994).

3.2.3 Heat shock factors

Heat shock transcription factors (HSFs) activate the transcription of heat shock protein genes by binding to HSEs in their target genes. HSFs from different organisms comprise a large protein family, and different eukaryotes harbor different numbers of HSFs. The HSF gene was originally identified in yeast as an essential gene for survival and the only HSF in yeast (Sorger and Pelham, 1988; Wiederrecht *et al*., 1988). Fruit fly was shown to have one HSF, as well (Westwood *et al*., 1991), required under normal growth conditions for oogenesis and early larval development (Jedlicka *et al*., 1997). In contrast, several members of HSF family have been found in vertebrates and plants. Human and mouse HSF1 and HSF2 were cloned almost simultaneously (Rabindran *et al*., 1991; Sarge *et al*., 1991; Schuetz *et al*., 1991), Subsequently, two more HSFs, HSF3 and HSF4, have been identified in vertebrates (Nakai and Morimoto, 1993; Nakai *et al*., 1997), of which HSF3 seems to be an avian-specific factor. Plants, in turn, harbor numerous HSFs; tomato more than 16 and wall cress (*Arabidopsis*) 21 putative HSFs (Nover *et al*., 2001).

All members of the heat shock factor family share a similar structure, comprising of an amino-terminal helix-turn-helix DNA-binding domain, an adjacent coiled-coil trimerization domain and with the exception of HSF4, a second coiled-coil domain, located toward the carboxyl-terminus of the protein (reviewed in Pirkkala *et al*., 2001).

Despite their similar structure, vertebrate HSFs exhibit different functions, target tissues and activation patterns. The vertebrate HSF1 and its yeast homologue Hsf1p can be considered as typical metazoan stress-inducible heat shock factors. Under physiological conditions, human HSF1 occurs as inactive monomers in the cytoplasm. Upon stress stimuli, HSF1 trimerizes, translocates into the nucleus, binds to target stress protein gene promoters and activates their transcription (reviewed in Voellmy, 2004). Yeast Hsf1p, in turn, is constitutively trimerized and bound to DNA (Sorger *et al*., 1987; Jakobsen and Pelham, 1988) and its DNA-binding is enhanced upon stress stimuli (Giardina and Lis, 1995). Phosphorylation of human HSF1 on serine residue 230 promotes its transcriptional activity (Holmberg *et al*., 2001), whereas phosphorylation on serine residues 303, 307 and 363 is involved attenuation of HSF1 activity subsequent to stress (Knauf *et al*., 1996; Kline and Morimoto, 1997). The amount of HSF1 varies greatly in different tissues (reviewed in Pirkkala *et al*., 2001). The most studied function of HSF1 is to activate hsp expression upon various stress stimuli that cause protein

misfolding, as described above. However, studies with HSF1 null mice suggest a role for HSF1 also in spermatogenesis, in early mammalian development and in apoptosis protection (McMillan *et al*., 1998; Xiao *et al*., 1999; Christians *et al*., 2000).

Functions of HSF1 and HSF2 seem to be distinct, but they display different specifities for different HSEs. Results from several studies on HSF2 suggest that it is a nonstress-responsive member of the HSF family, controlling hsp expression in development and differentiation (reviewed in Pirkkala *et al*., 2001). The functions of HSF2 are largely unknown. HSF2 is not activated by heat shock or stress stimuli, but during hemin-induced differentiation of K562 cells (Sistonen *et al*., 1992; Sistonen *et al*., 1994); see chapter below. Whereas HSF1 is activated within minutes upon stress, hemin-induced activation of HSF2, in turn, ranges from hours to days (Sistonen *et al*., 1992; Sistonen *et al*., 1994). In contrast to HSF1, activation of HSF2 seems not to be regulated by phosphorylation, but by an increase in protein levels (Sistonen *et al*., 1994) and by modification by SUMO-1, an ubiquitin-related protein (Goodson *et al*., 2001). In K562 cells, the inactive, non-DNA-binding form of HSF2 exists in the cytoplasm as dimers. Upon activation, HSF2 is trimerized and transported into the nucleus (Sistonen *et al*., 1994). Like HSF1, HSF2 also exists as two isoforms, HSF2-α and HSF2-β. Additional splicing of the *hsf2* transcript results in the smaller HSF2-β isoform, which lacks 18 amino acids present in the longer HSF2-α isoform (Goodson *et al*., 1995).

Traditionally, HSF2 has been viewed as a nonstress-activated heat shock factor, involved mainly in developmental processes, such as embryogenesis, organogenesis and spermatogenesis (Eriksson *et al*., 2000; Min *et al*., 2000). HSF2 is expressed in a cell-type dependent manner in the testis (Sarge *et al*., 1994; Alastalo *et al*., 1998) and the HSF2-α and HSF2-β isoforms are developmentally regulated in a stage-dependent manner (Alastalo *et al*., 1998).

Three groups reported simultaneously the generation of mice deficient in *hsf2*, with somewhat different results. All of the three knock-out mouse strains were viable. The *hsf2*-deficiency did not affect the expression pattern of hsps, but it was equivalent in wildtype and *hsf2*-deficient embryos both in normal conditions and after heat shock (McMillan *et al*., 2002; Wang *et al*., 2003). Two of the studies resulted in mice that exhibit several defects in meiosis, brain development and female hormone response (Kallio *et al*., 2002; Wang *et al*., 2003), whereas one group reported that their *hsf2*-deficient mice were normal in terms of fertility, brain development and cognitive and psychomotor function (McMillan *et al*., 2002). In contrast, disruption of both *hsf1* and *hsf2* leads to arrest in spermatogenesis and thus male infertility (Wang *et al*., 2004). The differencies between these mouse models remain enigmatic, but they can be speculated to partially depend on the different technical manners and genetic backgrounds, in which the *hsf2* knock-outs were made.

4 K562 cells as a model for hematopoietic differentiation

The K562 cell line was established in 1971 from a patient with chronic myeloid leukemia in the acute phase (Lozzio and Lozzio, 1975). K562 is a widely used model for studying gene expression during hematopoiesis, because these cells can be induced to differentiate along several lineages. Erythroid differentiation of K562

cells can be induced by a variety of agents, including hemin and sodium butyrate (reviewed in Tsiftsoglou *et al*., 2003). Treatment of K562 cells with the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induces them to differentiate along megakaryocytic lineage, in turn (reviewed in Alitalo, 1990).

Hemin, the synthetic chloride form of heme and a natural regulator of erythropoiesis, binds to hemin-binding proteins inside the cell and modulates the transcription factors binding to Gγ-globin gene promoter (reviewed in Tsiftsoglou *et al*., 2003). When induced towards the erythroid pathway of differentiation, K562 cells start the synthesis of red-cell specific proteins, including globins (Benz, Jr. *et al*., 1980) and glycophorin A (Gahmberg *et al*., 1979). Globin production occurs by transcriptional activation of the embryonic α- and β-like globin genes, ε and ζ, respectively, as well as the fetal γ-globin and adult α-globin genes (Charnay and Maniatis, 1983). Erythroid differentiation of K562 cells induced by hemin treatment does not, however, lead to terminal maturation (Dean *et al*., 1981).

During hemin-induced erythroid differentiation, K562 cells upregulate the expression of Hsp70, which is due to activation of HSF2 binding to the *hsp70* gene promoter (Theodorakis *et al*., 1989; Sistonen *et al*., 1992). In addition, the expression of thioredoxin is induced in K562 cells in response to hemin in a HSF2-dependent manner (Leppä *et al*., 1997a). The existence of two HSF2 isoforms (Goodson *et al*., 1995) adds complexity to the regulatory functions of HSF2 in response to hemin. HSF2-α is the predominantly expressed HSF2 isoform in K562 cells (Leppä *et al*., 1997b). Furthermore, the molar ratio of the isoforms α and β regulates HSF2 activity in hemin-treated K562 cells; overexpression of HSF2-β inhibits HSF2 activation and hemin-induced erythroid differentiation of K562 cells (Leppä *et al*., 1997b).

Megakaryoblastoid differentiation of K562 cells induced by TPA is characterized by loss of the erythroid properties and synthesis of several megakaryoblastoid markers such as platelet-derived growth factor, glycoprotein IIIa and TGF-β. In addition, TPA treatment enhances the expression of tromboxan A_2 receptors, normally found on platelets, on the surface of K562 cells (reviewed in Alitalo, 1990). TPA exerts its effect through PKC, a phospholipid/calcium-dependent serine-threonine kinase, which mediates activation of genes containing TPA-responsive elements in their upstream regulatory sequences (reviewed in Alitalo, 1990).

5 Yeast as a model organism for studies of stress responses

In addition to K562 cells, baker's yeast *Saccharomyces cerevisiae* was used as model in this study. This unicellular eukaryote is an outstanding experimental model to dissect and understand the biochemical mechanisms by which cells sense and respond to stress. Firstly, growing yeast cells in laboratory is fast, safe and non-expensive. Secondly, stable manipulation of the haploid genome of laboratory yeast strains is often fairly uncomplicated and does not pose ethical problems. Furthermore, *S. cerevisiae* genome was the first eukaryotic genome to be completely sequenced (Goffeau *et al*., 1996), and the availability of the yeast genome sequence has provided a remarkable resource for yeast biologists. Finally, as unicellular organisms live in direct contact with their environments, exposing yeast cells to highly defined conditions for studying stress responses is uncomplicated. Consequently, yeast stress responses for

instance to starvation, heat, pro-oxidants, heavy metals and changes in osmolarity or ionic balance are well-characterized (Hohmann and Mager, 1997). Moreover, yeast cells are known to carry out a wide range of physiological processes with high mechanistic similarity to human cells (Bassett, Jr. *et al*., 1996).

Despite the above mentioned benefits of yeast as a model organism, as a unicellular organism it is unsuitable for studying mechanisms that involve cell-to-cell interactions. Thus, for obvious reasons, processes that are based on specialized tissues such as organogenesis, neuromuscular functions or development of metastases cannot be studied using yeast.

AIMS OF THE STUDY

The aim of the present study was to characterize the regulation of different components of the heat stress response using two different model systems, human K562 erythroleukemia cell line and *Saccharomyces cerevisiae* yeast cells. The focus of the study was, however, in processes different from the classical heat shock response, namely differentiation in the case of human HSF2 and recovery from thermal insult in the case of yeast heat shock proteins.

Specific aims:

1) to characterize the regulation of human HSF2 in hemin-induced differentiation of K562 cells
2) to study the regulation of yeast chaperones, specifically Hsp104 and BiP/Kar2p, during recovery after thermal insult

MATERIALS AND METHODS

Experimental methods used in this work are summarized in **Table 1**. Detailed descriptions of the methods are given in the original publications or references therein. The *S. cerevisiae* yeast strains are listed in **Table 2** and the *KAR2* promoter-*lacZ* fusion constructs used in study III are schematically presented in **Figure 5.**

Table 1. Methods used in this study.

Method	Publication	Described in
Analysis of bulk protein synthesis	II	II
Antibody supershift analysis and competition experiment	I	I, Mosser *et al*., 1988
Bacterial transformation	II, III	Standard methods
β-galactosidase assay	III	III
Gel mobility shift analysis	I	I, Garner and Revzin, 1981; Mosser *et al*., 1988
Glucose consumption assay	II	II
Human cell culture	I	I
Northern analysis	I, II	I, II, Russo *et al*., 1993
Nuclear run-on analysis	I	I, Banerji *et al*., 1984
Plasmid construction	II, III	Standard methods
Pulse-labelling and immunoprecipitation of proteins	II, III	Saris *et al*., 1997
Real-time quantitative PCR	III	III
SDS-PAGE analysis	I-III	Standard methods
Site-directed mutagenesis by PCR	II, III	II, III
Thermotolerance assay	II	II
Transmission electron microscopy	II	Simola *et al*., 2000
Western analysis	I	I
Yeast cell culture	II, III	II, III
Yeast cell transformation	II, III	Standard methods

Table 2. *S. cerevisiae* strains used in this study.

Strain	Relevant mutation	Fusion construct	Publication	Source or reference
H1	none		II	R. Schekman
H4	*sec18-1*		II	R. Schekman
H7	none		III	J. Knowles
H245	none		II, III	Thomas and Rothstein, 1989
H335	none	HSP150Δ-β-lactamase	III	Simonen *et al*., 1994
H454	Δ*hsp104*		II	Sanchez and Lindquist, 1990
H720	Δ*ire1*		III	This study
H960	Δ*hog1*		II	Schüller *et al*., 1994
H1095	Δ*msn2* Δ*msn4*		II	Treger *et al*., 1998
H1097	none		II	Treger *et al*., 1998
H1138	STREI in *HSP104* promoter[1]		II	This study
H1139	STREII in *HSP104* promoter[1]		II	This study
H1140	STREIII in *HSP104* promoter[1]		II	This study
H1166	STREI-III in *HSP104* promoter[1]		II	This study
H1189	STREI,II in *HSP104* promoter[1]		II	This study
H1190	STREI,III in *HSP104* promoter[1]		II	This study
H1377	HSE in *HSP104* promoter[1]		II	This study
H1378	HSE+STREI-III in *HSP104* promoter[1]		II	This study
H1388	Δ*hog1 HOG1*$^+$		II	This study
H1765	none	pKTH5157[2]	III	This study
H1766	none	pKTH5158[2]	III	This study
H1767	none	pKTH5159[2]	III	This study
H1768	none	pKTH5160[2]	III	This study
H1769	none	pKTH5161[2]	III	This study
H1777	Δ*hac1*		III	Euroscarf
H1806	none	pKTH5163[2]	III	This study
H1807	Δ*ire1*	pKTH5163[2]	III	This study
H1808	Δ*hac1*	pKTH5163[2]	III	This study
H2004	none	pKTH5222[2]	III	This study

[1] The heat shock element (HSE) and the three stress response elements (STREs) in *HSP104* promoter were mutated individually and in combinations. The *HSP104* cDNAs with the different promoter variants were integrated into the genome of a deletion strain lacking *HSP104* (Δ*hsp104*; strain H454).
[2] *KAR2* promoter-*lacZ* fusion constructs, for schematic presentation see Figure 5.

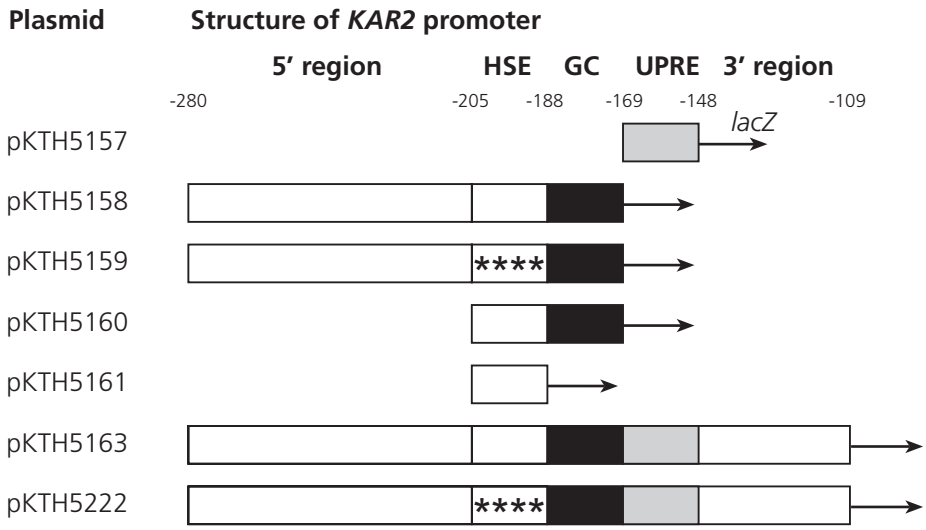

Figure 5. Schematic presentation of *KAR2* promoter-*lacZ* fusion constructs used in this study. HSE, heat shock element; GC, GC-rich region; UPRE, unfolded protein response element of *KAR2* promoter. Asterisks indicate mutation of the HSE.

RESULTS

1 Differentiation lineage-dependent regulation of human heat shock transcription factor 2 in K562 erythroleukemia cells (I)

1.1 HSF2 is activated and upregulated in K562 cells specifically within erythroid differentiation

The K562 erythroleukemia cell line has previously been shown be induced to differentiate along the erythroid lineage with hemin, and along the megakaryocytic lineage with 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Alitalo, 1990; Tsiftsoglou *et al.*, 2003). Erythroid differentiation of K562 cells is characterized by expression of several erythroid markers, such as globins (Benz, Jr. *et al*., 1980) and glycophorin A (Gahmberg *et al*., 1979). K562 cells induced to differentiate along the megakaryocytic lineage, in turn, lose their erythroid properties and instead, synthesize several megakaryoblastoid markers, such as platelet-derived growth factor (PDGF), glycoprotein IIIa and transforming growth factor β (TGF-β; Alitalo, 1990).

Transcriptional activation of genes encoding heat shock proteins is regulated by a family of heat shock transcription factors by binding to heat shock elements in the target gene promoters. HSF2 is one of the three human HSFs identified so far, suggested to function as a developmental regulator, expressed and active during mouse embryogenesis and spermatogenesis, and in embryonal carcinoma cells (Sarge *et al*., 1994; Murphy *et al*., 1994; Mezger *et al*., 1994a; Rallu *et al*., 1997; Alastalo *et al*., 1998).

Abundant expression of Hsp70 in K562 cells undergoing hemin-mediated erythroid differentiation (Theodorakis *et al*., 1989) was previously reported to be due to the HSE-binding activity of HSF2 (Sistonen *et al*., 1992; Sistonen *et al*., 1994). However, it was not known, if the activation of HSF2 was specific for K562 cells differentiating along the erythroid lineage. That is why we wanted to investigate the expression of HSF2 in K562 cells induced to differentiate along the megakaryocytic lineage.

Western and Northern analyses confirmed that hemin-treated K562 cells expressed the erythroid marker γ-globin, but not mRNA for the megakaryoblastoid marker PDGF-B (I, Fig. 1A), in accordance with earlier studies (Dean *et al*., 1981; Mäkelä *et al*., 1987). In contrast, TPA-treated cells expressed PDGF-B, but not γ-globin mRNA. Thus, K562 cells appeared to be able to differentiate along the erythroid and megakaryocytic lineages by hemin and TPA, respectively.

Activation of DNA-binding of HSF2 was inhibited in TPA-treated K562 cells, and, consistent with earlier results (Sistonen *et al*., 1992; Sistonen *et al*., 1994), activated in hemin-treated cells (I, Fig. 1B). Hemin treatment following TPA pretreatment could not induce DNA-binding of HSF2 (I, Fig. 1B). Likewise, TPA treatment following hemin pretreatment did not abolish activation of HSF2, suggesting that commitment of K562 cells to erythroid differentiation is irreversible.

Western analysis showed that HSF2 expression increased in response to hemin-induced erythroid differentiation of K562 cells (I, Fig. 1C). In contrast, brief TPA-treatment led to a decrease in, and prolonged treatment to a complete loss of HSF2 protein expression. The increase in HSF2 levels induced by hemin pretreatment could not be reversed by TPA, and the decrease in HSF2 levels induced by TPA pretreatment could not be reversed by subsequent hemin treatment (I, Fig. 1C),

suggesting that the differentiation lineage commitment of K562 cells is irreversible.

1.2 Upregulation of HSF2 is due to transcriptional activation and stabilization of HSF2 mRNA in hemin-treated K562 cells

Northern analysis of HSF2 mRNA levels confirmed the increase of HSF2 upon hemin treatment and decrease upon TPA treatment (I, Fig. 2). Nuclear run-on assay similarly showed transcriptional induction of the HSF2 gene (I, Fig. 3A). However, the transcriptional induction was 1.5- to 2-fold, whereas the increase in the mRNA level was 6-fold. This suggests that the modest transcriptional induction could not alone give rise to the prominent increase in steady-state mRNA levels. That is why the effect of hemin on the half-life of HSF2 was examined by treating the cells with actinomycin D, which prevents *de novo* transcription, and subsequently analyzing the HSF2 mRNA levels with Northern blotting. The results demonstrated that the half-life of HSF2 was longer in hemin-treated cells than in control cells, showing a marked stabilization of HSF2 mRNA by hemin (I, Fig. 2B). In addition, treatment of K562 cells with the protein synthesis inhibitor cycloheximide, in combination and without hemin, demonstrated that stabilization of HSF2 mRNA was independent of *de novo* protein synthesis (not shown).

1.3 TPA-induced downregulation of HSF2 is mediated *via* the HSF2 promoter

Megakaryoblast differentiation of K562 cells induced by TPA led to a decrease in HSF2 mRNA levels (I, Fig. 2B). By using stably transfected K562 cell clones overexpressing mouse HSF2-α or HSF2-β isoforms under the control of the human β-actin promoter, we wanted to investigate, whether the decrease in HSF2 mRNA in response to TPA occurred at the promoter level. Upon hemin treatment, the increase in HSF2 protein levels was observed in HSFα-overexpressing, but not in HSF2-β-overexpressing cells (I, Fig. 4), in agreement with earlier work (Leppä *et al*., 1997b). Upon TPA treatment, the levels of endogenous human HSF2 protein decreased in both K562 cells and in the transfected cell clones, as expected. On the contrary, the protein levels of mouse HSF2 isoforms expressed under the human β-actin promoter remained unaffected by TPA (I, Fig. 4). Thus, the downregulation of HSF2 in K562 cells induced to megakaryoblastoid differentiation with TPA appears to be regulated *via* the endogenous HSF2 promoter.

1.4 The differentiation lineage-dependent expression patterns of HSF2 are specific for K562 cells

HSF2 expression appears to be strictly differentiation lineage-dependent in K562 cells. The human cell lines Raji (Burkitt's lymphoma), Molt-4 (T-lymphoblast leukemia), and HeLa (cervical carcinoma) were treated with hemin or TPA, and HSF2 expression was analyzed with Western blotting. HSF2 protein expression occurred in all studied cell lines, but in varying amounts, Raji cells containing the highest and HeLa cells the lowest levels (I, Fig. 5). The increase in HSF2 expression in response to hemin, or the loss of HSF protein in response to TPA were, however, detected only in K562 cells. In the other cell lines, HSF2 protein expression remained unchanged after hemin or TPA treatments, suggesting that the differentiation lineage-dependent regulation of HSF2 is specific for K562 cells. In conclusion, during hemin-induced differentiation of K562 cells, HSF2 is upregulated, accompanied by activation of DNA-binding. This upshift was detected at the protein and at the transcriptional level,

and was shown to be due to mRNA stabilization, in addition to transcriptional induction. In contrast, megakaryocytic differentiation of K562 cells induced with TPA led to downregulation of HSF2 expression and DNA-binding activity. This downregulation occurred *via* the HSF2 promoter.

2 Yeast chaperones upregulated during recovery from thermal insult (II, III)

After investigating the regulation of HSF2 in human cells, the focus of the study was shifted to regulation of heat shock protein expression in another model organism, the yeast *Saccharomyces cerevisiae*. HSEs binding the yeast heat shock factor Hsf1p are found, among others, in the promoters of genes encoding for the yeast chaperones Hsp104 and BiP/Kar2p.

2.1 Hsp104 is upregulated during recovery from thermal insult (II and unpublished)

Hsp104 is a cytoplasmic chaperone of the Hsp100/ClpB family, required for refolding of damaged cytoplasmic and ER proteins (Parsell *et al*., 1994; Hänninen *et al*., 1999) and for acquisition of thermotolerance (Sanchez and Lindquist, 1990). A novel type of regulation of Hsp104 expression was identified in the present work. The experimental set-up was as follows. *S. cerevisiae* cells were grown at 24°C, preconditioned for an hour at 37°C, then shifted to 50°C for 20 min (thermal insult) and then back to physiological temperature 24°C (recovery; see II, Fig. 1A). The cells were labelled with ^{35}S-methionine-cysteine during successive 1 h time periods (indicated by bars in Fig. 1A), and thereafter lysed and immunoprecipitated with anti-Hsp104 antibody, followed by SDS-PAGE analysis (II, Fig. 1B) and quantitation by phosphorimager (Fig 1C).

In all experiments involving the above-described heat treatment scheme (both the work in this thesis and previous work in our laboratory), the basal expression level at 24°C (II, Fig. 1, lane a) is defined as the value to which all the other time points are compared to. This strategy was chosen because at 24°C, the cells have not undergone any treatments and this sample can thus be thought to reflect genuine basal expression.

Fig. 1 of publication II shows that upon shift to 37°C, Hsp104 was induced approximately 3-fold, in agreement with previous studies (Sanchez and Lindquist, 1990; Lindquist and Kim, 1996). After thermal insult at 50°C and shift back to 24°C, Hsp104 expression was negligible for some hours. Thereafter it increased to levels higher than after shift from 24°C to 37°C. The maximal expression level, 4- to 5-fold, was reached at approximately 3 h of recovery at 24°C (II, Fig. 1B-C).

Western analysis with Hsp104 antibody (Fig. 6 of this thesis) showed that, upon shift to 37°C, the levels of Hsp104 increased several fold, in agreement with earlier work (Sanchez and Lindquist, 1990; Lindquist and Kim, 1996). Unlike the *de novo* protein synthesis, which was shut off after thermal insult, the elevated level of Hsp104 persisted after thermal insult and increased further at 3 h of recovery at 24°C (Fig. 6 of this thesis). The upregulation of Hsp104 during recovery after thermal insult was not due to an overall upregulation of protein synthesis, but was specific for Hsp104. Cells were exposed to heat treatments and metabolic labelling as before, but total protein was precipitated from the cell lysates with trichloroacetic acid

(II, Fig. 1D). After thermal insult at 50°C and shift back to 24°C, protein synthesis was at first negligible (lane c). Then, it gradually recovered, reaching the physiological level 5-6 h after thermal insult (II, Fig. 1D). When the cells were only preconditioned at 37°C and shifted back to 24°C, omitting the thermal insult at 50°C, no delayed upregulation of Hsp104 occurred (II, Fig. 1E). In conclusion, thermal insult at 50°C resulted in a slow upregulation of Hsp104 expression, although the cells were maintained at 24°C. This novel type of regulation was designated delayed upregulation, or DUR, for short.

2.2 Delayed upregulation of Hsp104 at the mRNA level (II and unpublished)

Northern blot hybridization experiments confirmed the upregulation of *HSP104* mRNA during recovery from thermal insult (II, Fig. 2). The increase in mRNA preceded the increase of *de novo* protein synthesis. Because the kinetics of DUR of Hsp104 was remarkably slow, the induction of *HSP104* mRNA at 37°C was analyzed for reference. Fig. 7 of this thesis shows that at 24°C, the *HSP104* mRNA level was barely detectable, in agreement with earlier reports (Sanchez and Lindquist, 1990). At 37°C, *HSP104* expression was strongly induced after 5 minutes and reached its maximal level after 15 minutes. After subsequent thermal insult at 50°C and shift back to 24°C, the *HSP104* mRNA expression started to increase after 1 hour of recovery at 24°C. These results show that upregulation of *HSP104* mRNA occurs slowlier during recovery from thermal insult than at 37°C.

2.3 Chaperones of the endoplasmic reticulum and mitochondria are also subject to DUR (III)

The next subject of study was to investigate, whether DUR is specific for Hsp104 only. BiP/Kar2p is a member of the 70-kDa heat shock protein (Hsp70) family, resident in the ER. To study the expression of BiP/Kar2p after thermal insult, the metabolic labelling experiment, described above for Hsp104, was performed for BiP/Kar2p (III, Fig. 1). BiP/Kar2p expression was negligible for 1.5 hours after shift back to 24°C, but increased

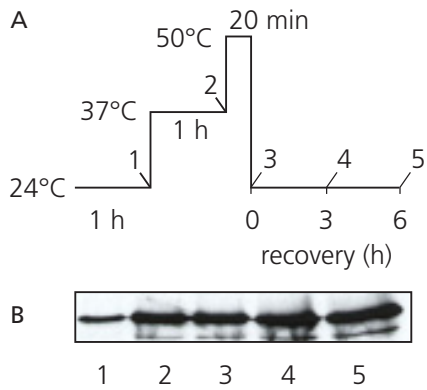

Figure 6. The steady-state level of Hsp104 remains elevated after thermal insult. *S. cerevisiae* cells, grown at 24°C, were preconditioned for 1 hour at 37°C, exposed to thermal insult at 50°C for 20 minutes, and thereafter allowed to recover at 24°C. Cells were collected at the indicated time points (panel A), and the cell lysates were resolved in SDS-PAGE, followed by Western blotting with Hsp104 antibody (panel B).

thereafter to a higher level than after shift from 24°C to 37°C. The expression was maximal 2-3 h after thermal insult and returned to normal after 6 h of recovery (III, Fig. 1B). When only the preconditioning at 37°C was given and the cells were returned to 24°C, no delayed upregulation of BiP/Kar2p occurred (III, Fig. 1D). Thus, DUR is not specific for Hsp104, or cytosolic chaperones, but also the ER chaperone BiP/Kar2p is subject to DUR.

Real-time quantitative PCR (qPCR) using *KAR2* primers on total RNA demonstrated upregulation of the *KAR2* gene at the mRNA level (III, Fig. 2B). Next, we repeated the qPCR experiment for another ER chaperone, Lhs1p, and for the mitochondrial chaperone Hsp78. Lhs1p is 70-kDa heat shock protein, involved in refolding of lumenal ER proteins and acquisition of thermotolerance (Saris *et al*., 1997; Saris and Makarow, 1998) and was recently shown to interact with BiP/Kar2p (Steel *et al*., 2004). The 100-kDa family protein Hsp78, in turn, is required for mitochondrial thermotolerance and for reactivation of denaturated mitochondrial proteins and respiratory function after thermal insult (Schmitt *et al*., 1996; Krzewska *et al*., 2001). qPCR experiments showed that Lhs1p and Hsp78 are also subject to DUR (III, Fig. 2C, D). This demonstrates that chaperones are upregulated during recovery after thermal insult in the cytosol, in the ER and in mitochondria.

2.4 The promoter element required for DUR of *HSP104* and *KAR2* after thermal insult (II, III and unpublished)

Next, we wanted to study, which elements of the *HSP104* and *KAR2* gene promoters are responsible for delayed upregulation after thermal insult. The upstream sequence of *HSP104* contains two heat shock element

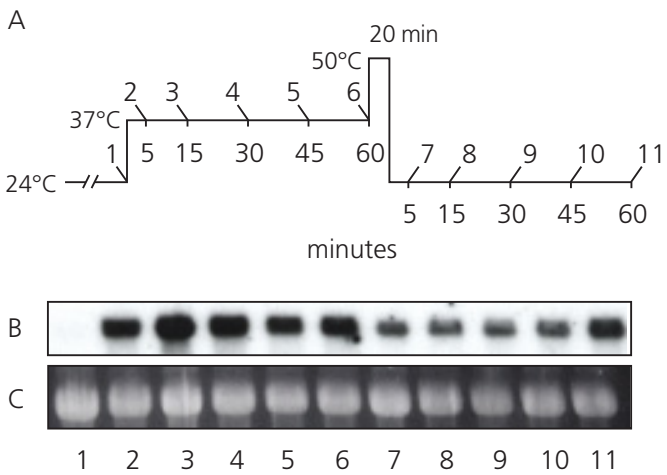

Figure 7. Different kinetics of *HSP104* upregulation at 37°C and during recovery after thermal insult. *S. cerevisiae* cells, grown at 24°C, were preconditioned for 1 hour at 37°C, exposed to thermal insult at 50°C for 20 minutes, and thereafter allowed to recover at 24°C. Total RNA was extracted from the cells collected at the indicated time points (panel A), resolved in a formaldehyde-agarose gel, followed by Northern blotting with a *HSP104* probe (panel B). Ethidium bromide staining of ribosomal RNA, used as a loading control (panel C).

(HSE) sequences repeated *in tandem*, recognized by the transcription factor Hsf1p, and three stress response elements (STRE) recognized by the transcription factors Msn2p and Msn4p (II, Fig. 3). Both Hsf1p and Msn2/4p have been shown to contribute to the stress-induced expression of *HSP104* (Treger *et al*., 1998; Amorós and Estruch, 2001; Grably *et al*., 2002). We mutated these elements individually and in combinations, as indicated in Fig. 3 of publication II, and integrated the *HSP104* cDNAs with the mutated promoter variants into the genome of a *S. cerevisiae* strain, where the *HSP104* gene had been deleted (Δ*hsp104*; see II, Table 1 for genotype). Metabolic labelling experiments performed as in II, Fig. 1A-C showed that in the absence of a functional HSE, the upregulation of Hsp104 at 37°C was reduced, as expected. Furthermore, the upregulation of Hsp104 during recovery from thermal insult was

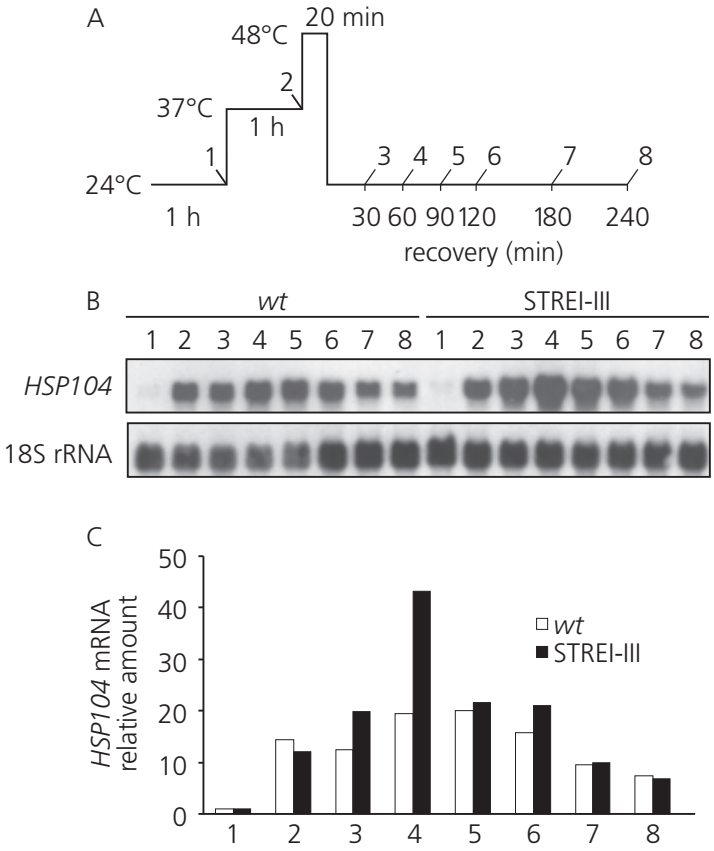

Figure 8. Analysis of *HSP104* mRNA in the STREI-III mutant. *wt* (*S. cerevisiae* strain H1) and STREI-III mutant cells (strain H1166), grown at 24°C, were preconditioned for 1 hour at 37°C, exposed to thermal insult at 48°C for 20 minutes, and thereafter allowed to recover at 24°C. Total RNA was extracted from the cells collected at the indicated time points (panel A), resolved in a formaldehyde-agarose gel, followed by Northern blotting with *HSP104* and *RDN18-1* probes (18S rRNA used as a loading control; panel B). *HSP104* mRNA from *wt* cells (white columns) and the STREI-III mutant (black columns) was quantified by Phosphoimager (panel C).

abolished, showing that HSE was required for DUR of Hsp104 (II, Fig. 4A). The HSE mutant was able to acquire thermotolerance; it formed colonies at 24°C after thermal insult as efficiently as control cells (II, Fig. 5A). Furthermore, the HSE mutant strain remained metabolically active, consuming glucose (not shown) and synthesizing proteins (II, Fig. 4C) as efficiently as control cells during the 6-h recovery period after thermal insult. Thus, the residual expression of Hsp104 in the HSE mutant was sufficient to ensure survival of the strain. Furthermore, the absence of DUR of Hsp104 in the HSE mutant was not due to an impaired survival of the strain.

Mutagenization of the three STREs of the *HSP104* promoter individually or in combination had little or no effect on DUR. In the STREI-III mutant, DUR of Hsp104 was in average fourfold (II, Fig. 4), suggesting that the STREs were dispensable. The metabolic labeling experiment with the STREI-III mutant was performed twice, of which the average is shown in II, Fig. 4. The DUR of Hsp104 was 3.7- and 4.4-fold, respectively (not shown). In turn, the metabolic labeling experiment with the *wt* strain H1 was repeated five times. In these experiments, DUR of Hsp104 was in average 5.2-fold, ranging from 2.5- to 8.7-fold (not shown). Due to the variation between single metabolic labeling experiments, we confirmed the result from the STREI-III mutant using Northern blot hybridisation. Fig. 8 of this thesis shows that DUR of *HSP104* mRNA was 43-fold in the STREI-III mutant, whereas in the *wt* strain, DUR was 20-fold. This Northern experiment was performed twice with similar results. In conclusion, mutagenisation of the STREs in the *HSP104* promoter did not decrease DUR of Hsp104.

In contrast, mutagenization of all three STREs and the HSE in combination abolished Hsp104 expression completely, leading to severely impaired ability to form colonies after thermal insult (II, Fig. 5A) and to resume protein synthesis (II, Fig. 4C) after thermal insult.

The *KAR2* promoter contains one heat shock element (HSE), an unfolded protein response element (UPRE), recognized by Hac1p, and a GC-rich region between them. *KAR2* is an essential gene and disturbance of its upstream regions is potentially lethal. That is why we used Δ*ICL1-lacZ* fusion constructs, where the *E.coli* β-galactosidase gene was placed under different *KAR2* promoter variants (III, Fig. 3; Zimmer *et al.*, 1999). Strain H1806 contains the *KAR2* promoter fragment sufficient for *KAR2* expression, nucleotides -280 to -109 (Mori *et al.*, 1992), directly upstream of the *lacZ* reporter (Zimmer *et al.*, 1999). H1806 cells were exposed to the heat treatments and metabolic labelling, and β-galactosidase expression was monitored by immunoprecipitation (III, Fig. 3). Enzymatic assays were used only for measuring basal activity at 24°C due to thermal inactivation of the enzyme by the heat treatments. Under the control of this *KAR2* promoter variant, β-galactosidase expression was regulated similarly after thermal insult as that of authentic genomic BiP/Kar2p (III, Fig. 3B). Thus, this assay system was feasible for investigating *KAR2* promoter elements in DUR.

Next, the four consecutive GAA repeats in the HSE of the *KAR2* promoter were mutated (strain H2004; III, Table 1). Metabolic labelling experiments showed that in strain H2004, the upregulation of β-galactosidase at 37°C (III, Fig. 3, column HS) was reduced, as expected (Mori *et al.*, 1992; Kohno *et al.*, 1993) but that DUR occurred similarly as in the reference strain H1806 (III, Fig. 3D).

The GC-rich region has been shown to regulate basal expression of *KAR2* (Mori *et al*., 1992). Next, we investigated a strain harbouring a construct, where the 5' region and the GC-rich region were deleted in addition to the HSE (strain H1765; III, Fig. 3E). In this strain, the induction at 37°C was abolished, and DUR dramatically reduced (III, Fig. 3E). This suggests that the 5' and the GC-rich regions backed up the HSE when the latter was absent, because mutation in the HSE abolished DUR of *KAR2* only in combination with deletion of the 5' and GC-rich regions.

The next question was, whether the GC-rich region could be responsible for DUR in the abscence of the HSE. In strain H1767, harbouring a construct where the 5' and the GC-rich regions were the only functional *KAR2* promoter elements, β-galactosidase expression was undetectable in both immunoprecipitation and enzymatic assays (III, Fig. 3I). Thus, the 5' and GC-rich regions failed to drive DUR alone. This result is in agreement with the work of Zimmer and co-workers (1999), who reported lack of β-galactosidase activity from the same construct under ER stress conditions produced by artificially misfolded cytochrome P450. Finally, results from strain H1769 showed that HSE alone was sufficient for evoking DUR (III, Fig. 3H).

Kar2p is a target of the unfolded protein response (UPR), activated *via* the unfolded protein response element (UPRE) in its promoter, when unfolded proteins accumulate in the ER (Mori *et al*., 1992; Kohno *et al*., 1993). The role of the UPRE in DUR was studied using a strain harboring a construct, where the *KAR2* promoter lacked the UPRE (III, Fig. 3F; strain 1766). In this strain, both induction at 37°C and DUR were unaffected, showing that the unfolded protein response element UPRE was dispensable for DUR. This was likewise the case for strains H1768 (III, Fig. 3G) and H1769 (III, Fig. 3H), harboring shorter variants of the UPRE-deleted *KAR2* promoter.

The UPR signal is transmitted from the ER to the cytosol by the transmembrane kinase Ire1p, which causes splicing of *HAC1* mRNA (Fig. 3 of this thesis). Hac1p is the transcription factor, which activates the UPR target genes (Fig. 3 of this thesis). To investigate the role of these UPR key players in DUR of *KAR2*, strains lacking the *IRE1* or *HAC1* genes were transformed with the *lacZ* reporter construct, resulting in strains H1807 and H1808, respectively (III, Fig. 3J-K). Both deletions prevent the activation of UPR pathway (Cox *et al*., 1993; Mori *et al*., 1996). In these strains, DUR occurred as in the reference strain H1806 (III, Fig. 3C), demonstrating that the UPR pathway was dispensable for DUR (III, Fig. 3J-K). Thus, our studies have revealed a novel regulatory mechanism for the *KAR2* gene, which is unrelated to UPR.

In conclusion, DUR occurred via the HSE of *HSP104* and *KAR2* promoters, and the other stress-responsive elements in these gene promoters were dispensable for DUR.

2.5 Delayed upregulation of Hsp104 after thermal insult requires the transcription factors Msn2p and Msn4p (II)

The transcription factors Msn2p and Msn4p activate stress-dependent expression of STRE-containing target genes. Their role in DUR of Hsp104 was examined by using a strain lacking the genes for *MSN2* and *MSN4* (Δ*msn2/4*; II, Table 1). In this strain, only a slight upregulation of Hsp104 expression during recovery was detected (II, Fig. 6). Because the STREs in the *HSP104* promoter were dispensable for DUR, it appears that some other target genes of the transcription factors Msn2p and Msn4p are required to induce DUR of Hsp104. These unknown

proteins also contribute to acquisition of thermotolerance, because it was impaired in the Δ*msn2/4* strain (II, Fig. 5B). However, total protein synthesis was normal in this strain (II, Fig. 6C).

2.6 The MAP kinase Hog1p is required for DUR of Hsp104 (II)

Hog1p is a MAP kinase implicated in upstream regulation of transcription factors Msn2p and Msn4p upon osmotic stress (Alepuz *et al*., 2001) and shown to be activated also upon heat stress at 39°C (Winkler *et al*., 2002). In a strain lacking the *HOG1* gene (Δ*hog1*; II, Table 1), delayed upregulation of Hsp104 after thermal insult was abolished, whereas the 24°C-to-37°C induction persisted (II, Fig. 6). The Δ*hog1* strain displayed normal bulk protein synthesis (II, Fig. 6) and glucose consumption (not shown) at least for 6 hours of recovery at 24°C, but its capability of acquiring thermotolerance was impaired (II, Fig. 5).

Because Hog1p was required for thermotolerance, we wanted to study the effect of Hog1p on another biological function of Hsp104, solubilization of heat-aggregated proteins (Parsell *et al*., 1994). Transmission electron microscopy showed that after growth at 24°C, the morphology of Δ*hog1* cells was normal (II, Fig. 7A). However, after preconditioning at 37°C and thermal insult for 20 min at 50°C (II, Fig. 7B), the cytosol of Δ*hog1* cells was full of aggregate-like structures that persisted even after 6 hours of recovery (II, Fig. 7C). In normal cells treated the same way, the heat-induced aggregates dissolved after 6 hours of recovery at 24°C (Parsell *et al*., 1994; Simola *et al*., 2000). In conclusion, Hog1p was required for DUR of Hsp104, for efficient acquisition of thermotolerance, and for solubilization of heat-induced cytosolic aggregates.

2.7 Biological functions of BiP/Kar2p after thermal insult (III)

Kar2p functions in the translocation of polypeptides into the ER, in facilitating of protein folding, and in retention of misfolded proteins in the ER (for review, see Gething, 1999). BiP/Kar2p synthesis was abolished by the thermal insult and resumed only after two hours of recovery (III, Fig. 1). To study the biological functions of BiP/Kar2p, the translocation kinetics of a secretory reporter protein, Hsp150Δ-β-lactamase, was followed. The 150Δ fragment, a signal-peptide-containing N-terminal fragment of the natural yeast secretory glycoprotein Hsp150 (Russo *et al*., 1993) assists the β-lactamase portion to fold properly in the yeast ER (Simonen *et al*., 1994). The Hsp150 signal peptide confers post-translational translocation (Paunola *et al*., 1998). The cytosolic, ER and mature forms of the reporter can be distinguished by their different extent of O-glycosylation (Paunola *et al*., 1998). In SDS-PAGE, the unglycosylated cytoplasmic form of Hsp150Δ-β-lactamase migrates like a 66-kDa protein, the ER-resident form like a 110-kDa protein, and the fully glycosylated, mature form like a 145-kDa protein (Paunola *et al*., 1998; Fatal *et al*., 2004). Hsp150Δ-β-lactamase was expressed under the heat-activated *HSP150* promoter (Russo *et al*., 1993). Six parallel samples undergoing the thermal treatments described above were labelled with ^{35}S methionine/cysteine (III, Fig. 4A; bars) followed by chase for different periods (III, Fig. 4B). The growth media were separated from the cells, and immunoprecipitation with β-lactamase antiserum was performed for the cell lysates and the media, followed by SDS-PAGE and autoradiography.

After the pulse at 24°C (III, Fig. 4B, graph a), 60% of β-lactamase was found in the cytoplasmic form (triangles), 13% in the ER

form (squares), and 26% in the fully glycosylated, mature form (circles). The fraction of mature molecules increased with increasing chase periods. The fraction of ER-resident β-lactamase (Fig. 4B; graph a; squares) remained relatively constant at roughly 10% for the first time points, which probably reflects equal flow of translocation and of ER exit. Finally, after 30 minutes of chase, practically all of the labelled reporter protein was in the mature form (circles), meaning it had exited the ER and become glycosylated in the Golgi. After preconditioning at 37°C and thermal insult at 50°C, a 5-minutes pulse failed to label β-lactamase molecules (not shown), due to overall halt of protein synthesis (II, Fig. 1D; Jämsä *et al*., 1995). After 1 hour of recovery at 24°C (Fig. 4B; graph b), a 5-minutes pulse followed by different chase times showed that a sufficient amount of the reporter protein was produced that the experiment could be performed. However, the reporter remained in the cytoplasm and had thus failed to translocate even after 40 minutes of chase (triangles). This was the case also after 2 hours of recovery at 24°C (graph c). Only after 3 hours of recovery the newly synthesized reporter molecules started to translocate. The cytoplasmic pool decreased from 90% to 40% (graph d; triangles), while the ER-resident pool increased from 5% to 20% (squares). However, the mature pool only increased from 3% to 35% (circles), suggesting that the reporter did not efficiently exit the ER. After 4 hours of recovery, the translocation velocity of labelled β-lactamase molecules was only slightly slower than before the thermal insult (graph e). Finally, when the reporter was pulse-labelled after 6 hours of recovery, practically all reporter molecules were in mature form after 40 minutes of chase (graph f; circles), showing that the translocation kinetics had recovered to the same level as at 24°C before the heat treatments. In conclusion, ER translocation resumed concomitantly with enhanced synthesis of BiP/Kar2p after 3 hours of recovery, whereafter ER exit and protein secretion also were resumed.

DISCUSSION

1 Differentiation lineage-dependent expression of human HSF2

Eukaryotic heat shock gene expression is mediated by a family of heat shock transcription factors, which bind to heat shock promoter elements in their target gene promoters. In vertebrates, four members of this family, HSF1-4, have been identified (Rabindran *et al*., 1991; Sarge *et al*., 1991; Schuetz *et al*., 1991; Nakai and Morimoto, 1993; Nakai *et al*., 1997). The existence of multiple HSFs raises the question about their specific or overlapping functions, and possible divergent or convergent signaling pathways leading to their activation.

1.1. The role of HSF2 in developmental processes and heat shock response

It has been known previously that human HSF2 is upregulated during hemin-induced differentiation of human K562 erythroleukemia cells. In this work, this upshift was detected at the protein level and at the transcriptional level, and was shown to be due to mRNA stabilization, in addition to transcriptional induction. The upregulation of HSF2 was accompanied by activation of DNA-binding of HSF2. Furthermore, the results of the present study indicate that HSF2 expression and activation were specific for K562 cells induced to differentiate along the erythroid lineage. In contrast, in cells undergoing megakaryocytic differentiation, HSF2 expression and DNA-binding was abolished in a HSF2 promoter-dependent manner.

HSF2 has traditionally been offered a developmental role among the vertebrate heat shock factors. Earlier work has shown that HSF2 is activated in mouse embryonic carcinoma cells, during the blastocyst stage of mouse embryogenesis, during spermatogenesis and during heart development (Sarge *et al*., 1994; Murphy *et al*., 1994; Mezger *et al*., 1994a; Rallu *et al*., 1997; Alastalo *et al*., 1998; Eriksson *et al*., 2000). However, recent reports expand the picture by suggesting a role for HSF2 in heat stress, as well. HSF2 was shown to interact physically with HSF1 in specialized sub-nuclear structures called stress granules, and thereby to influence the localization of HSF1, which is a regulatory mechanism of HSF1-mediated heat shock response (Jolly *et al*., 1999; Alastalo *et al*., 2003). Furthermore, HSF2 was also suggested to form hetero-complexes with HSF1 both upon heat shock and hemin treatment (He *et al*., 2003; Trinklein *et al*., 2004). Moreover, heat stress enhanced activation of the *hsp70* promoter by these hybrid trimers (He *et al*., 2003). Further studies are needed to elucidate the possible interaction of HSF1 and 2 in developmental processes and heat shock response.

1.2 Regulation of HSF2 activation in hemin-induced K562 cells

In agreement with earlier work, an increase in HSF2 expression and DNA-binding was detected in hemin-treated K562 cells (Theodorakis *et al*., 1989; Sistonen *et al*., 1992; Sistonen *et al*., 1994). In contrast, in cells induced to differentiate along the megakaryocytic lineage with TPA, HSF2 expression and DNA-binding were abolished. Furthermore, the expression pattern of HSF2 in K562 cells committed to one of these lineages could not be reversed.

The signals leading to up- or downregulation of HSF2 in K562 cells are still unknown. Hemin has been shown to activate the expression of globins *via* heme response

promoter elements, which resemble the consensus binding sites for the erythroid-specific transcription factor NF-E2 (Ney *et al*., 1990; Solomon *et al*., 1993; Palma *et al*., 1994; Inamdar *et al*., 1996). These binding sites are identical to the antioxidant response element and the heme-responsive element (Johnsen *et al*., 1998). An analysis of human HSF2 promoter showed the presence of putative binding sites for NF-E2, suggesting an explanation for the hemin-induced upregulation of HSF2 mRNA (Nykänen *et al*., 2001). No HSEs are present in the HSF2 promoter, indicating that HSF2 does not regulate its own transcription (Nykänen *et al*., 2001). Future studies will reveal, whether the heme responsive element-like sequences of HSF2 promoter are functional. So far, the possible role HSF2 in erythropoiesis remains elusive.

We showed here that the increase in HSF2 protein seen previously in hemin-treated K562 cells (Theodorakis *et al*., 1989; Sistonen *et al*., 1992; Sistonen *et al*., 1994) was due to stabilization of HSF2 mRNA. The transcriptional induction observed for HSF2 was only 1.5- to 2-fold, whereas the increase in steady-state mRNA was 6-fold. When hemin-treated cells were treated with the transcription inhibitor actinomycin D, HSF2 mRNA levels remained higher in than in control cells, indicating a prolonged half-life of the mRNA molecules. In another study, luciferase reporter protein expression controlled by a 950-bp HSF2 promoter did not increase upon hemin treatment, further suggesting that the augmentation in HSF2 upon hemin occurs posttrancriptionally (Nykänen *et al*., 2001).

In K562 cells, the inactive, non-DNA-binding form of HSF2 exists in the cytoplasm as dimers. Upon activation, HSF2 is trimerized and transported into the nucleus (Sistonen *et al*., 1994). In contrast, constitutive HSE-binding activity of HSF2 has been reported in various cell types (Mezger *et al*., 1989; Murphy *et al*., 1994; Mezger *et al*., 1994a; Mezger *et al*., 1994b). In addition to trimerization, the DNA-binding activity of HSF2 has been shown to be enhanced by attachment of SUMO-1, an ubiquitin-related protein with a number of different functions (Goodson *et al*., 2001), and by heterotrimerization with HSF1 (He *et al*., 2003). Furthermore, the HSF2-α isoform is transcriptionally more active than the β isoform (Goodson *et al*., 1995; Leppä *et al*., 1997b). Consistent with earlier work, our results showed that hemin-induced accumulation of HSF2 protein occurred in HSF2-α- but not in HSF2-β-overexpressing cells (Leppä *et al*., 1997b). The molar ratio between the isoforms seems to modify the transcriptional activity of HSF2, because overexpression of HSF2-β was shown to repress the hemin-mediated increase of HSP70 expression in K562 cells (Leppä *et al*., 1997b). Furthermore, the ratio of HSF2-α and HSF2-β isoforms varies significantly between different mouse tissues, further implicating that regulation of isoform expression could represent a mechanism of regulating HSF2 activity (Goodson *et al*., 1995).

1.3 Expression and function of HSF2

Cell differentiation involves a complex expression program of spatially and temporally organized factors. The results of this study suggest that DNA-binding of HSF2 increases upon hemin-induced differentiation of K562 cells. This result was in agreement with some previous reports (Sistonen *et al*., 1992; Sistonen *et al*., 1994), but inconsistent with another (Yoshima *et al*., 1998). The latter implicates that HSF1, instead of HSF2, is the primary factor binding to HSE in the HSP70 promoter and activating its transcription in hemin-treated K562 cells. One explanation to this apparent

contradiction might be the heterogeneity of K562 cells, which exist in several subtypes presumably exhibiting different HSF activation patterns. Another explanation could be the affinity differences in antibodies raised against mouse, human and chicken HSFs, used in different laboratories to detect human HSF-HSE complexes (Pirkkala and Sistonen, 1999).

HSF2 activity was shown to be required for the erythroid differentiation of K562 cells (Leppä *et al*., 1997b). The cellular role and the target genes of HSF2 in hemin-induced K562 cells remain, however, still unclear. Trinklein and co-workers recently reported about 9 genes, whose expression was induced 10-fold or more by hemin treatment, and about 6 genes, whose promoters were occupied by HSF2 upon hemin treatment. However, the extent of HSF2 occupation did not correlate with the range of induction of these genes by hemin treatment (Trinklein *et al*., 2004), suggesting that DNA-binding of HSF2 and target gene activation may not be coupled. Consistently with this work, during mouse embryogenesis and rat spermatogenesis, induced heat shock gene expression was shown to be independent of HSF2 expression and DNA-binding activity (Rallu *et al*., 1997; Alastalo *et al*., 1998; Eriksson *et al*., 2000).

In conclusion, human HSF2 was upregulated during hemin-induced differentiation of human K562 erythroleukemia cells. This upshift was detected at the protein level and at the transcriptional level, and was shown to be due to mRNA stabilization, in addition to transcriptional induction. The upregulation of HSF2 was accompanied by activation of DNA-binding of HSF2. In contrast, megakaryocytic differentiation of K562 cells induced with 12-O-tetradecanoyl-phorbol-13-acetate led to downregulation of HSF2 expression and DNA-binding activity. This downregulation occurred *via* the HSF2 promoter.

These results support a role for HSF2 in development and differentiation, underlining the divergent functions of the multiple HSFs in vertebrates. The differentiation lineage-specificity of HSF2 could possibly represent an early marker for differentiating pathways of multipotent hematopoietic progenitor cells.

2 Novel regulation mechanism of yeast chaperones

2.1 Delayed upregulation of yeast chaperones during recovery from thermal insult

In this study, a novel regulatory mechanism of yeast chaperones was discovered. When *S. cerevisiae* cells grown at 24°C, were preconditioned at 37°C, exposed to a brief thermal insult at 50°C and thereafter returned to 24°C, chaperone expression was upregulated 3 hours after the thermal insult, although the cells were maintained at 24°C. We designated this novel regulation mechanism Delayed Upregulation (DUR). The heat shock proteins Hsp104 (cytosol), BiP/Kar2p and Lhs1p (ER), and Hsp78 (mitochondria), were subject to DUR. This shows that several chaperone genes are subject to DUR and DUR occurs in various compartments of the yeast cell. For both Hsp104 and BiP/Kar2p, the heat shock promoter element (HSE) was necessary and sufficient for DUR. In the case of Hsp104, the stress response elements (STRE) were dispensable. The STRE-specific transcription factors Msn2p and Msn4p appeared to affect DUR of Hsp104 *via* other unknown target proteins. The MAP kinase Hog1p was also required for DUR of Hsp104 and for removing cytosolic heat-aggregated proteins after thermal insult. In the case of BiP/Kar2p,

the unfolded protein response element (UPRE) and the unfolded protein response pathway were dispensable for DUR. Thermal insult abolished the biological activities of BiP/Kar2p. They were resumed after excessive amounts of new BiP/Kar2p molecules were synthesized due to DUR.

2.2 The promoter element and transcription factors involved in DUR

We identified the *KAR2* and *HSP104* promoter element responsible for DUR. In both cases, the HSE was essential and sufficient for DUR. In contrast to the HSEs, the results show for both genes that the other types of stress-inducible promoter elements, the STREs and the UPRE, in *HSP104* and *KAR2* promoter, respectively, were dispensable for DUR. DUR appears to be a regulatory mechanism, where the transcription factor of HSE, Hsf1p, has an instrumental role, as no other transcription factor is known to operate *via* the HSE. Future studies should address the activation of Hsf1p after thermal insult.

Hsp104 is required for acquisition of thermotolerance of yeast cells (Sanchez and Lindquist, 1990). Although mutation of the HSE in the *HSP104* promoter abolished DUR of Hsp104, a basal level of Hsp104 expression was still present in the HSE mutant after thermal insult. The HSE mutant acquired thermotolerance, demonstrating that even the residual amount of Hsp104 present in this strain was adequate to guarantee thermoprotection. It is known from previous studies that the *HSP104* promoter elements are capable of backing each other up (Amorós and Estruch, 2001; Grably *et al*., 2002). In contrast to the HSE, all the three STREs in the *HSP104* promoter could be destroyed without decreasing DUR or the ability of the strain to acquire thermotolerance. The mutations from AGGGG to ACGAG that we introduced to the STREs in *HSP104* promoter, have been reported to abolish stress-induced expression through STRE, which confirms that our mutated elements truly are unfunctional (Marchler *et al*., 1993). However, deletion of the HSE in combination with all the three STREs resulted in the same phenotype as that caused by deletion of *HSP104*, namely lack of Hsp104 expression and failure to survive the thermal insult. Surprisingly, although the STREs of the *HSP104* promoter were dispensable for DUR, deletion of the genes for the STRE-specific transcription factors Msn2/4p led to a decreased DUR of Hsp104 and an impaired ability to acquire thermotolerance. This apparent contradiction suggests that some other STRE-regulated genes are involved in DUR of Hsp104. One candidate could be the bZIP transcription factor Yap4, whose induction under osmotic and oxidative stress has been shown to require Msn2p (Nevitt *et al*., 2004a; Nevitt *et al*., 2004b). *YAP4* mRNA is upregulated under various stress conditions, including heat shock at 37°C, oxidative and osmotic stress (Posas *et al*., 2000; Rep *et al*., 2000; Gasch *et al*., 2000; Nevitt *et al*., 2004b). A promoter search for putative Yap4 recognition sites in the *HSP104* promoter would imply, whether Msn2p and Yap4 could build a transcription factor activation chain contributing to DUR of Hsp104.

2.3 DUR is different from upregulation at 37°C

The results from this work demonstrated that DUR occurs *via* the HSEs in the *HSP104* and *KAR2* promoters. The HSE has been previously thought to be responsible for the well-known upregulation of all hsps upon shift of the cells from physiological temperature to 37°C. However, DUR differs from upregulation upon shift to 37°C in several regards. Firstly, upregulation of both Hsp104 and BiP/Kar2p at 37°C occurs within

minutes (Fig. 7 of this thesis; Kohno *et al*., 1993; Gasch *et al*., 2000), whereas maximal DUR within hours. Secondly, the Hsf1p/HSE regulon of the *HSP104* promoter is not essential for activation at 37°C (Treger *et al*., 1998; Amorós and Estruch, 2001; Grably *et al*., 2002), but essential and sufficient for DUR. Thirdly, activation at 37°C can be driven *via HSP104* STREs alone (Grably *et al*., 2002), whereas the STREs had no role in DUR. Fourthly, Mns2/4p-regulated proteins have no significant role in activation at 37°C (Boy-Marcotte *et al*., 1999), whereas they do contribute to DUR. And finally, Hog1p is required only for DUR of Hsp104 but not for the upregulation of Hsp104 at 37°C. Thus, DUR is a novel regulatory mechanism different from upregulation of hsps upon shift to 37°C.

What could be the molecular mechanisms leading to the remarkable slowness of DUR? Slow induction of stress proteins has been reported for other severe stresses. Upon severe osmotic shock (1.4 M NaCl), nuclear accumulation and phosphorylation of the MAP kinase Hog1p is delayed compared with mild stress (0.4 M NaCl; Van Wuytswinkel *et al*., 2000). Severe osmotic shock renders the active and nuclear-localized Hog1p transiently unable to induce transcription of osmotic stress-responsive genes. Furthermore, there is reasonable evidence that many different stress conditions, even when mild, transiently arrest the yeast cell cycle. Moderate heat shock and experimental accumulation of misfolded proteins cause G_1 arrest *via* a common Hsf1p-dependent mechanism (Bedard *et al*., 1980; Johnston and Singer, 1980; Shin *et al*., 1987; Trotter *et al*., 2001). Likewise, moderate osmotic and oxidative shocks cause a cell cycle delay, as well (Lee *et al*., 1996; Bellí *et al*., 2001). Thus, the remarkable slowness of heat shock protein induction during recovery from thermal insult may be caused by delay in nuclear transport and phosphorylation of some crucial factor(s), and/or delay in progression of the cell cycle.

2.4 DUR and signaling pathways

2.4.1 The high-osmolarity glycerol pathway

The high-osmolarity glycerol (HOG) MAP kinase pathway is one of five MAPK pathways regulating a variety of biological responses in yeast. Upon osmotic stress, the MAP kinase Hog1p is phosphorylated and transported to the nucleus (Reiser *et al*., 1999), where it has been shown to be recruited by Msn2/4p to the *CTT1* and *HSP12* promoters (Reiser *et al*., 1999; Alepuz *et al*., 2001). Hog1p mediates its effect through at least five different transcription regulators, amongst them Msn2p and Msn4p. The results of this study showed that DUR of Hsp104 was dependent on the MAP kinase Hog1p. In a Δ*hog1* deletion strain, bulk protein synthesis and the rate of glucose consumption were normal for at least six hours of recovery from thermal insult, whereas DUR and acquisition of thermotolerance were abolished. Moreover, the Δ*hog1* strain was incapable of removing cytosolic aggregates induced by thermal insult. In normal cells, the heat-induced aggregates dissolved during recovery from thermal insult. The precise involvement of Hog1p in DUR remains, however, elusive. There are some studies that suggest a connection between the HOG pathway and cellular responses to thermal stress. Winkler and co-workers (2002) showed that, in addition to osmotic stress, also heat stress (shift from 23 to 39°C) increased the kinase activity of Hog1p. Moreover, Hog1p was not essential at 39°C, but the recovery from heat stress of a Δ*hog1* strain was, however, slower than that of normal cells (Winkler *et al*.,

2002). Furthermore, the Hog1p homologue of fission yeasts, Spc1/Sty1, is known to be activated in response to heat and oxidative stress, in addition to osmotic stress (Degols *et al*., 1996). In another study, osmosensitivity of a Δ*hog1* strain was reduced when grown at 37°C, further implicating that the HOG pathway plays a role in thermoprotection (Siderius *et al*., 2000). More studies are needed to elucidate the role of the HOG pathway in thermal stress responses.

2.4.2 The unfolded protein response pathway

Induction of BiP/Kar2p expression in response to perturbation of protein folding in the ER lumen activates the unfolded protein response pathway UPR and occurs *via* the UPRE in the *KAR2* promoter. The UPR signal is transferred from the ER lumen to the cytosol by the transmembrane kinase Ire1p (Fig. 3). Ire1p causes splicing of *HAC1* mRNA in the cytosol. Hac1p is the transcription factor that recognizes the UPRE in the target genes (Fig. 3). In the present study, experiments with strains lacking the genes for Hac1p or Ire1p showed that these mediators of UPR are not involved in DUR of BiP/Kar2p. These results demonstrate that DUR is a regulatory mechanism different from UPR.

2.5 The biological function of DUR

In this work it was demonstrated that expression of chaperones is upregulated after thermal insult in the cytosol, in the ER and in the mitochondria. After thermal insult, aggregated proteins accumulate in the cell. Could the accumulation of heat-denatured aggregates elicit a chain of regulatory events leading to DUR? We propose that the increased chaperone amount in the cell serves to repair the heat-induced damage to cellular proteins. Earlier it was shown in our laboratory that proteins, denatured by thermal insult and aggregated inside the ER, were renatured during recovery at physiological temperature by an ATP-dependent mechanism (Jämsä *et al*., 1995). Both Lhs1p and BiP/Kar2p were found to be associated with the aggregates (Jämsä *et al*., 1995; Saris *et al*., 1997). In the present work, an upregulation of yeast chaperone expression after thermal insult was observed, although the cells were maintained at physiological temperature. Accumulation of misfolded proteins containing the amino acid analogue azetidine 2-carboxylic acid in place of proline has been shown to activate Hsf1p and induce expression of heat shock factor-regulated genes in the absence of temperature changes (Trotter *et al*., 2001; Trotter *et al*., 2002). Under ER stress conditions, overexpression of BiP/Kar2p has been reported to restore a cell growth defect caused by overproduction of Δpro, a mutated fungal secretory proteinase, which accumulates as misfolded aggregates The extra BiP/Kar2p associated directly with the aggregates (Umebayashi *et al*., 1999).

Yeast as a unicellular organism cannot rely on its neighbouring cells or sacrifice damaged cells by apoptosis. In their natural habitat, on the surface of fruit, yeast cells are exposed to greatly varying conditions in temperature, hydration and nutrient balance. In the Mediterranean climate zone, the origins of most wine yeast strains, temperatures ranging to 40°C are not unusual. Although the temperature changes in nature are not as sudden as the heat treatments applied in this study, on a summer day the local temperature on the surface of a wine grape could be 24°C in the morning, 37°C during the midday, and 45°C and even above in the afternoon sun. These extreme temperatures challenge the proper conformation of cellular proteins of

yeast cells living on the surface of this grape. In the evening, when the temperature sinks to 24°C, yeast cells may need an augmented expression of hsps to repair the cellular damage caused by the heat stress during the day. In conclusion, we propose that DUR represents a mechanism to ensure survival after severe heat stress by enhancing conformational repair of heat-denatured proteins.

2.5.1 Biological functions of BiP/Kar2p during recovery from thermal insult

BiP/Kar2p is involved in the translocation of polypeptides into the ER, in facilitating of protein folding, and in retention of misfolded proteins in the ER (for review, see Gething, 1999). The results of this study showed that after thermal insult, newly synthesized reporter proteins failed to translocate, as they were detected in the cytosolic unglycosylated form. At the same time after thermal insult, BiP/Kar2p synthesis was abolished. Thus, BiP/Kar2p molecules that existed prior to thermal insult were not able to perform the translocation function, or other components required for ER translocation were inactivated at 50°C. After 4 hours of recovery at 24°C, the reporter protein was translocated and glycosylated, it exited the ER and was secreted to the medium. Earlier, it was found that intracellular transport of vacuolar carboxypeptidase Y and cell wall invertase also resumed after 3-4 h of recovery (Saris *et al.*, 1997; Saris and Makarow, 1998). Concomitantly, BiP/Kar2p expression was upregulated due to DUR after 3 hours of recovery. After 6 hours of recovery, translocation, ER exit and secretion had recovered to normal, meaning that functions of Bip/Kar2p were resumed. Resumption of the functions of BiP/Kar2p during the recovery period may have resulted from newly synthesized BiP/Kar2p due to DUR, repair of BiP/Kar2p molecules which existed prior to thermal insult and were damaged at 50°C, or both. Enhanced synthesis of BiP/Kar2p molecules due to DUR may serve to ensure resumption of translocation and ER exit functions.

CONCLUDING REMARKS

Heat shock proteins (hsps) are needed both in stress conditions and in physiological conditions. They exist in all organisms studied so far, and in all cellular compartments. This study characterized the regulation of different components of the heat stress response, specifically the human heat shock factor HSF2 and the yeast chaperones Hsp104 and BiP/Kar2p. The focus of the study was, however, in processes different from the classical heat shock response, namely differentiation in the case of human HSF2 and recovery from thermal insult in the case of yeast hsps.

The results concerning the regulation of HSF2 in differentiating K562 human erythroleukemia cells suggest an erythroid-specific role for HSF2. For the megakaryocytic differentiation of K562 cells, HSF2 appeared to be dispensable. However, the molecular mechanisms behind the lineage-dependent regulation of HSF2 are unknown. During hemin-induced differentiation of K562 cells, HSF2 was shown to be regulated transcriptionally and by mRNA stabilization. Despite the relatively numerous studies on HSF2 in recent years, HSF2 still remains rather mysterious. Identification of HSF2 target genes and factors regulating HSF2 expression will hopefully reveal the biological role of HSF2.

When studying recovery of yeast *S. cerevisiae* from thermal insult, a novel regulatory mechanism of yeast heat shock proteins was identified. This regulatory mechanism, which we designated Delayed Upregulation (DUR), turned out to concern several chaperones functioning in different compartments of the yeast cell. The results from this study show that DUR occurs exclusively *via* the heat shock element (HSE) in heat shock gene promoters. However, DUR is regulated differently than the well-known upshift of yeast hsps upon shift of the cells from physiological temperature to 37°C. Detailed understanding of the precise biological role of DUR still requires further studies. Two large areas of future research needed are determination DUR target genes and the signaling pathways leading to its activation. Yeasts are unicellular organisms living in highly dynamic environments in nature. Yeast cells cannot rely on apoptosis to sacrifice damaged cells, but need elaborate repair machineries to refold vital proteins. We propose that after thermal insult, which denatures proteins in all compartments of the cell, enhanced production of chaperones is a mechanism which ensures survival.

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