Interaction of Heat Shock Proteins with Splicing Machinery Components During and After Thermal Stress in *Saccharomyces cerevisiae*.

By

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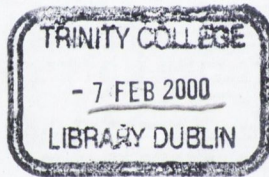
The Trip to Happiness

Here, under the emotional influence of the Joshua,

I choose to wander,

Seeking to find what I'm looking for,

I walk with a world in front of me,

A world of many different faces,

Stumbling on the stones of adolescence,

Blocked by the mist of conformity,

But with unbounded energy, I wish to fly,

Above the mist and away at great speed,

The mountains of life, like stones in the distance,

I'm free, no man's actions can trip me now,

I have found what I'm looking for.

April 1993.

Dedicated to my family and friends.

Declaration

This is to certify that the experimentation recorded herein represents my own work, unless otherwise stated, and has not been submitted for higher degree at this or any other university. This thesis may be lent at the discretion of the librarian, Trinity College Dublin.

Adrian Bracken.

Summary

This thesis is a study of the ameliorative effects of Heat Shock Proteins (Hsps) on the cellular process of mRNA splicing during and after thermal stress in *Saccharomyces cerevisiae*. Splicing is a vital process that is known to be extremely sensitive to thermal inactivation, but can be protected by pre-treatment of cells under conditions that are known to induce Hsps. This latter phenomenon is known as splicing thermotolerance. In this thesis I correlate splicing inactivation upon heat shock with multiple alterations in the splicing machine components, the small nuclear ribonucleoprotein particles (snRNPs). Following a heat shock, dramatic reductions in the tri-snRNP (U4/U6.U5), the free U6 snRNP and the Lhp1p/U6 pre-U6 snRNP complex, coupled with alterations in the U1, U2, U5 and U4/U6 snRNP profiles are observed. Evidence is presented that pre-forms of several snRNPs accumulate in heat shocked cells, indicating that blockage of snRNP assembly may be the primary reason for splicing inactivation upon heat shock. Supporting this hypothesis is the fact that the reassembly of snRNPs is correlated with the recovery of mRNA splicing. In addition, both Hsp104 and the Ssa members of the yeast Hsp70 family are required for this reassembly. Furthermore, I correlate "splicing thermotolerance" with the protection of a sub-set of snRNPs by Ssa proteins but not Hsp104. Lastly, my results suggest that Ssa proteins also play a role in snRNP assembly, even under normal physiological conditions.

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Contents

Chapter 1:

General Introduction.

1. 1   CELLULAR STRESS

All organisms, from bacteria to humans, are constantly challenged by conditions that cause acute and chronic cellular stress (Parsell and Lindquist, 1994). These cellular stresses can be categorised into two major types; environmental stresses and pathophysiological stresses. Examples of environmental stress include, exposure to high temperatures, UV light, ethanol, drugs, heavy metals, amino acid analogues and toxic chemicals. Pathophysiological stress is unique to multicellular organisms such as man. Examples include, infection by bacteria and viruses, ischemia, fever, inflammation, ageing and cancer. Severe stresses can ultimately lead to cell death due to irrepairable cellular damage caused. A key concept in the understanding of cellular stress is that both the magnitude and duration of the stress determine the amount of damage incurred, and cells die when a threshold of damage is exceeded.

Exposing cells to high temperatures, termed 'heat shocking', is the most commonly used method to study cellular stress biology. Heat shocking cells is known to cause damage to a wide variety of cellular structures and metabolic processes (Laszlo, 1992, Nover, 1991). One of the most immediate effects in higher eukaryotes is the collapse of the cytoskeleton coupled with an aggregation of actin filament around the nucleus (Parsell and Lindquist, 1994, Welch and Feramisco, 1985, Welch and Suhan, 1986, Welch and Suhan, 1985). The nucleus itself, as well as the nucleolus, are also observed to have considerable morphological alterations in heat shocked cells (Parsell and Lindquist, 1994, Welch and Suhan, 1986). In addition, several cytoplasmic organelles are also affected.

For instance, the golgi apparatus and the endoplasmic reticulum are observed to disrupt and fragment. Mitochondria swell and the number of lysosomes increase dramatically. The permeability of the plasma membrane has also been reported to increase significantly (Piper, 1995).

Several cellular processes have been shown to be detrimentally affected by heat shocking, including pre-RNA processing and mRNA splicing (Bond, 1988, Czarnecka, et al., 1988, Ghoshal and Jacob, 1996, Kay, et al., 1987, Yost and Lindquist, 1986). Care must be taken at this point, so as not to confuse heat induced damages with the controlled cellular reorganisations, such as transcription redirection, that are discussed later in this chapter in the context of the cell's response to cellular stress. Probably the best studied cellular process that is sensitive to heat shock is the process of mRNA splicing, in which intronic sequences are removed from pre-mRNA. A number of previous studies have shown that this process is reversibly inactivated upon heat shock in a variety of eukaryotic cell types, including human (Bond, 1988), *Drosophila melangaster* (Yost and Lindquist, 1986, Corell and Gross, 1992), *Caenorhabditis elegans* (Kay, et al., 1987), soybean (Czarnecka, et al., 1988), trypanosomes (Muhich and Boothroyd, 1988) and *Saccharomyces cerevisiae* (Vogel, et al., 1995, Yost and Lindquist, 1991). In all cases, a treatment of cells at sub-lethal heat shock temperatures for an hour or more results in a complete but reversible inactivation of mRNA splicing. This is actually the focus of this thesis and will be discussed in much greater detail later in this chapter.

On a molecular level, DNA, lipids and proteins have all been demonstrated to be damaged by heat shock (Parsell and Lindquist, 1994,

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Piper, 1995). However, proteins are the most severely affected and have been shown to denature and aggregate in several organisms (Parsell and Lindquist, 1994). Significantly, other environmental stresses, such as exposure to heavy metals, such as copper-chelating agents and arsenite, are known to cause conformational changes in proteins (Nover, 1991). While ethanol and UV light both ultimately result in the accumulation abnormal proteins (Piper, 1995, Sanchez, et al., 1992). In addition, many pathophysiological stresses ultimately result in the accumulation of abnormal proteins. For example, ischemia, a condition caused by a blockage of blood flow, results in oxygen deprivation and consequently results in free radical damage to proteins (Ferreira, et al., 1996, Higashi, et al., 1995). The significance of this abnormal protein accumulation in stressed cells is discussed below.

1. 2   THE CELL'S STRESS RESPONSE:

In response to cellular stress, all organisms react to counteract its potentially detrimental effects (Lindquist, 1986, Mathew and Morimoto, 1998, Morimoto, et al., 1997, Schoffl, et al., 1998, Segal and Ron, 1998). It is commonly held that the primary signal for this 'stress response' is actually the accumulation of abnormal or non-native proteins in the cytosol (Bukau, 1993, Hightower, 1991, Kampinga, 1993, Morimoto, et al., 1994, Segal and Ron, 1998, Yura, et al., 1993). Initial experiments using frog oocytes demonstrated that by microinjecting denatured proteins, it was possible to observe an activation of a specific set of genes, coding for 'Stress Proteins' or 'Heat Shock Proteins' (Hsps) (Ananthan, et al., 1986).

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However, it is also known that various stresses modulate the activity of protein kinase signalling pathways. Specifically, certain Mitogen Activated Protein kinases (MAP kinases) have been shown to be activated by phosphorylation in response to stress in both mammals (Bendinelli, et al., 1995, Venetianer, et al., 1995) and *S. cerevisiae* (Kamada, et al., 1995). These MAP kinases are thought to be involved in the complex network of signalling pathways which ultimately result in the transcription of stress related genes such as Hsps (Bensaude, et al., 1996). Intriguingly, the primary sensors which trigger the phosphorylation of these kinases are actually located in the cell's plasma membrane (Faux and Scott, 1996). It is tempting to speculate that these molecules are activated in response to alterations in the plasma membrane's fluidity. Supporting this hypothesis is the fact that in *S. cerevisiae*, the membrane lipid composition has been shown to be a determinant of the temperature at which the heat shock response is induced (Carratu, et al., 1996). So, while the accumulation of damaged proteins may be the signal inside the cell, membrane bound receptors likely function as the signalling link between the cytosol and conditions outside the cell.

The heat shock response itself is regulated at many different levels. At the transcriptional level, the set of genes which code for Hsps are rapidly induced immediately following exposure of cells to stressful conditions (Georgopoulos, et al., 1994, Morimoto, et al., 1997, Morimoto, et al., 1996). In prokaryotic organisms such as *Escherichia coli*, the transcriptional activator is the specialised sigma factor σ32 (Georgopoulos, et al., 1994). This factor is present in very low amounts under normal

conditions. However, it's levels are transiently increased during stressful conditions as a result of increased translational efficiency and decreased rate of turnover.

In eukaryotic organisms, 'Heat Shock Factors' or 'HSFs' are responsible for the transcriptional activation of *hsp* genes (Morimoto, et al., 1997, Morimoto, et al., 1996). Multiple forms of HSF occur in humans, mice and chickens, but in the yeast *S. cerevisiae*, HSF is encoded by a single essential gene (Sorger and Pelham, 1987). In contrast to prokaryotes, these transcriptional activators are pre-existent in unstressed cells in large amounts and therefore don't require further synthesis. In higher eukaryotes, HSFs are present in both the cytoplasm and the nucleus in monomeric forms that have no DNA binding activity. In response to heat shock and other cellular stresses, they assemble into trimeric complexes, acquire DNA binding activity and translocate to the nucleus. These active forms of HSFs bind in a sequence-specific manner to heat shock elements (HSEs) which are located in the promoter regions of heat-shock genes, and consequently result in elevated transcription (Fernandes, et al., 1994). This trimerisation, relocation and subsequent *hsp* transcription induction occurs extremely rapidly upon exposure of cells to stressful conditions (Perisic, et al., 1989, Sarge, et al., 1993, Westwood, et al., 1991).

In *S. cerevisiae*, HSFs are actually constitutively expressed and bound to many HSEs even under normal growing conditions (Sorger, et al., 1987). It is actually the phosphorylation of these DNA bound HSFs during stressful conditions, leading to a more efficient interaction with the transcriptional machinery, that is the trigger for *hsp* transcription (Gross,

et al., 1993, Sorger, 1990, Sorger, et al., 1987, Sorger and Pelham, 1988). HSFs have also been shown to bind several additional HSEs during stressful conditions in *S. cerevisiae*, causing the transcription of additional stress related genes (Giardina and Lis, 1995). In addition, two other major positive transcriptional elements are known to be activated by stress in *S. cerevisiae* (Attfield, 1997). These are the Stress Related Elements (STREs) and the AP-1 Responsive Elements (AREs) which are regulated by the Msn2/4 and Yap1/2 transcriptional activators, respectively (Attfield, 1997). It is now known that HSEs, STREs and AREs have overlapping but separable functions (Mager and Kruijff, 1995). For example, most Hsps have an HSE element, but some genes like those coding for Hsp104, Hsp26 and Hsp12 have STREs.

In higher eukaryotes at least, certain Hsps have been shown to actually regulate their own gene expression by an autoregulatory loop (Abravaya, et al., 1992, Duina, et al., 1998, Silverstein, et al., 1998, Zou, et al., 1998). Under stressful conditions, an increase in the levels of abnormal proteins, which are substrates for Hsps, depletes the free Hsp pool, which in turn results in trimerisation of HSF. This HSF is in an active form and consequently can induce *hsp* transcription (Morimoto, et al., 1996). During recovery of cells in normal, non stressful conditions, the levels of abnormal proteins decrease gradually and consequently, the free pool of Hsps increases. These Hsps can interact with HSF to break the trimer into monomeric particles, thereby inactivating it and turning off *hsp* gene expression. From this autoregulatory loop model, it is possible to explain how Ananthan *et al.* observed a dramatic transcriptional activation of *hsp*

genes in frog oocytes in response to rises in the cellular levels of abnormal or denatured proteins.

In addition to the rapid transcriptional induction of *hsp* genes, the heat shock response is also regulated at a post-transcriptional level. For example, during stressful conditions in the yeasts *Schizosaccharomyces pombe* and *S. cerevisiae*, poly(A)$^+$ RNA is observed to accumulate within the nucleus (Liu, et al., 1996, Saavedra, et al., 1996, Tani, et al., 1996). In contrast, heat shock mRNAs are efficiently exported from the nucleus by a selective export pathway during the stress (Saavedra, et al., 1997). These mRNAs are subsequently translated into heat shock proteins in the cytoplasm. Another level of control in the heat shock response is the alterations in mRNA half-lives. The half life's of *hsp* mRNAs are maintained or prolonged during exposure to thermal stress (Moore, et al., 1987, Saavedra, et al., 1996) while, in contrast, non *hsp* mRNA half-live's are thought to decrease (Laroia, et al., 1999, Saavedra, et al., 1996).

1. 3   THE HEAT SHOCK PROTEINS

The first report on Hsps appeared in 1962 after *Drosophila melanogaster* salivary gland cells were heat shocked at 37°C for 30 min and then returned to normal temperature of 25°C for recovery (Ritosa, 1962). A "puffing" of genes was found to have occurred in the chromosomes of the recovering cells, accompanied by an increase in the expression of proteins

Table 1.1   Summary of Heat Shock Protein Family Members

	Organism	Hsp	Localisation	Functions
Hsp70	*S. cerevisiae*	Ssa1-4	Cytosol/Nucleus	Stress Tolerance, protein translocation and folding.
		Ssb1, Ssb2	Cytosol	Removal of nascent polypeptides from ribosome.
		Kar2	ER	Translocation of proteins into endoplasmic reticulum.
		Ssc1	Mitochondria	Translocation of proteins into mitochondrium.
	E. coli	DnaK	Cytosol	Stress tolerance and Protein folding.
	Human	Hsc70/Hsp70	Cytosol/Nucleus	Stress Tolerance, protein translocation and folding.
		Bip	ER	Translocation of proteins into endoplasmic reticulum.
		mHsp70	Mitochondria	Translocation of proteins into mitochondrium.
Hsp100/Clp	*S. cerevisiae*	Hsp104	Cytosol/Nucleus	Stress Tolerance.
	E. coli	ClpA, ClpX	Cytosol	Proteolysis and Regulation.
		ClpB	Cytosol	Stress tolerance.
Hsp90	*S. cerevisiae*	Hsp83/Hsc83	Cytosol	Signal Transduction
	Human	Hsp90	Cytosol	Signal transduction and steroid hormone function.
Hsp60	*S. cerevisiae*	Hsp60	Mitochondria	Protein folding and stress tolerance in mitochondria
	E. coli	GroEL	Cytosol	General protein folding functions
	Human	Hsp60	Mitochondria	Same as *S. cerevisiae* Hsp60
Small Hsps	*S. cerevisiae*	Hsp27	Cytosol	Stress tolerance
	Human	Hsp27	Cytosol	Stress tolerance and cell growth and differentiation

with distinctive molecular masses, now known as Hsps (Tissieres, et al., 1974). Since then, several other Hsps, induced by a multitude of stress types in many different organisms have been identified (Ferreira, et al., 1996, Morimoto, 1993, Morimoto, et al., 1997, Morimoto, et al., 1996, Morimoto and Santoro, 1998, Schoffl, et al., 1998). The degree of Hsp induction depends on the level and duration of exposure to stress. The increase is transient, but how long it persists varies in different cell types.

Detailed analysis of the actions of Hsps under normal growing conditions reveals that these proteins transiently bind to stretches of polypeptides rich in hydrophobic amino acids such as those found in unfolded proteins, thus facilitating cellular processes such as protein folding and translocation across membranes (Craig, et al., 1993, Craig, et al., 1994, Hendrick and Hartl, 1993, Morimoto, et al., 1994, Netzer and Hartl, 1998). In an analogy of their human counterpart, they have been named molecular chaperones (Ellis, 1987). Hsps are also known to have several functions during and following stressful conditions, including the protection, reactivation and even degradation of damaged proteins (Mathew and Morimoto, 1998, Parsell and Lindquist, 1993).

There are at least five major families of heat shock proteins; the Hsp100s, Hsp90s, Hsp70s, Hsp60s and the small Hsps, categorised on the basis of their approximate molecular mass (in kilodaltons). In the next sections, each family is described in detail with examples of highly conserved individual members from both prokaryotic and eukaryotic organisms. Interestingly, several eukaryotic organisms possess organelle-located members, for example the *S. cerevisiae* mitochondrial Hsp70, Ssc1,

which are actually more homologous to their bacterial counterparts than their cytosolic neighbours (Craig, et al., 1994). In the next sections most of the emphasis is on the cytosolic/nuclear Hsp family members, and in particular, the *S. cerevisiae* Hsp100 member, Hsp104 and the Hsp70 members, Ssa1-4. These proteins are vital for stress tolerance in this organism and are central to the work in this thesis.

1. 3. 1 The Hsp70 Family

The Hsp70 family is highly conserved and demonstrates a 60-80% identity among eukaryotic cells and a 40-60% identity between eukaryotic Hsp70 and *E. coli* DnaK (Caplan, et al., 1993, Gupta and Singh, 1992). Hsp70s have a diversity of biological functions, including, the translocation of proteins across membranes (Baxter, et al., 1996, Becker, et al., 1996, Caplan, et al., 1993), the folding of nascent polypeptides on ribosome's (Nelson, et al., 1992, Teter, et al., 1999) and the prevention of protein denaturation during stressful conditions (Craig, et al., 1993, Nollen, et al., 1999, Parsell and Lindquist, 1993). All of these diverse functions depend on the ability of the Hsp70 peptide binding domain to transiently associate with partially unfolded proteins by binding to specific motifs consisting of four or five hydrophobic residues (Rüdiger, et al., 1997, Rüdiger, et al., 1997). This association requires the hydrolysis of ATP (Cyr, et al., 1994, Greene, et al., 1998, McCarty, et al., 1996). It is thought that the association of Hsp70 with unfolded protein substrates actually prevents their aggregation by shielding the exposed hydrophobic residues. Interestingly, both its peptide and ATP binding sites have been shown to

not actually determine the substrate specificity of each Hsp70 (James, et al., 1997). Instead, functional specificity is thought to be determined by an interaction between one or more Hsp70 domains and other components of the cellular machinery. Indeed, several members of the Hsp40 family of co-chaperones have been shown to associate with specific Hsp70 members and are now thought to be associated with Hsp70 specificity (Becker, et al., 1996, Caplan, et al., 1993, Caplan, et al., 1992, Cyr, et al., 1994, Frydman, et al., 1994, Greene, et al., 1998, Michels, et al., 1997, Minami, et al., 1996, Schumacher, et al., 1996). This occurs through direct interaction of different Hsp70 and Hsp40 protein pairs that appear to be specifically adapted to each other.

1. 3. 1. 1   *Multiple Hsp70s with Diverse Functions in Eukaryotic Cells:*

In bacteria, a single Hsp70, called DnaK, aided by its co-chaperone Hsp40, called DnaJ, functions in the binding of proteins in the intermediate stages of folding, assembly, and translocation across membranes (Gaitanaris, et al., 1994, Motohashi, et al., 1999, Segal and Ron, 1998, Teter, et al., 1999). In contrast, all eukaryotic cells possess multiple Hsp70s to carry out such functions. The extensively studied Hsp70 family of *S. cerevisiae* contains at least fourteen members (Baxter, et al., 1996, Craig, et al., 1994, Craig, et al., 1994, Craven, et al., 1996). Eight of these members are located in organelles such as the endoplasmic reticulum and mitochondria and perform similar functions to bacterial DnaK (Craig, et al., 1994, Craig, et al., 1993, Craig, et al., 1994). The Ssa and Ssb subfamilies are located in the nucleus and cytoplasm (Lopez-Buesa, et al., 1998, Shulga,

et al., 1999). The Ssa subfamily has four members: Ssa1-4; the Ssb subfamily has two members: Ssb1 and Ssb2, which are 99% identical. These two subfamilies are functionally distinct, despite sharing 60% amino acid identity (Craig and Jacobsen, 1985, James, et al., 1997, Lopez-Buesa, et al., 1998). The essential Ssa proteins, which are most closely related to the major cytolsolic Hsp70s of mammalian cells, Hsp70/Hsc70, are essential for vegetative growth. At least one member must be expressed at high levels to ensure cell survival (Werner-Washburne, et al., 1987). It is now known that they are involved in the translocation of proteins across membranes and in stress tolerance (Craig, et al., 1994, Craig, et al., 1994). In addition, they have been shown to be involved in the folding of certain proteins after translation (Kim, et al., 1998).

The Ssb1 and Ssb2 proteins appear to be much more functionally specialised. They have been shown to associate with translating ribosomes and can be cross-linked to nascent polypeptide chains (Nelson, et al., 1992, Pfund, et al., 1998). This association, in addition to the fact that strains lacking Ssb are hyper-sensitive to certain inhibitors of protein synthesis, suggests that this class of Hsp70s may be involved in the chaperoning of the nascent polypeptide out of the ribosome.

1. 3. 1. 2   *Stress Tolerance Function:*

Substantial evidence indicates that Hsp70 proteins are capable of protecting cells, tissues, organs, and even complete organisms from a multitude of stress types (Barbe, et al., 1988, Currie, et al., 1993, Marber, et al., 1995, Mestril, et al., 1994, Plumier, et al., 1995, Stojadinovic, et al., 1995).

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On a molecular level, Hsp70 is thought to function during cellular stress by binding to hydrophobic surfaces of denatured proteins thereby inhibiting their aggregation. Evidence for this comes from *in vitro* studies in which purified members of the Hsp70 family have been found to contribute to the protection of several substrate proteins from thermal treatments. Human Hsp70 alone has been shown to protect Topoisomerase I and DNA Polymerase enzymes from heat inactivation (Ciavarra, et al., 1994, Ziemienowicz, et al., 1995). In addition to this, recent *in vivo* studies have demonstrated that the levels of Hsp70 in the cell are found to correlate precisely with the degree of protection conferred on firefly luciferase during a thermal treatment (Nollen, et al., 1999).

Evidence is accumulating that Hsp70 may also be involved in the reactivation of damaged proteins. *In vivo* experiments have demonstrated that Hsp70 on its own is sufficient to reactivate heat denatured luciferase in the cytoplasm (Nollen, et al., 1999). However, Hsp70 alone was not sufficient to yield a complete reactivation of heat denatured luciferase in the nucleus. An earlier report demonstrated that co-expression of Hsp40 (or Hdj1) enhanced Hsp70-mediated reactivation of nuclear located heat-inactivated luciferase (Michels, et al., 1997). *In vitro* evidence suggests that Hsp40 is required in the nucleus to stabilise the binding of Hsp70 to substrates by stimulating hydrolysis of the Hsp70-bound ATP. Hsp70, Hsp40 and ATP either directly enhance refolding of heat denatured nuclear proteins or can keep them in a folding competent state at high temperatures (Freeman and Morimoto, 1996, Schumacher, et al., 1996, Schumacher, et al., 1994). In addition, Hsp70 has been shown to associate

with HSFs in mammalian cells and is consequently very important in the autoregulation of *hsp* genes, discussed above (Abravaya, et al., 1992).

1. 3. 2 The Hsp100/Clp Family

The Hsp100 or Clp proteins are a distinct, highly conserved family, widely distributed among prokaryotes and eukaryotes (Horwich, 1995, Schirmer, et al., 1996, Wawrzynow, et al., 1996). The first member with discernible biochemical function was the *E. coli* ClpA protein, originally identified in 1987 as a mediator of casein proteolysis (Caseinolytic protease, Clp) (Hwang, et al., 1987, Katayama, et al., 1988). Since, then several other members, including the *S. cerevisiae* Hsp104 protein, have been identified and characterised into what is now called the Hsp100/Clp family. Members of this family have a very wide variety of roles, from tolerance to extreme heat stress, proteolysis, DNA replication, control of gene expression, and inheritance of prion-like factors (reviewed by Schirmer, et al., 1996).

1. 3. 2. 1   Classification, Structure and Biochemical Properties.

The Hsp100 family is divided into two major classes based on whether they possess one or two nucleotide binding domains (NBDs) (Schirmer, et al., 1996). Members of class one contain two NBDs which are flanked by amino terminal, middle and carboxy-terminal regions. The two NBDs are highly conserved in all members of this class, but in contrast with most other proteins containing two NBDs, they have very different amino acid sequences. Examples of class one Hsp100s include Hsp104 of *S. cerevisiae*; its bacterial homologue, ClpB, found ubiquitously in various

bacteria (Celerin, et al., 1998, Eriksson and Clarke, 1996, Grandvalet, et al., 1999, Ingmer, et al., 1999, Squires, et al., 1991, Woo, et al., 1992); ClpA of *E. coli* and ClpC of *B. subtilis* (Horwich, 1995, Kruger, et al., 1994, Levchenko, et al., 1997, Parsell, et al., 1994, Sanchez and Lindquist, 1990, Schirmer, et al., 1996, Squires and Squires, 1992, Wawrzynow, et al., 1996). A common characteristic of class one members, is their assembly into higher order oligomers (Parsell, et al., 1994, Singh and Maurizi, 1994, Wawrzynow, et al., 1995). It is thought that one NBD contributes to oligomerisation, while the other contributes to ATPase activity (Kessel, et al., 1995). In the absence of nucleotides, ClpA from *E. coli* and Hsp104 from *S. cerevisiae* exist as monomers, dimers or trimers. However, in the presence of ATP or ADP, both proteins assemble into ring-like, homo-hexameric structures (Parsell, et al., 1994, Thompson and Maurizi, 1994). It is thought that this toroidal arrangement of subunits is central to the various functions of class one Hsp100s.

Members of class two Hsp100s are shorter in length, containing only a single NBD and the carboxy-terminal region (Schirmer, et al., 1996). Apart from the *E. coli* ClpX protein, identified in 1993, little is known about this class (Wojtkowiak, et al., 1993). Despite this limited knowledge about class 2 Hsp100s, members have been identified by sequence analysis in organisms as diverse as *E. coli* and humans (Schirmer, et al., 1996). A ClpX homologue has been identified in the mitochondria of *S. cerevisiae*, called Mcx1p (for Mitochondrial ClpX) (Dyck, et al., 1998). However, to date nothing has been reported about its function. In addition, very recently, a

ClpX homologue has been identified in mouse mitochondria (Santagata, et al., 1999).

1.3.2.2   _Stress Tolerance Functions of Members of The Hsp100 Family._

Some members of class 1 Hsp100s have been associated with stress tolerance functions. In _S. cerevisiae_, the Hsp104 protein plays a vital role in tolerance to severe stress (Iwahashi, et al., 1997, Lindquist and Kim, 1996, Parsell, et al., 1994, Parsell and Lindquist, 1993, Piper, 1995, Sanchez and Lindquist, 1990, Sanchez, et al., 1992). It is present in very low amounts during normal physiological conditions, but is induced to about 2% of total protein during stressful conditions, such as heat shock, exposure to heavy metals or ethanol (Piper, 1995, Sanchez, et al., 1992). Cells carrying a deletion in _hsp104_ grow as well as wild-type cells at temperatures up to 38.5°C, the highest temperature at which standard laboratory strains can grow (Sanchez and Lindquist, 1990). However, when cells are exposed to extreme temperatures of 50°C, _hsp104_ mutants die at 100 to 1000 times faster than wild-type cells. Similarly, _hsp104_ cells are much more sensitive to ethanol exposure (Sanchez, et al., 1992).

A second class 1 Hsp100 protein, homologous to Hsp104, has recently been identified in _S. cerevisiae_ mitochondria and is called Hsp78 (Schmitt, et al., 1996). This protein, if expressed in the cytosol can substitute for Hsp104 in mediating stress tolerance, which suggests a conserved mode of action of both proteins. Furthermore, the heat induced Hsp100 family members from different eukaryotic organisms exhibit a conserved stress-

tolerance function. For example, both the *Arapidopsis* and the soybean Hsp100 proteins can restore tolerance to high temperatures in yeast cells lacking Hsp104 (Lee, et al., 1994, Schirmer, et al., 1994). Stress tolerance related class 1 Hsp100 proteins have also been identified in bacteria. For example, the *E. coli* ClpB and the *B. subtilis* ClpC proteins are rapidly induced by heat and other stresses and have been shown to be required for stress tolerance (Kruger, et al., 1994, Squires, et al., 1991).

As mentioned above, members of the Hsp70 family are thought to function during stressful conditions to prevent the aggregation of denatured proteins. However, Hsp70, and most other Hsps are ineffective in the resolubilisation of aggregates once they have formed. A unique property of certain members of the Hsp100 family is their ability to promote the resolubilisation of stress induced protein aggregates (Motohashi, et al., 1999, Parsell, et al., 1994). When yeast cells are subjected to a moderate to severe thermal stress, protein aggregates are seen to accumulate both in the nucleus and in the cytoplasm of both wild type and *hsp104* mutant cells. However, upon return to normal temperatures, aggregates are cleared in wild-type cells but not in cells lacking Hsp104. Very recently, it has been shown, by electron microscopy, that Hsp104 actually colocalises and physically associates with aggregated bodies formed in both the nucleus and the cytoplasm during thermal stress (Kawai, et al., 1999).

A model for the actual mechanism of action of Hsp100/Clp members in stress tolerance has been proposed, based on work done on Hsp104 (Parsell, et al., 1994, Parsell, et al., 1994, Sanchez and Lindquist,

1990, Sanchez, et al., 1992). It is thought that the toroidal arrangement of Hsp100 sub-units in hexameric rings, allows for the display of active surfaces at a high local concentration, thereby permitting multiple simultaneous interactions with substrates. Such interactions, combined with the co-ordinated use of ATP, are thought to be required to overcome the forces stabilising collapsed or assembled conformations of substrates and promote dissaggregation, unfolding or disassociation.

As described above, most cellular stresses are linked by the fact that they ultimately result in the accumulation of abnormal or denatured proteins. Therefore, during such stressful conditions, it is very likely that the rate of abnormal protein accumulation and subsequent aggregation, actually outpaces the ability of the cells protective systems, such as Hsp70, to prevent aggregation. Thus Hsp104 provides an additional type of survival strategy, i.e. recovery and repair of damage done as opposed to protection from damage.

The functional relationships between members of Hsp70 and Hsp100 families in stress tolerance are just beginning to be understood. The picture that is emerging is that Hsp70, aided by its co-chaperone, Hsp40, and ATP, can function to inhibit the aggregation of proteins during stressful conditions. However, during recovery after stress, Hsp100 proteins are required in addition, forming what has been called the Hsp100-Hsp70-Hsp40 chaperoning machine. This machine is capable of disaggregating large protein aggregates. For example, in the bacterium *Thermus thermophilus*, a functional co-operativity between a Hsp70 (DnaK) and a Hsp100 (ClpB) has very recently been demonstrated *in vitro*

(Motohashi, et al., 1999). It was found that the Hsp40 in co-operation with the Hsp70 could suppress the aggregation of proteins in an ATP dependant manner. However, the Hsp100 was also required for the reactivation of heat damaged proteins. In addition Glover *et al.* demonstrated that *S. cerevisiae* Hsp104, Hsp70 and Hsp40 (Ydj1), function co-operatively *in vitro* in the reactivation of chemically denatured and aggregated proteins (Glover and Lindquist, 1998).

This *in vitro* data is supported by *in vivo* observations in *S. cerevisiae* where Hsp104 can partially compensate for Ssa proteins in growth at high temperatures, while Ssa proteins can partially compensate for Hsp104 in the recovery from severe heat stress (Parsell and Lindquist, 1993, Sanchez, et al., 1993). In addition, deletion of Hsp78, the *S. cerevisiae* mitochondrial member of the Hsp100 family results in synthetic lethality on non fermentable carbon sources in mutant strains carrying temperature-sensitive alleles of Ssc1, the *S. cerevisiae* mitochondrial Hsp70 (Moczko, et al., 1995, Schmitt, et al., 1996).

1. 3. 2. 3 *Non Stress Tolerance Functions of Hsp100/Clp Members*

In addition to stress tolerance functions, several Clp/Hsp100 family members are known to have both proteolytic and regulatory functions (reviewed by Gottesman, 1996, Horwich, 1995, Horwich, et al., 1999, Hoskins, et al., 1998, Pak and Wickner, 1997, Schirmer, et al., 1996, Squires and Squires, 1992, Wawrzynow, et al., 1996). These functions, are similar to the stress tolerance functions of other Hsp100/Clp members in that they also rely on the ability of their oligomeric structures to disaggregate protein

conformers (Weber-Ban, et al., 1999). In bacteria, two family members, ClpA and ClpX, have been very well characterised (Gottesman, 1996). Both proteins form hexameric rings and are known to be involved in the disassembly of specific multimeric protein complexes into their component subunit complexes to trigger a biological response. For example, in *E. coli*, ClpA is specifically required for the conversion of inactive P1 Replication-initiation protein (RepA) dimers to active monomers (Pak and Wickner, 1997, Wickner, et al., 1994). This regulatory function of ClpA results in a 100-fold increase in the DNA binding activity of RepA. Analogous regulatory functions have also been described for ClpX (Levchenko, et al., 1995, Wojtkowiak, et al., 1993).

In addition to their regulatory functions, both ClpA and ClpX are known to be directly involved in the degradation of proteins that have been recognised for proteolysis (Chung, 1993, Gottesman, 1996, Weber-Ban, et al., 1999). Both ClpA and ClpX form large heter-oligomeric complexes structures with the ClpP peptidase. These are composed of fourteen subunits of ClpP with either ClpA or ClpX hexameric rings at one or both ends, forming structures which very closely resemble the eukaryotic 26S proteasome (Grimaud, et al., 1998, Kessel, et al., 1995, Thompson, et al., 1994). It is now known that both ClpA and ClpX associate with proteins that have carboxy-terminal peptide tails added by the SsrA-tagging system (Gottesman, et al., 1998). These proteins are unfolded by the Hsp100/Clp hexamers and subsequently translocated to ClpP peptidase in a process that requires the hydrolysis of ATP (Hoskins, et al., 1998, Pak and Wickner, 1997, Weber-Ban, et al., 1999). Thus ClpA and ClpX, through their

interactions with both substrate and ClpP, act as gatekeepers, actively translocating specific substrates into the proteolytic chamber of ClpP where degradation occurs.

No proteolytic role has been detected for the *S. cerevisiae* Hsp104 protein. However, a regulatory role, in which it is involved in the alteration of the conformation of the yeast prion, has been reported (Chernoff, et al., 1995).

1. 3. 3 The Hsp90 Family

The Hsp90 family is a relatively small family of chaperones (For reviews see Buchner, 1999, Caplan, 1999, Mayer and Bukau, 1999). It has members in eubacteria, plants, *S. cerevisiae* and mammals (See Table 1.1). In eukaryotic cells, Hsp90 is actually one of the most abundant cytosolic proteins, amounting to ~1% of soluble protein, even in the absence of stress (Welch and Feramisco, 1982). However, its abundance is significantly increased by stress treatments. Despite this heat inducibility, Hsp90 has been shown to be essential in *S. cerevisiae* (Borkovich, et al., 1989) and *D. melanogaster* under normal growing conditions (Cutforth and Rubin, 1994). These features have led to the belief that Hsp90 has both stress related and house keeping functions. This has been substantiated by the recent identification of a variety of cellular targets for Hsp90 under normal and stress conditions (reviewed in Buchner, 1999).

Hsp90 has been shown to form an elongated dimer (monomers are 80-90 kDa). Recently, an ADP/ATP binding site in *S. cerevisiae* Hsp90 has been identified and characterised (Prodromou, et al., 1997). In addition, the

binding and hydrolysis of ATP have been shown to be essential for *in vivo* function (Panaretou, et al., 1998). Hsp90 is a very selective molecular chaperone associated with the folding of signal-transducing proteins, such as steroid hormone receptors and protein kinases (Buchner, 1999, Craig, et al., 1993, Cutforth and Rubin, 1994, Nathan and Lindquist, 1995, Nathan, et al., 1997, Silverstein, et al., 1998). Recently, this small substrate range has been expanded to include nitric oxide synthase (Garcia-Cardena, et al., 1998), Telomerase (Holt, et al., 1999) and chloride channel (Loo, et al., 1998). Recently, it has been found that Hsp90 forms discrete sub-complexes, each containing distinct groups of co-chaperones that function together in various different folding pathways (Buchner, 1999, Mayer and Bukau, 1999).

Human Hsp90 has been shown also to co-operate with Hsp70 and it's co-chaperone Hsp40, in the repair of denatured proteins after stress (Freeman and Morimoto, 1996) However, loss of Hsp90 function in *S. cerevisiae*, using a conditional temperature sensitive mutant, revealed no increase in the level of aggregated proteins after heat shock (Nathan, et al., 1997). Despite this, recent studies have suggested that mammalian Hsp90 may be an important negative regulator of heat shock gene induction as interactions between it and HSF have been reported (Duina, et al., 1998, Silverstein, et al., 1998, Zou, et al., 1998).

1. 3. 4 The Hsp60 or Chaperonin Family:

The Hsp60 family members are found in the cytosol of bacteria and in the mitochondria and chloroplasts of eukaryotic organisms where they

share nearly 60% amino acid identity (Buchner, 1996, Bukau and Horwich, 1998, Netzer and Hartl, 1998, Parsell and Lindquist, 1993). They also share a large oligomeric structure composed of 60 kDa subunits arranged as two stacked heptameric rings with a central cavity. Although stress induces expression in bacteria and eukaryotic mitochondria, Hsp60s are constitutively expressed and are essential for growth under all conditions. The Hsp60s are known to co-operate with smaller stress inducible proteins, called Hsp10's. These 'co-chaperonins' are also highly conserved. They have a subunit size of approximately 10 kDa and form single heptameric rings that bind Hsp60 (Hartman, et al., 1992, Lubben, et al., 1990).

The Hsp60 of *E. coli*, GroEL, and its Hsp10, GroES, are the best characterised 'chaperonin team' and have been shown to be involved in the folding of newly made bacterial proteins (Horwich, et al., 1993). It is thought that GroEL, in cooperation with its cochaperonin, GroES, functions in assisting a large number of proteins to reach native form through the actions of binding and folding in its large central cavity (Mayhew, et al., 1996). In *S. cerevisiae*, the Hsp60 or Chaperonin-60 of the mitochondria was originally found to be essential for the assembly of proteins newly imported into mitochondria (Cheng, et al., 1989). Since then, it has been shown to function in the refolding of mitochondrial proteins only after they have been translocated across the mitochondrial membrane by Hsp70 proteins (Bukau and Horwich, 1998, Dyck, et al., 1998).

In addition to roles at normal temperatures, both prokaryotic and eukaryotic members are vital in stress tolerance. GroEL is extremely strongly induced by heat, increasing to a remarkable 10-15% of total

cellular protein (Neidhardt, et al., 1984). This increase is essential for growth of bacteria at high temperatures. Chaperonin-60 of eukaryotes have been shown to associate with proteins and prevent their denaturation in mitochondria during heat stress (Martin, et al., 1992).

1. 3. 5 The Small Hsp Family:

Small Hsps (sHsps) are a poorly conserved ubiquitous chaperone family which range in size from 12 to 30 kDa (Jakob, et al., 1993, Landry and Landry, 1994, Lindquist and Craig, 1988). The major characteristic feature of all sHsps is a conserved central domain, referred to as the α-crystallin domain (Landry and Landry, 1994). Another common feature of sHsps is their oligomeric quaternary structure of up to 32 sub-units forming complexes of up to 800 kDa (Landry and Landry, 1994, Rogalla, et al., 1999). They are found in all organisms from bacteria to mammals and are induced during heat shock conditions (Landry and Landry, 1994). In addition, sHsps have even been reported to be induced during oxidative or ischemic conditions (Mestril, et al., 1994, Preville, et al., 1999).

Of the several families of Hsps, sHsps have probably been the least characterised in terms of function. Firstly, unlike the higher molecular weight Hsp90, Hsp70 and Hsp60 proteins, there is no evidence that sHsps are essential for normal cellular function. Rather, the sHsps appear to primarily involved in the stress response. In heat shocked plants, sHsps are characteristically abundant and comprise an array of about 20 different proteins, localised to virtually every cellular compartment (Waters and Schaal, 1996). In contrast, *S. cerevisiae* and mammalian cells synthesise

only a single sHsp which is localised primarily in the cytosol (Landry and Landry, 1994). Furthermore, in mammalian cells, Hsp27 has been shown to stabilise cytoskeletal elements such as actin during heat shock (Lavoie, et al., 1993, Lavoie, et al., 1993, Lavoie, et al., 1995). Experiments *in vitro* have demonstrated that mammalian Hsp27 can function as a molecular chaperone by preventing thermal aggregation of other proteins as well as enhancing their refolding after heat or chemical denaturation (Buchner, 1996, Jakob, et al., 1993, Merck, et al., 1993).

Several sHsps have been shown to have a high binding capacity to virtually any denatured protein substrate (Ehrnsperger, et al., 1997, Horwitz, 1992, Lee, et al., 1997, Rogalla, et al., 1999). Recently, the crystal structure of a sHsp from *Methanococcus jannaschii* has been solved (Kim, et al., 1998). Twenty four monomers are found to form a hollow spherical complex with a total of fourteen 'windows' that may allow polypeptides to enter the complex. It is now thought that sHsps may function during stressful conditions to actually hold heat denatured proteins in a form which they can later refold in conjunction with other chaperones, such as Hsp70 (Dillmann, 1999). Thus sHsps may provide a store of denatured proteins that can be reactivated upon return to normal physiological temperatures. An additional fate of sHsp-bound substrates could potentially involve proteolytic turnover, in which case prevention of aggregation and maintenance of substrates in an unfolded conformation could facilitate their subsequent degradation.

1. 4   THE PHENOMENON OF INDUCED THERMOTOLERANCE

Hsps have for a long time been associated with the phenomenon of 'Induced Thermotolerance'. This is defined as the ability of cells to survive severe elevated temperatures if first briefly pre-treated at a moderately elevated temperature (Parsell and Lindquist, 1993, Parsell and Lindquist, 1994). This increased resistance is observed in virtually every cell type from bacteria to man. Hsps have been shown to be induced and synthesised during such pre-treatments. Furthermore, the gradual decay of thermotolerance after heat shock correlates with a reduction in Hsp levels (Parsell and Lindquist, 1993, Stege, et al., 1995). In addition, several studies have demonstrated that certain *hsp* mutants are significantly restricted in their ability to induce thermotolerance. For example, when *S. cerevisiae* cells are grown at 25°C and pre-treated at 37°C for 30 minutes to induce thermotolerance, they survive exposure to 50°C significantly better than non pre-treated cells (Sanchez and Lindquist, 1990). However, cells carrying mutations in the *HSP104* gene and given the same pre-treatment, die at 100 to 1,000 times the rate of wild type cells (Sanchez and Lindquist, 1990). In addition, the Ssa proteins have been shown to partially compensate for this in *HSP104* mutants (Sanchez, et al., 1993). Experiments in mouse cells demonstrated that by artificially increasing the levels of Hsp27, a thermoresistance was developed approaching that which could be attained by pre-treatment at mild heat shock temperatures (Lavoie, et al., 1993). Despite all of this data, very little is known about how Hsps actually protect the cellular processes from heat stress.

1.5 TOWARDS AN UNDERSTANDING OF THE AMELIORATIVE EFFECTS OF HSPS ON THE CELLULAR PROCESS OF MRNA SPLICING

Previous workers have demonstrated that the process of mRNA splicing is protected from thermal inactivation at temperatures that would otherwise inactivate it, if cells are first subjected to a mild heat pre-treatment (Yost and Lindquist, 1991). This phenomenon, referred to as 'splicing thermotolerance', to clearly distinguish it from total cell protection or 'survival thermotolerance', has been reported in human (Bond, 1988), *S. cerevisiae* (Yost and Lindquist, 1991) and *D. melanogaster* (Yost and Lindquist, 1986, Corell and Gross, 1992). While protein synthesis is required for splicing thermotolerance in *D. melanogaster*, in *S. cerevisiae* it can be attained even if cells are treated with cycloheximide (Yost and Lindquist, 1991). This latter result prompted Yost to suggest that the protective factors, most probably heat shock proteins, must be present in the requisite quantities at normal temperatures in *S. cerevisiae*. Whereas, in *D. melanogaster* cells, such factor(s) must have to be induced during the pre-treatment. Hsp70 was thought to be the most likely candidate, because constitutive members of the Hsp70 family, Ssa1 and Ssa2, are present at normal temperatures in *S. cerevisiae*, but no Hsp70 forms exist in *Drosophila* under such conditions. It was proposed that while factors responsible for splicing thermotolerance are present in the cell, a pre-treatment is required to 'activate' or relocate them to the nucleus (Yost and Lindquist, 1991).

Despite the fact that no direct evidence exists to show that Hsps actually protect the process of mRNA splicing during heat shock in

thermotolerant *S. cerevisiae*, two studies have shown that Hsps definitely play a vital role in the reactivation of the process following a heat shock (Vogel, et al., 1995, Yost and Lindquist, 1991). Careful analysis of cells carrying mutations in the yeast small heat shock protein gene, *HSP26*, or the genes coding for the two members of the yeast Hsp90 family, *HSC82* and *HSP82*, revealed no defects in mRNA splicing recovery (Yost and Lindquist, 1991). In contrast, a *hsp104* mutation was shown to have a dramatic detrimental effect on the rate of splicing recovery after a heat shock. Vogel *et al.* went on to reproduce this observation and to demonstrate that the heat inducible, cytosolic members of the Hsp70 family, Ssa1, Ssa3 and Ssa4, are also required for splicing recovery, but to a lesser extent than Hsp104 (Vogel, et al., 1995). Furthermore, they observed that, although the recovery of splicing was impaired by the lack of Hsp104 and Ssa proteins, transcription was not. They concluded from this that the effect of the mutations on the recovery of splicing was specific, and not a general effect of heat toxicity on all vital processes.

These workers subsequently observed that Hsp's restore splicing activity to *in vitro* heat shocked extracts (Vogel, et al., 1995). However, this is not surprising since it is known that heating native cell extracts *in vitro* causes protein denaturation. It is very well established from several previous studies that Hsps are capable of restoring protein activity when added *in vitro* to extracts that have been heated in test tubes (Lee, et al., 1997, Schumacher, et al., 1994). However, any assumption that this is in any way reflective of the *in vivo* situation is likely to be an over simplification of what is probably a much more complex situation. To

begin to appreciate this point, it is vital to actually understand the complicated and compartmentalised nature of the process of mRNA splicing and the multitude of factors involved in its functioning.

1. 6   mRNA SPLICING

1. 6. 1 The Process

Most human genes and up to 3% of *S. cerevisiae* genes are interrupted by non-coding stretches of DNA known as introns. After transcription, these introns must be excised from the pre-mRNA and the flanking exons joined together. This process, called mRNA splicing, was first discovered in 1977 (Berget, et al., 1977, Chow, et al., 1977). The splicing apparatus, or the spliceosome, is a large highly conserved multi-component complex (Staley and Guthrie, 1998). It is constituted of several small nuclear ribonucleoprotein particles (snRNPs) and other associated protein factors (Beggs, 1995, Will and Lührmann, 1997). Each snRNP is generally composed of a single small nuclear RNA (U1, U2, U4, U5 and U6 snRNAs) and a multitude of proteins (See Table 1.2) (Staley and Guthrie, 1998). The exception to this composition is the U4/U6 snRNP which contains two snRNAs, U4 and U6. The formation of the spliceosome is an ordered process that involves the stepwise interaction of snRNPs with the pre-mRNA (See Figure 1.1) (Kramer, 1996, Staley and Guthrie, 1998). The U1 and U2 snRNPs interact with the pre-mRNA as single particles while the tri-snRNP (U4/U6.U5) is first assembled from the U4/U6 and the U5 snRNPs before entry into the spliceosome. The primary function of snRNPs within the spliceosome is to facilitate mRNA splicing by

juxtaposing the spatially separated 5' and 3' splice sites through specific base-pairing interactions between snRNAs and the pre-mRNA and through protein-protein interactions involving snRNP proteins and other splicing factors (Newman, 1998, Newman, 1994, Staley and Guthrie, 1998).

Figure 1.1   mRNA splicing and the spliceosomal snRNPs. The splicing process involves the elimination of intervening sequences (introns) in mRNA precursors and the subsequent ligation of their flanking regions (exons) to produce mature mRNAs (See text).
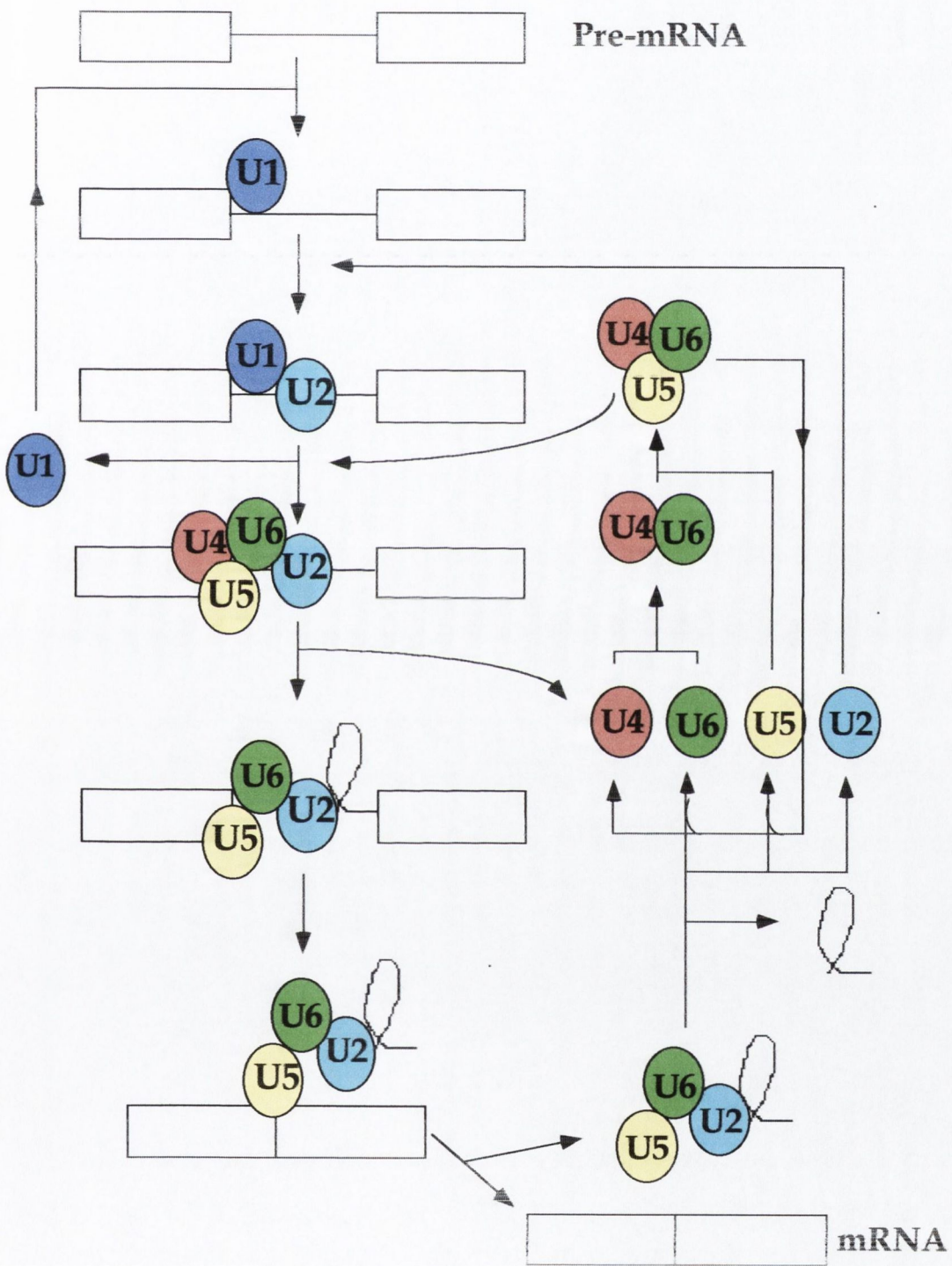
1. 6. 2 The Composition and Biogenesis of snRNPs

The U1, U2, U4 and U5 snRNAs are transcribed by RNA Polymerase II and are known to migrate to the cytoplasm where they bind in an ordered process to several proteins, commonly referred to as Sm or core proteins (Kastner, et al., 1990, Mattaj and Robertis, 1985, Raker, et al., 1999, Raker, et al., 1996). These proteins were originally discovered to be produced by patients with systemic lupus erythematosus (Lerner and Steitz, 1979). In humans cells, there are seven Sm proteins: SmB/SmB', SmD1, SmD2, SmD3, SmE, SmF and SmG (Hermann, et al., 1995, Lerner and Steitz, 1979). *S. cerevisiae* cells possess an homologous set of seven Sm proteins (See Table 1.2) (Gottschalk, et al., 1998, Neubauer, et al., 1997). Recently, the seven human Sm proteins have been shown to form a ring like or doughnut shaped structure that is thought to be capable of binding snRNAs within its central cavity (Kambach, et al., 1999). The binding of these Sm proteins is required for the subsequent hyper-methylation of the snRNA 5' cap (Mattaj, 1986) and import of the assembled core snRNP into the nucleus (Fischer, et al., 1997, Mattaj, et al., 1988, Nelissen, et al., 1994). Upon import of core snRNPs, it is thought that the snRNP specific proteins assemble on to ultimately form the mature U1-U5 snRNP species (Zieve and Sauterer, 1990). Recently, in human cells, a group of four U5 snRNP specific proteins have been shown to form a complex which is stable in the absence of snRNA or Sm proteins (Achsel, et al., 1998). It is likely that these four proteins assemble onto imported U5 core snRNP as a single unit. Furthermore, since three of these proteins have U5 specific

homologues in *S. cerevisiae* (Brr2, Snu114 and Prp8), it is also possible that an analogous process of U5 snRNP biogenesis occurs in this organism.

In contrast to the U1-U5 snRNAs, the U6 snRNA gene is transcribed by RNA Polymerase III and consequently the U6 snRNA does not leave the nucleus (Moenne, et al., 1990). In human cells, the La protein has long been identified as the first protein to bind to all newly transcribed RNA Polymerase III transcripts (Chambers, et al., 1983, Hendrick, et al., 1981, Rinke and Steitz, 1985, Rinke and Steitz, 1982). An homologous protein, Lhp1p (La homologous protein 1), has been identified to function similarly in *S. cerevisiae* (Yoo and Wolin, 1994). In addition, recently Lhp1p has been shown to be involved in the stabilisation of newly synthesised U6 RNA (Pannone, et al., 1998). This stabilisation may facilitate the biogenesis of the U6 snRNA into the U6 snRNP. Very recently, seven Sm like proteins, termed Lsm2-8 (Like sm), have been shown to be part of the U6 snRNP in both *S. cerevisiae* (Mayes, et al., 1999) and human cells (Gottschalk, et al., 1999, Stevens and Abelson, 1999). In addition, in human cells, these 7 proteins have been shown to form a doughnut shaped structure, analogous to the Sm ring structure, and are thought to play an important role in the biogenesis of the U4/U6 snRNP (Achsel, et al., 1999).

Since both U6 snRNP biogenesis and the latter stages of U1-U5 snRNP biogenesis occur in the nucleus, it follows that the biogenesis of the U4/U6 and U4/U6.U5 snRNPs likely occur there also. There are at least 14 U4/U6.U5 tri-snRNP specific proteins (Gottschalk, et al., 1999, Stevens and Abelson, 1999), 9 U1 snRNP specific (Gottschalk, et al., 1998, Neubauer, et al., 1997) and 9 U2 snRNP specific proteins (Caspary and Seraphin, 1998,

Ruby, et al., 1993, Wells, et al., 1996, Yan, et al., 1998) in *S. cerevisiae* (See Table 1.2). In addition, there are also many non-snRNP proteins which interact with the snRNPs during the process of spliceosome assembly (Table 1.2) (reviewed by (Beggs, 1995, Kramer, 1996)). For example, the Prp24 protein is required for the association of the U4 and U6 RNPs in *S. cerevisiae* extracts (Ghetti, et al., 1995, Raghunathan and Guthrie, 1998).

Table 1.2 *Saccharomyces cerevisiae* **Splicing Proteins**

	Protein Name(s)	M_r (kDa)	References
U1-U5 Core Proteins	SmB	22	(Gottschalk, et al., 1998)
	SmD1	16	(Rymond, 1993)
	SmD2	13	(Neubauer, et al., 1997)
	SmD3	11	(Roy, et al., 1995)
	SmE	10	(Bordonne and Tarassov, 1996)
	SmF	10	(Séraphin, 1995)
	SmG	9	(Séraphin, 1995)
U6 Core Proteins	Lsm2	11	(Gottschalk, et al., 1999, Stevens and Abelson, 1999)
	Lsm3	10	(Séraphin, 1995)
	Lsm4	21	(Cooper, et al., 1995)
	Lsm5	10	(Gottschalk, et al., 1999, Stevens and Abelson, 1999)
	Lsm6	14	(Gottschalk, et al., 1999, Stevens and Abelson, 1999)
	Lsm7	12	(Gottschalk, et al., 1999, Stevens and Abelson, 1999)
	Lsm8	15	(Pannone, et al., 1998)
U1 Specific Proteins	Snp1	34	(Kao and Siliciano, 1992, Smith and Barrell, 1991)
	Mud1	37	(Neubauer, et al., 1997)
	yU1-C	32	(Neubauer, et al., 1997)
	Snu56/Mud10	52	(Gottschalk, et al., 1998)
	Nam8/Mud15	57	(Gottschalk, et al., 1998)
	Snu65/Mud16/Prp42	57	(Gottschalk, et al., 1998)
	Prp39	69	(Lockhart and Rymond, 1994)
	Prp40	69	(Kao and Siliciano, 1996)
	Snu71	77	(Gottschalk, et al., 1998)

Table 1.2 Continued.

U2 Specific Proteins	Yib9		(Caspary and Seraphin, 1998)
	Lea1		(Caspary and Seraphin, 1998)
	Rse1/scSAP154	148	(Caspary, et al., 1999)
	Prp5		(Ruby, et al., 1993)
	Prp9	63	(Ruby, et al., 1993)
	Prp11		(Ruby, et al., 1993)
	Prp21		(Ruby, et al., 1993)
	Cus1		(Wells, et al., 1996)
	Cus2		(Yan, et al., 1998)
U4/U6.U5 Specific Proteins	Prp8	280	(Lossky, et al., 1987)
	Brr2/Snu246	246	(Lauber, et al., 1996, Lin and Rossi, 1996, Noble and Guthrie, 1996)
	Snu114	114	(Fabrizio, et al., 1997)
	Prp6	104	(Abovich, et al., 1990)
	Snu66	66	(Gottschalk, et al., 1999, Stevens and Abelson, 1999)
	Prp31	56	(Weidenhammer, et al., 1997)
	Prp3	56	(Anthony, et al., 1997)
	Prp4	52	(Banroques and Abelson, 1989, Bjørn, et al., 1989)
	Spp381	34	(Gottschalk, et al., 1999, Lybarger, et al., 1999)
	Prp38	28	(Blanton, et al., 1992)
	Prp18	25	(Horowitz and Abelson, 1993)
	Snu23	23	(Gottschalk, et al., 1999, Stevens and Abelson, 1999)
	Dib1/Snu16	16	(Gottschalk, et al., 1999)
	Snu13	13	(Gottschalk, et al., 1999)
Non snRNP Proteins	Prp24		(Shannon and Guthrie, 1991)
	Prp2		(Vijayraghavan, et al., 1989)
	Prp22		(Company, et al., 1991)
	Prp43		(Arenas and Abelson, 1997)

1. 7 PROJECT BACKGROUND AND OBJECTIVES

While the process of mRNA splicing is known to be inactivated by heat stress, the components within the splicing apparatus affected have not been thoroughly examined. Previous data from HeLa cells, has attributed splicing inactivation to defects in the assembly of the active spliceosome (Bond, 1988, Utans, et al., 1992). These defects were correlated with alterations of the snRNP components of the spliceosome. This included a complete disruption of the U4/U6.U5 tri-snRNP, as well as slight reductions in the levels of U1 and U2 snRNP species. In addition, Bond also reported the accumulation of a faster migrating U2 hybridising species in heat shocked cells (Bond, 1988). It is now known that the 17S human U2 snRNP has at least two precursor or intermediate forms, of 12S and 15S respectfully (Behrens, et al., 1993, Kramer, et al., 1999). It is possible that the altered U2 species, previously observed by Bond in heat shocked HeLa cells, is actually one of these two precursor forms.

Other types of ribonucleoprotein (RNP) complexes are also observed to be affected in heat shocked cells. In HeLa cells and *D. melangaster* cells, redistributions of heteronuclear ribonucleoproteins (hnRNPs) and alterations of hnRNP particles have also been observed after heat shock (Buchenau, et al., 1997, de Graaf, et al., 1992, Gattoni, et al., 1996, Lutz, et al., 1988, Mahé, et al., 1997, Mähl, et al., 1989, Mayrand and Pederson, 1983). This suggests that all RNP particles may be sensitive to heat shock. However, despite these pleiotropic effects of heat on RNPs, Utans *et al.* demonstrated that *in vitro* splicing activity, in extracts prepared from heat shocked HeLa cells, can be restored by the addition of a protein fraction

consisting minimally of five proteins (Utans, et al., 1992). These five proteins are components of the U4/U6.U5 tri-snRNP complex and are of sizes 90, 60, 27, 20, and 15.5 kDa, most of which have been subsequently characterised (Fetzer, et al., 1997, Lauber, et al., 1997, Teigelkamp, et al., 1998). Together, they are capable of reassembling the U4/U6 and the U5 snRNPs into the U4/U6.U5 tri-snRNP suggesting that the primary splicing defect in heat shocked HeLa cells may be the lack of a functional tri-snRNP. Thus, in HeLa cells at least, splicing inactivation may result from, either the thermal deactivation or unavailability of essential tri-snRNP proteins.

While mRNA splicing is known to be inactivated by heat shock in yeast cells, and Hsp104 and Ssa proteins are required for it's subsequent reactivation upon return to normal temperatures, the actual components affected by the heat stress within the spliceosome have not been characterised to date. Therefore, the first aim of this thesis, as outlined in Chapter 3, was to characterise the effects of heat shock on the snRNP components of the S. cerevisiae spliceosome. In this chapter multiple alterations in several snRNPs are described and in some cases, precursor snRNP forms are observed to accumulate.

Having established that snRNPs are indeed altered in a manner similar to that described previously in mammalian cells, it was then possible to use the powerful genetics of S. cerevisiae to decipher both the role played by Hsps in the restoration of normal snRNP profiles following a heat shock and their protective role in splicing thermotolerant cells. The role of Hsp104 and the Ssa proteins in snRNP restoration was investigated

in Chapter 4 using *S. cerevisiae* mutant strains lacking the genes coding for these proteins (Table 2.1). The results demonstrate that splicing reactivation after heat shock correlates with snRNP reassembly and is dependent on the presence of these Hsps. In Chapter 5, splicing thermotolerance is correlated with a physical association of Ssa proteins with snRNPs to confer protection. Surprisingly, despite the snRNP protections observed, splicing thermotolerance itself appears to be independent of the Ssa proteins and Hsp104.

Chapter 2:

Materials and Methods.

2. 1   YEAST STRAINS.

Saccharomyces cerevisiae mutant strains lacking specific *HSP* genes and their isogenic wild type parent strains, used in this study, are listed in Table 2. 1.

2. 2   CULTURE AND HEAT SHOCK CONDITIONS.

Yeast strains were cultured at 25°C in YEPD (1.0% yeast extract, 2.0% Bacto Peptone, 2.0% glucose) to mid-logarithmic phase (4.0×10^6 and 1.0×10^7 cells/ml or OD_{600} 0.4-0.7). This was done to avoid the accumulation of Hsp104 and other stress tolerance factors such as trehalose, which are known to be induced as cells approach stationary phase (Lindquist and Kim, 1996). For the initial heat shock experiments (3. 2. 1 to 3. 2. 3), 500ml aliquots of cells, in 2 L culture flasks, were transferred directly from an air incubator at 25°C to a heated shaker waterbath for one hour at the temperatures (+/- 0.5°C) indicated in the text. The temperature of the culture medium usually reached that of the waterbath within approximately 10 min by this heating regime. For all subsequent heat shock experiments, (3. 2. 4 to 5. 5. 2), 500ml aliquots of cells were centrifuged in a Sorvall GSA rotor at 7000 r.p.m. for 5 min at 25°C, resuspended in pre-warmed supernatent media in 2 L culture flasks and heat shocked for one hour at the temperatures (+/- 0.5°C) indicated in the text. This latter heating regime ensured an instantaneous heat shock. Recovery experiments involved immediate transfer of heat shocked cells to waterbaths at 25°C following the heat shock treatment. Mild heat pre-

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treatments were performed by transferring cells to pre-warmed media at 37°C and incubating with shaking for 45 min before immediately transferring to 42°C for one hour. After all heat treatments, cultures were chilled on ice, centrifuged in a Sorvall GSA rotor at 5000 r.p.m. for 5 min at 4°C, quick frozen on dry ice and stored at -70°C for subsequent splicing extract and RNA preparations. In general 450ml and 50ml aliquots of cultures were used for the preparation of splicing extracts and RNA respectively.

2. 3   RNA ISOLATION.

Total RNA was isolated by the Hot-Phenol Method, as previously described (Krieg, 1996). The protocol was scaled down for smaller volumes as follows. Aliquots of 25ml cells (OD_{600} 0.4-0.7) pelleted and frozen at -70°C were thawed on ice for 1 hour. Cells were then washed with 10ml of ice-cold, sterile dH_2O and centrifuged in a Sorvall SS-34 rotor at 7,000 r.p.m. for 5 min at 4°C. These cells were then washed with 10ml ice-cold AE buffer (50mM sodium acetate pH5.3, 10mM EDTA) and centrifuged again. The cell pellets were then transferred to 1.5ml Eppendorf tubes by resupending in 500µl fresh AE buffer. 50µl 10% SDS and 550µl AE buffered phenol were then added to each pellet. Eppendorfs were incubated at 65°C for 30 min with 10 second vortexing at 5 min intervals. Eppendorfs were then centrifuged in a micro-centrifuge at 14,000 r.p.m. for 5 min. The aqueous layer was carefully removed and extracted once more with AE

buffered Phenol and once with Chloroform before being precipitated in a final concentration of 300mM sodium acetate with 3 volumes of ethanol at -20°C. RNA was resupended in 50μl DEPC-treated dH$_2$O. The concentration and relative purity of the RNA was determined measuring the absorbence readings at OD$_{260}$ and OD$_{280}$. In general, the ratio was approximately 1.8.

2. 4   FORMALDEHYDE GEL ELECTROPHORESIS.

RNA was separated on 0.9% formaldehyde-agarose gels containing 0.5μg/ml ethidium bromide as described previously (Maniatis, et al., 1989). RNA samples (10-30μg) were brought up to 10μl with DEPC treated dH$_2$O. 20μl Formaldehyde Gel loading buffer (2.75M formaldehyde, 15mM MOPS pH7.0, 22mM sodium acetate, 70% (v/v) formamide) was added. Samples were mixed well, quick spun and incubated at 65°C for 15 min to denature RNA. 3μl loading dye (0.1% Bromophenol Blue, 0.1% Xylene Cyanol, 50% (w/v) glycerol) was added before loading samples and running overnight at 50V in 1X Formaldehyde gel running buffer (20mM MOPS, 30mM sodium citrate pH7.0). Gels were run overnight to ensure adequate separation of actin pre-mRNA and mRNA species and consequently the buffer had to be re-circulated during electrophoresis. The gel was photographed under UV light and subsequently washed twice for 30 min in DEPC treated 10X SSC (1.5M NaCl, 150mM sodium acetate, pH7.0) before being capillary blotted to Nytran membranes (Schleicher & Schuell). Blots were UV cross-linked for 2 min at 150mJ by a Bio-Rad GS Gene Linker prior to hybridisation.

2. 5 NORTHERN HYBRIDISATION ANALYSIS.

The plasmid pT7Act, containing a fragment of the actin (Act1) gene, was kindly provided by Christine Guthrie (University of California, San Francisco). This plasmid was originally created by Art Zaug by first cloning the Alu-Alu fragment of Act1 into the Sma site of pSP65 (Promega). The actin containing fragment was then removed from this vector and cloned into pT7 (USB) to create pT7Act. Digoxygenin-dUTP (Roche Molecular Biochemicals) labelled DNA probes specific for the Act1 gene were generated by standard PCR amplification from this plasmid template using the primers, 5'-TAATACGACTCACTATAGGG-3' and 5'-GGCTGCAGGTC GACTCTAGA-3'. Digoxygenin-dUTP to dTTP ratios varied from 1:2 to 1:6 but yielded similar results. Blots were prehybridised for 1-3 hours in EasyHyb buffer (Roche Molecular Biochemicals) at 50°C, and hybridised in bottles for 15-20 hours at 50°C with approximately 25ng labelled probe per ml of EasyHyb solution. After hybridisation, stringency washes were performed. This was done by immediately transferring blots to bottles containing 2X wash solution (2X SSC (0.3M NaCl, 30mM sodium acetate, pH7.0), 0.1% SDS) at 68°C and washing twice for 15 to 20 min each time. A second stringency wash, this time in cleaned glass baking trays, was performed twice with 0.5X wash solution (0.5X SSC (75mM NaCl, 7.5mM sodium acetate, pH7.0), 0.1% SDS) at room temperature for 15 to 20 min each time. After stringency washing, the blots were washed in Maleic Acid Buffer or MAB (0.1M Maleic Acid, 0.15M NaCl, pH 7.5) with 0.3% (v/v)

Tween-20 for 10 min in a fresh glass baking tray. Blots were next blocked in MAB with 1% (w/v) Casein for at least 40 min and then incubated with anti-DIG-AP conjugate (Roche Molecular Biochemicals) diluted 1:10,000 in MAB with 1% (w/v) Casein for exactly 30 min. Blots were then washed for 5 min in MAB with 0.3% (v/v) Tween-20 before being equilibrated in Detection Buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 2 to 5 min before being incubated in a sealed hybridisation bag with $1ml/200cm^2$ of either of the chemiluminescent substrates, CSPD or CDP-Star (Roche Molecular Biochemicals) diluted 1:100 in Detection Buffer for 1 or 5 min respectively. These were subsequently exposed to autorad film. Usually CSPD detected blots required 10 to 120 min for optimum exposure whereas CDP-Star required 1 to 15 min.

2. 6 PREPARATION OF YEAST SPLICING EXTRACTS.

Cell pellets, frozen from heat shock experiments, were thawed on ice and washed with 10ml of ice-cold, sterile distilled water and then suspended in 3.5mls of extraction buffer A (10mM HEPES-KOH pH 7.5, 1.5mM $MgCl_2$, 10mM KCl and 0.5mM DTT added just prior to use). Cells were subsequently broken by vortexing with 3mls of acid washed glass beads in Corex tubes, periodically for 30 secs each time, in a cold room as previously described (Séraphin and Rosbash, 1989). This vortexing was continued until 70-90% cell breakage was obtained as judged by microscopic examination (Usually 15-18 vortex's). Cell debris and glass beads were pelletted by centrifugation in a SS-34 rotor at 7,000 r.p.m. at 4°C for 5 min. The supernatants were then mixed with a 1/9th volume of 2M KCl and

allowed to mix gently at 4°C for 30 min. These extracts were then ultra-

centrifuged in a Beckman TL-100 rotor at 56,000 r.p.m. at 4°C for 20 min.

The supernatant was transferred to dialysis tubing, taking care to avoid the

lipid layer at the top of the tube. Dialysis was performed against 2L of

Buffer D (20mM HEPES-KOH 7.5, 0.2mM EDTA pH 8.0, 50mM KCl, 20%

(w/v) glycerol and 0.5 mM DTT added just prior to use) for 3 hours at 4°C.

2. 7 GLYCEROL GRADIENT ANALYSIS.

Glycerol gradient analysis was performed as described previously

(Anthony, et al., 1997, Ayadi, et al., 1997, Banroques and Abelson, 1989,

Brown and Beggs, 1992, Galisson and Legrain, 1993). 80µl of splicing

extracts were diluted with 150µl buffer R (150mM Tris-HCl pH 7.4, 25mM

KCl, 5mM $MgCl_2$) and layered on 10-40% continuous glycerol gradients

prepared in buffer R. These were sedimented in a SW 40 Ti rotor at 37,000

r.p.m. for 14 hours at 4°C. The gradient was fractionated into 400µl aliquots

and RNA was extracted from each with an equal volume of phenol-

chloroform, ethanol precipitated and analysed by Northern blotting.

2. 8 NORTHERN BLOTTING ANALYSIS OF snRNAS.

RNA from gradient fractions was separated on 6%

acrylamide:bisacrylamide (40:2), 7M urea gels in 1X TBE (100mM Tris,

90mM Boric Acid, 1mM EDTA pH 8.0) and electroblotted onto nylon

membranes (Micron Separations Inc./ Schleicher & Schuell) at 40V for 3

hours at 4°C. Plasmids of pUC18 derivation and containing snRNA

sequences (Ares, 1986, Kretzner, et al., 1987, Patterson and Guthrie, 1987, Siliciano, et al., 1987), were kindly provided by Bertrand Séraphin (EMBL, Heidelberg). Digoxygenin labelled DNA probes specific for U4, U5 and U6 snRNAs were generated by PCR from these pUC18 derived plasmids using the standard cloning vector, 24-mer forward 5′-CGCCAGGGTTTTCCCAGT CACGAC-3′ and reverse 5′-AGCGGATAACAATTTCACACAGGA-3′ primers. Northern analysis was perform exactly as described above for Act1 experiments.

2. 9   NATIVE GEL ANALYSIS OF snRNP COMPLEXES.

Splicing extracts (40-60μg total protein/lane) were separated on 4% acrylamide:bisacrylamide (80:1) non-denaturing gels in TG buffer (50mM Tris base, 50mM glycine, pH 8.9) using the BioRad Protean II xi Cell system, with glass plates of 20cm X 20cm and spacer width of 0.5mm (Bond, 1988, Konarska and Sharp, 1986). Gels were pre-electrophoresed for 20-40 min at 230V at 4°C. Samples were then loaded and electrophoresed for 3 to 4 hours under the same conditions. To observe upward shifts in snRNPs, the gels were electrophoresed for 7 hours. Gels were electroblotted to Nytran membranes for 3 hours at 40V (Schleicher & Schuell) and hybridised with PCR generated Digoxygenin labelled DNA probes specific for U1, U2, U4, U5 and U6 snRNAs as indicated. Hybridisations were performed as described above. For densitometric analysis, blots were scanned using a BioRad imaging densitometer Model GS-670, and were analysed using the accompanying Molecular Analyst software.

2. 10 PROTEIN ANALYSIS.

2. 10. 1 Determination of Protein Concentration.

The total protein concentration of the splicing extracts was determined using a modification of the Bradford method (Bradford, 1976). A standard curve was prepared by using a dilution series of bovine serum albumin at concentrations (w/v) of 25µg/ml, 12.5µg/ml, 6.3µg/ml, 3.1µg/ml, and 1.6µg/ml. 200µl of Dye Reagent Concentrate (BioRad Chemicals) was added to 800µl of each of the dilution series samples and vortexed to ensure adequate mixing. Samples were allowed to sit at room temperature for 20 min before reading absorbances at OD_{595}. After plotting a standard curve, appropriate volumes, usually 2 or 3µl, of splicing extracts were diluted to 800µl and assayed for absorbance at OD_{595} exactly as described above. Total protein concentrations of splicing extracts were determined using the standard curve.

2. 10. 2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins.

Splicing extracts (40-100µg total protein/lane) were electrophoresed on 10% acrylamide:bisacrylamide (30:0.8) SDS-polyacrylamide gels as previously described (Maniatis, et al., 1989). Samples were mixed with an equal volume of 2X Protein Loading Dye (125mM Tris-HCl pH 6.8, 20% glycerol, 4.0% SDS, 288mM β-Mercaptoethanol, 0.12% Bromophenol Blue), heat

denatured at 95°C for 3 min, quick chilled on ice and loaded on gel. Pre-stained molecular weight markers (Sigma Chemical Co.) were loaded to determine approximate protein size. The gel was electrophoresed in 1X Gel Running Buffer (25mM Tris base, 192mM glycine, 0.1% SDS) at 100V until Bromophenol blue dye reached the bottom of the gel (Usually 60-90 min for 8cm X 10cm gels and 3-4 hours for 20cm X 20cm gels).

2. 10. 3   Western Immunoblot Analysis.

After electrophoresis, gels for western analysis were electroblotted to Protran nitrocellulose membranes (Schleicher & Schuell) in 1X Transfer Buffer (25mM Tris-HCl pH 8.3, 192mM glycine) at 100V for 1 hour for 8cm X 10cm gels or at 40V for 3 hours, for 20cm X 20cm gels. The membranes were blocked in MAB with casein (100mM maleic acid, 150mM NaCl, 1.0% casein, pH 7.5) for 1 hour and subsequently incubated with primary antibody, diluted in fresh MAB with casein, for 2 hours. The primary antibodies used were rabbit anti-Hsp104 (A kind gift from Professor Susan Lindquist, University of Chicago), anti-Ssa (A kind gift from Dr. Elizabeth Craig, University of Wisconsin) and anti-Prp8 (A kind gift from Professor Jean Beggs, University of Edinburgh) at working concentrations of 1/10,000, 1/5,000 and 1/10,000 respectfully. Membranes were then washed 3 times, for 5 min each, in MAB with Tween (100mM maleic acid, 150mM NaCl, 3.0% Tween-20) before a 1 hour incubation with HRP-conjugated goat anti-rabbit antibody (Sigma Chemical Co.) at a dilution of 1/3,000 in MAB with casein. Membranes were then washed 6-8 times, for 5 min each, in MAB with Tween. Membranes were detected with the

chemiluminescent substrate, SuperSignal (Pierce Chemicals) exactly as described for Northern blot detections.

2.11 IMMUNOPRECIPITATION ANALYSIS

Immunoprecipitation of snRNPs with anti-Prp4 antibody was performed based on a protocol originally described by Banroques and Abelson (Banroques and Abelson, 1989). 6µl of anti-Prp4 antibody (A kind gift from Dr. Jossette Banroques, Centre de Génétique Moléculaire du CNRS, Paris) was coupled to 10µl Pierce Ultralink Immobilised Protein A/G, with a binding capacity of 26mg human IgG/ml gel, at 4°C for 1.5 h in 1.0 ml of NET-2 buffer (50 mM Tris HCl, pH 7.4, 0.05% Nonidet P-40, 150 mM NaCl). The pellets were then washed three times with 1ml NET-2 buffer. 500µg of total splicing extract was then incubated with antibody bound beads in 1ml of fresh NET-2 buffer for 1.5 h at 4°C with gentle mixing. The resin was then washed 6 times with 1ml amounts of NET-2. Hsp70 was immunoprecipitated with anti-Ssa antibody (A kind gift from Prof. E. Craig, University of Wisconsin) using this exact protocol. Samples were separated on 10% SDS-Polyacrylamide gels and western blotted with anti-Ssa antibody (See above).

TABLE 2. 1. *S. cerevisiae* strains used

Strain	Genotype	Source
SL303-1A	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2*	(Vogel, et al., 1995)
SL304A	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (hsp104::LEU2)*	(Vogel, et al., 1995)
SL324-1B	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (ssa1::HIS3) (ssa3::TRP1) (ssa4::LYS1)*	(Vogel, et al., 1995)
SL325-1B	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (hsp104::URA3) (ssa1::HIS3) (ssa3::TRP1) (ssa4::LYS1)*	(Vogel, et al., 1995)
JN55	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2*	(Becker, et al., 1996)
JN516	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (ssa2::LEU2) (ssa3::TRP1) (ssa4::LYS2)*	(Becker, et al., 1996)
JN519	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (ssa1-45BKD) (ssa2::LEU2) (ssa3::TRP1) (ssa4::LYS2)*	Elizabeth Craig. (University of Wisconsin)

Chapter 3:

Correlation of the Thermal Inactivation of mRNA Splicing in *S. cerevisiae* with Multiple Alterations in Spliceosomal Components.

3. 1   INTRODUCTION.

The primary aim of this chapter was to thoroughly characterise, for the first time, the effects of thermally inactivating mRNA splicing on snRNPs in *S. cerevisiae*. To do this, it was first necessary to establish the minimum heat shock conditions required for splicing inactivation in this organism. Previous workers had reported inactivation after a heat shock of one hour at 41.5°C (Vogel, et al., 1995). In order to assess the effects of the heat shock on snRNPs, it was decided to employ glycerol gradient analysis of extracts from heat shocked and non-heat shocked cells. This methodology has been used by several previous researchers to determine the effects of temperature sensitive mutations of yeast Prp proteins on snRNP integrities (Anthony, et al., 1997, Ayadi, et al., 1997, Banroques and Abelson, 1989, Brown and Beggs, 1992, Galisson and Legrain, 1993). In this chapter, alterations in the snRNP profiles of heat shocked cells are analysed by both this method and by the more informative method of native gel analysis, developed later on. In an attempt to determine if Hsps have an ameliorative effect on snRNPs during heat shock, extracts from the mutant strain SL325-1B, which lacks the genes coding for Hsp104 and the heat inducible members of the Ssa sub-family, are also analysed. Finally, the state of both the Prp8 protein and the U1-U6 snRNA snRNP components in heat shocked cells are compared to their states in non-heat shocked cells

3. 2   RESULTS.

3. 2. 1 Determination of the Temperature Required to Inactivate mRNA Splicing.

To begin to optimise experimental conditions capable of inactivating mRNA splicing, exponentially growing wild type yeast cells at 25°C were subjected to heat shock at various temperatures. This was done by transferring culture flasks directly to heated shaking waterbaths for one hour before collecting cells and subsequently preparing RNA. Inactivation of mRNA splicing was monitored by the appearance of intron containing pre-mRNA species on Northern blots hybridised with an actin-specific DNA probe (Figure 3.1). No inactivation of splicing was observed when cells were incubated at 40°C (Figure 3.1, lane 2). Partial inactivation of splicing was observed when cells were incubated at 41.5°C (Figure 3.1, lane 3) and complete inactivation was observed at 43°C (Figure 3.1, lane 4).

To determine the absolute minimum temperature required to inactivate splicing in both wild type and the mutant strain SL325-1B (*hsp104, ssa1, ssa3, ssa4*), heat shocking was performed at 42°C as described above (Figure 3. 2). Complete inactivation of splicing is observed in both wild type and SL325-1B cells at 42°C (Figure 3.2, lanes 2 and 5). Inactivation was also observed at 43.5°C in both strains (Fig
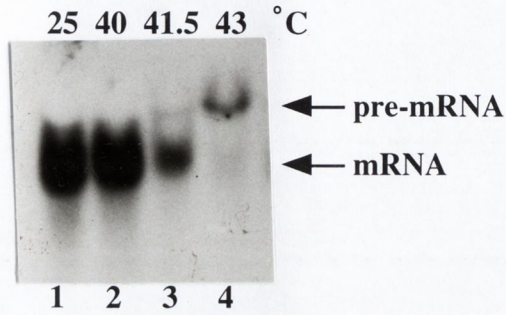
ure 3.2, lanes 3 and 6).

Figure 3. 1   mRNA Splicing is Partially Inactivated by Heat Shocking at 41.5°C in Wild Type *S. cerevisiae.*

Wild type yeast cultures were heat shocked for one hour at the indicated temperatures. RNA extracted following the treatment was separated on 0.9% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridised with a Digoxygenin-UTP labelled DNA probe specific to the actin gene (ACT1). The position of the mature mRNA and the pre-mRNA are indicated by arrows.

Figure 3. 2   mRNA Splicing is Completely Inactivated in Both Wild Type and SL325-1B strains at Temperatures of 42°C or Greater.

Both wild type and SL325-1B yeast cultures were heat shocked in parallel for one hour at the indicated temperatures. RNA extracted following the treatment was separated on 0.9% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridised with a Digoxygenin-UTP labelled DNA probe specific to the actin gene (ACT1). The position of the mature mRNA and the pre-mRNA are indicated by arrows.
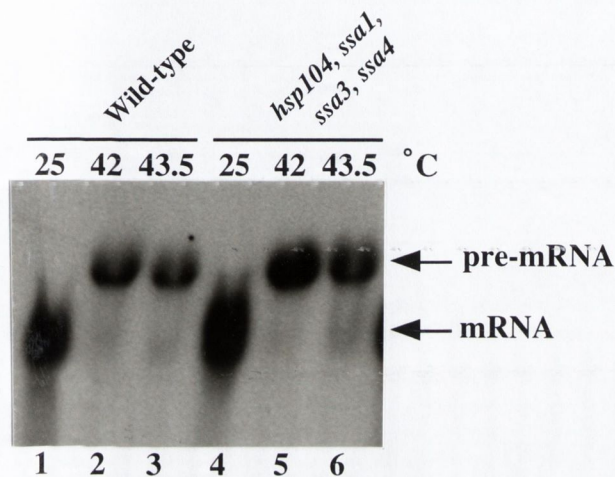
3. 2. 2 Splicing Inactivation is Correlated with Alterations in U4 and U6 Containing snRNPs on Glycerol Gradients.

Having established conditions for the inactivation of mRNA splicing, we next examined the state of the snRNPs to determine if these are altered as a result of the heat shock. Yeast splicing extracts prepared from the wild type and SL325-1B (*hsp104, ssa1, ssa3, ssa4*) cells, heat shocked at 42°C and 43.5°C to inactivate splicing, as described in section 3. 2. 2 (See Figure 3.2), were separated on 10-40% glycerol gradients and RNA isolated from each fraction. The fractions were probed for the presence of U4 and U6 snRNAs, to determine the position of the U4/U6.U5 tri-snRNP, U4/U6 di-snRNP, and free U6 snRNP species on the gradients (Figure 3.3; for wild type extracts; and Figure 3.4; for SL325-1B extracts). Under normal growing conditions in both strains (Figure 3.3A and Figure 3.4B), the U4/U6.U5 tri-snRNP sediments in fractions 20-30 and peaks in fraction 22. This was verified in both strains by hybridisation with a U5 probe (data not shown). The U4/U6 snRNP sediments in fractions 10-14 while the free U6 snRNP sediments in fractions 4-10.

In the wild type strain, following heat shock at 42°C, the level of the tri-snRNP decreases slightly but it's sedimentation position on the gradient remained unchanged (Compare Figure 3.3A and 3.3B, lanes 20-26). A more pronounced decrease in the level of the tri-snRNP is observed at 43.5°C but there appears to be no major alteration in sedimentation profile with the exception of a slight shift in the peak from fraction 22 to fraction 20 (Figure 3.3C). A distinct shift in the fractionation profile of the

U4 snRNA is evident in the region of the gradient corresponding to the U4/U6 snRNP in heat shocked cells (fractions 10-14). In cells heat shocked at 42°C, an upward shift in the U4 snRNA fractionating profile is observed (Figure 3.3B). This altered U4 snRNA fractionating profile is more pronounced at the higher temperature of 43.5°C (Figure 3.3C), suggesting a continuum of U4/U6 snRNPs in fractions 6-14. A major shift in the fractionation profile of the U6 snRNA is not observed following heat shock (Figure 3.3B and 3.3C). However, resolution at this point of the gradient is at a minimum and therefore slight alterations may not be detected.

In strain SL325-1B (*hsp104, ssa1, ssa3, ssa4*), alterations in the U4 and U6 snRNA containing snRNPs are more pronounced (Figure 3.4). Following heat shock at 42°C, the level of the tri-snRNP decreases dramatically at 42°C, but again it's sedimentation position on the gradient remained unchanged (Compare Figure 3.4A and 3.4B, lanes 20-26). An even more pronounced decrease in the level of the tri-snRNP is observed at 43.5°C but there still appears to be no major alteration in sedimentation profile (Figure 3.4C). Again, a more dramatic shift in the fractionation profile of the U4 snRNA is evident at 42°C (Figure 3.4B), and is even more pronounced at the higher temperature of 43.5°C (Figure 3.4C), suggesting a continuum of U4/U6 snRNPs in fractions 6-14. Again, no major shift in the fractionation profile of the U6 snRNA is observed following heat shock (Figure 3.4B and 3.4C). However, this is likely due to co-

sedimentation of altered U4/U6 containing species in heat shocked extracts.

A 25°C Sed →
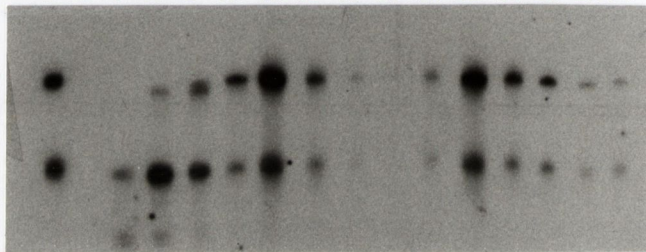
T 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
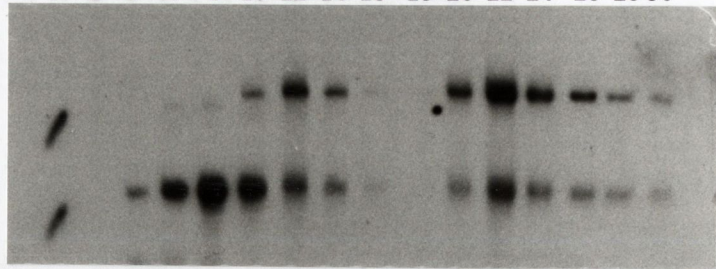
← U4

← U6

Free U6   U4/U6   U4/U6.U5

B 42°C Sed →

T 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
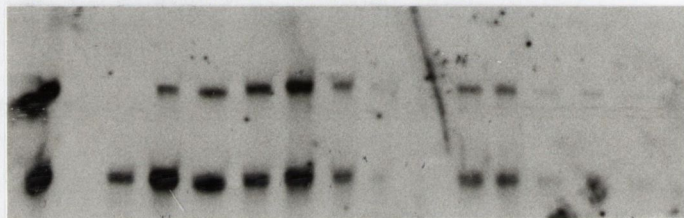
← U4

← U6

C 43.5°C Sed →

T 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

← U4

← U6

60

Figure 3. 3   Glycerol Gradient Analysis of U4 and U6 Containing snRNPs in Heat Shocked Wild Type Cells.

Wild type yeast cells were grown at normal temperatures, 25°C, (A) or heat shocked for one hour at 42°C (B), or 43.5°C (C). Total cell extracts were prepared and were sedimented through 10-40% glycerol gradients as described in the Materials and Methods section. RNA was extracted from alternate fractions, separated on denaturing polyacrylamide/urea gels and transferred to nylon membranes. The membranes were probed with Digoxygenin-UTP labelled DNA probes specific to U4 and U6 snRNAs. The bands corresponding to U4 and U6 snRNAs are indicated by arrows and the positions of the free U6 snRNP, the U4/U6 snRNP and the U4/U6.U5 snRNP are shown in panel A. T: total RNA extracted from wild type cells grown at 25°C.

Figure 3. 4   Glycerol Gradient Analysis of U4 and U6 Containing snRNPs in Heat Shocked SL325-1B Cells.

SL325-1B cells (*hsp104, ssa1, ssa3, ssa4*) were grown at normal temperatures, 25°C, (A) or heat shocked for one hour at 42°C (B), or 43.5°C (C). Total cell extracts were prepared and were sedimented through 10-40% glycerol gradients as described in the Materials and Methods section. RNA was extracted from alternate fractions, separated on denaturing polyacrylamide/urea gels and transferred to nylon membranes. The membranes were probed with Digoxygenin-UTP labelled DNA probes specific to U4 and U6 snRNAs. The bands corresponding to U4 and U6 snRNAs are indicated by arrows and the positions of the free U6 snRNP, the U4/U6 snRNP and the U4/U6.U5 snRNP are shown in panel A. T: total RNA extracted from wild type cells grown at 25°C.

3. 2. 3 Confirmation of Glycerol Gradient Analysis by Native Gel Electrophoresis.

To confirm the results of the glycerol gradient analysis and to more clearly determine the alterations to U4 and U6 containing snRNPs in heat shocked cells, the same cell extracts from section 3. 2. 2 were electrophoresed on a non-denaturing polyacrylamide gels which allow the separation of protein-RNA complexes (Raghunathan and Guthrie, 1998; Cheng and Abelson, 1987). Following transfer to nylon membranes, blots were hybridised with a U4 specific DNA probe (Figure 3.5). The tri-snRNP migrates as a single complex as judged by hybridisation to U4, U5 and U6 probes (data not shown). The level of this complex decreases with increasing temperature in heat shocked wild type cells (Figure 3.5; Compare lane 1 with lanes 2 and 3). Consistent with the gradient analysis, this decrease in level is more dramatic with increasing temperature in strain SL325-1B (Figure 3.5; Compare lane 4 with lanes 5 and 6). Significantly however, no alteration in tri-snRNP mobility is observed on these gels, again confirming the results obtained from the glycerol gradient analysis.

Figure 3.5 also confirms that the U4/U6 snRNP appears to be altered. Two complexes (designated as U4/U6) hybridise to both U4 and U6 probes in untreated cells (Figure 3.5, lane 1). Following a heat shock at 42°C, both species are reduced and altered, faster migrating form(s) appear (Figure 3.5, lanes 2, 3, 5 and 6). This is consistent with the altered sedimentation profile observed on glycerol gradients.

When the same samples are probed with a U6 specific probe, both the reduction in the level of the tri-snRNP and the alteration of the U4/U6 snRNP species are confirmed (data not shown). Additionally, the U6 probe detects two forms of free U6 snRNP (See inset Figure 3.5). The levels of this snRNP are drastically reduced following heat shock. This reduction was not apparent from the glycerol gradient analysis due to the co-sedimentation of altered U4/U6 species.

A

Wild-type   hsp104, ssa1,
             ssa3, ssa4
25 42 43.5   25 42 43.5   °C

← U4/U6.U5

U4/U6

1   2   3   4   5   6

B

1   2   3   4   5   6

66
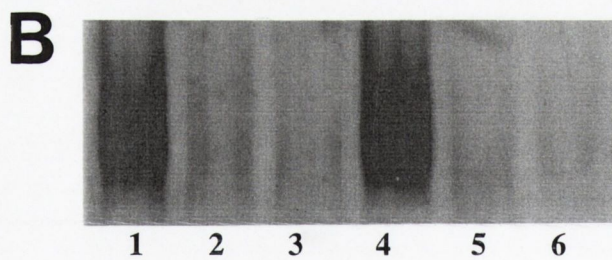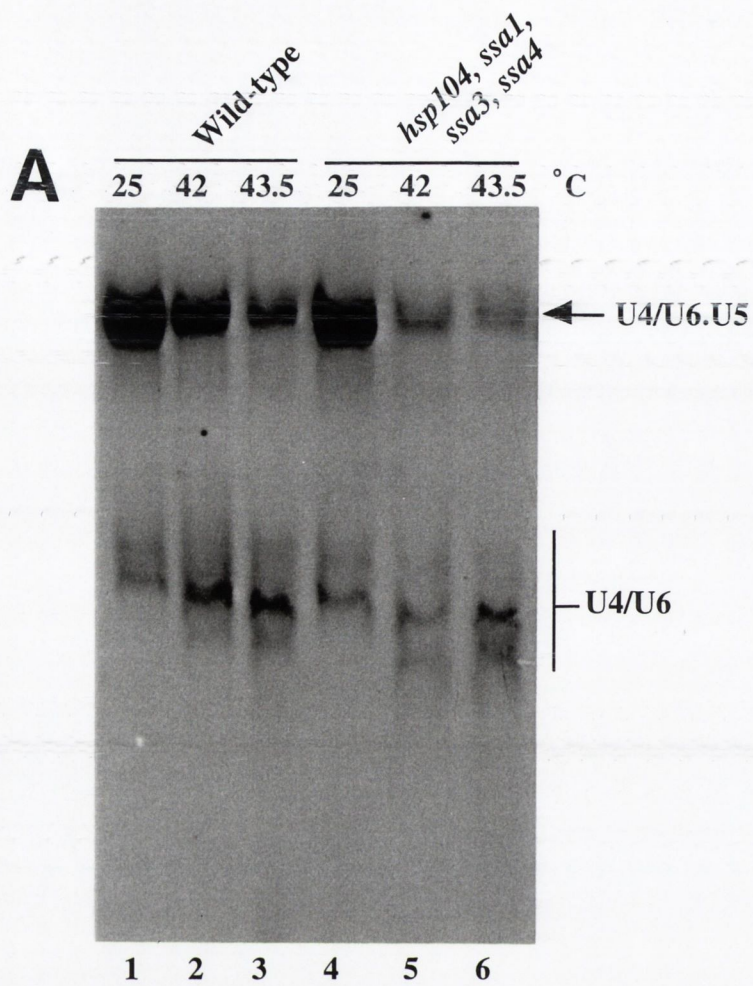
Figure 3. 5   Native Gel Analysis of U4 and U6 Containing snRNPs From Heat Shocked Yeast Cells.

The same extracts as shown in Figures 3.3 and 3.4 were separated on a non-denaturing polyacrylamide gel as described in the Materials and Methods section and transferred to nylon membranes. (A) Effects of heat shock on U4 containing snRNP species. The samples were probed with a Digoxygenin-UTP labelled U4 specific DNA probe. This probe detects snRNPs containing the U4 snRNA. The position of the U4/U6.U5 snRNP is indicated by an arrow and the U4/U6 snRNPs are indicated by a line. (B) Effects of heat shock on the free U6 snRNP species. The same extracts probed with a U6 specific probe. The section of the gel corresponding to the two forms of free U6 snRNP is shown. Lane 1; extracts from cells grown at 25°C. Lane 2; extracts from cells heat shocked at 42°C for one hour. Lane 3; extracts from cells heat shocked at 43.5°C for one hour.

3. 2. 4 Native Analysis Also Correlates Splicing Inactivation with Alterations in all the Other Essential snRNPs (U1, U2 and U5).

It was thought at this point that the slow heating regime adopted in previous experiments likely allows a significant accumulation of Heat Shock Proteins and/or other protective factors, such as trehalose, which could act to ameliorate the effects of the heat shock on the snRNPs. Therefore, it was decided to improve the heat shocking regime, such that the heating of cultures from 25°C to 42°C would be almost instantaneous. To do this, cells were collected by centrifugation and subsequently resuspended in pre-warmed supernatant media for heat shocking (See Materials and Methods).

The snRNP profiles in cells following a heat shock were analysed by native gel electrophoresis as described above. Wild type yeast splicing extracts, prepared from heat shocked and non-heat shocked cells, were electrophoresed on non-denaturing polyacrylamide gels and Northern hybridised with U4, U5 and U6 specific DNA probes (Figure 3.6A). As described in section 3. 2. 3, the tri-snRNP migrates as a single complex as judged by hybridisation to all three probes (Figure 3.6A, lanes 2, 5 and 8). A much greater decrease in the level of this complex is observed in wild type cells heat shocked by the more instantaneous heat shocking regime (Compare Figure 3.6A, lanes 3, 6 and 9 with Figure 3.4, lane 2). Significantly however, as described above, no alteration in its mobility is observed (Figure 3.6A, lanes 3, 6 and 9). Quantitation of levels of the tri-snRNP from a number of separate experiments indicates that on average,

levels decreased to 5-15% of the normal level after an instantaneous heat shock at 42°C.

Figure 3.5A very clearly shows that the U4/U6 snRNP is altered upon heat shock. Two complexes (designated as U4/U6) hybridise to both U4 and U6 specific probes in untreated cells (Figure 3.6A, lanes 2 and 8). Following a heat shock at 42°C, both species are reduced although the slower migrating species appears to be most affected. Two faster migrating species, which hybridise to the U4 specific probe, are also evident in the heat shocked samples (Figure 3.6A, lane 3). Lower levels of these two species are also evident in the untreated samples (Figure 3.6A, lane 2) suggesting that these species are U4 but not U6 snRNA containing precursors of U4/U6 and/or the tri-snRNP (See arrowheads).

The U6 probe detects two forms of free U6 snRNP in addition to the U4/U6 and the tri-snRNP complexes (Figure 3.6A, lanes 8). The level of this snRNP is significantly reduced following heat shock (Figure 3.6A, lane 9). It has recently been shown that the U6 snRNA firstly associates with the yeast La protein before assembly into the U6 snRNP (Pannone, et al., 1998). This complex, designated Lhp1p/U6 RNP, is also detected on this gel system and is reduced following a heat shock (Figure 3.6A, lane 9).

A number of U5 snRNA-containing RNP complexes are also resolved in this gel system (Figure 3.6A, lanes 5). The predominant band corresponds to the tri-snRNP as judged by its hybridisation to U4 and U6 probes. The exact composition of the previously unreported, multiple U5-hybridising complexes is not presently known. However, it appears that

the U5-snRNP profile is only very moderately altered, following heat shock (Figure 3.6A, lane 6).

The effects of heat shock on the other major snRNPs, namely U1 and U2, were also investigated. At least two distinct U1-containing species are observed in cells under normal physiological conditions (Figure 3.6B, lane 2). Following a heat shock at 42°C, the levels of both U1-containing snRNPs are reduced, although the slower migrating species appears to be the most affected (Figure 3.6B, lane 3). We also observe at least two U2-containing species in cells grown at 25°C (Figure 3.6B, lane 5) and again there is a significant reduction in the level of the slower migrating species following a heat shock while the faster migrating species appears to be less affected (Figure 3.6B, lane 6).

A

U4

RNA 25 42

← U4/U6.U5

U4/U6

1   2   3

U5

RNA 25 42

← U4/U6.U5

U5 RNPs

4   5   6

U6

RNA 25 42

← U4/U6.U5

U4/U6

U6 snRNP

← Lhp1p/
U6 RNP

7   8   9

B

U1

RNA 25 42

1   2   3

U2

RNA 25 42

4   5   6
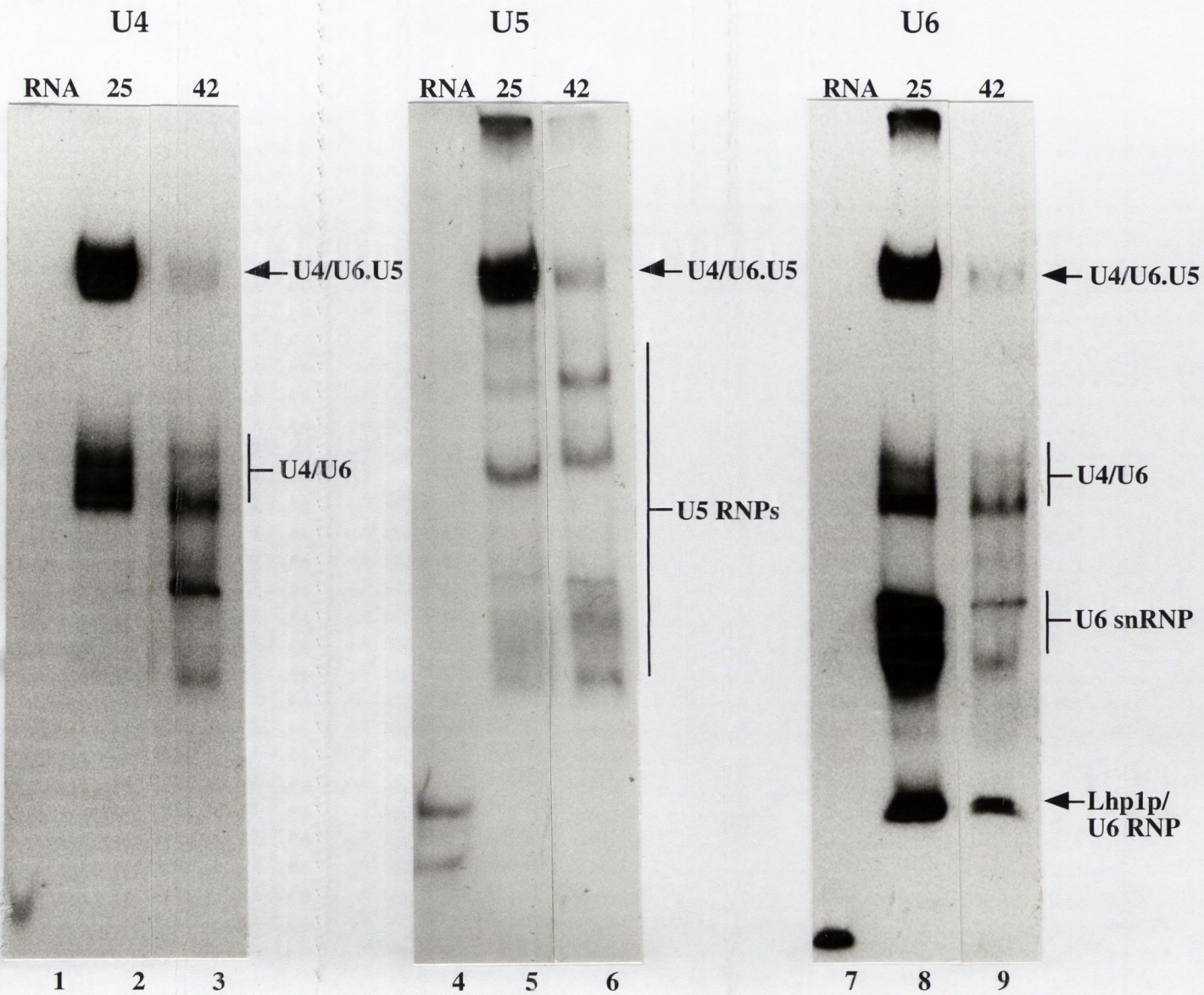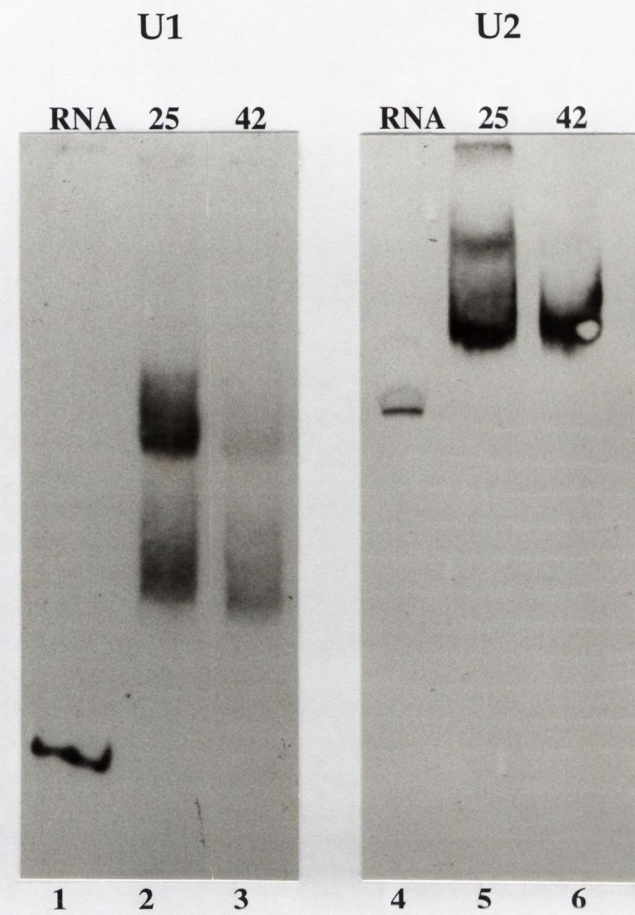
Figure 3. 6   Alterations in all snRNPs in Heat Shocked Yeast Cells.

Cell extracts were separated on non-denaturing polyacrylamide gels as described in the Materials and Methods section and transferred to nylon membranes. The membranes were probed with Digoxygenin-UTP labelled U1, U2, U4, U5 and U6 specific DNA probes as indicated. (A) Effects of heat shock on U4, U5 and U6 containing snRNPs. The position of the U4/U6.U5 snRNP and the Lh1p1/U6 RNP are indicated by arrows. The U6, U4/U6 and the U5 snRNPs are indicated by lines. Lanes 1, 4, 7; deproteinised RNA. Lanes 2, 5, 8; extracts from cells grown at 25°C. Lanes 3, 6, 9; extracts from cells heat shocked at 42°C for one hour. (B) Effects of heat shock on the U1 and U2 containing snRNPs. Lanes 1 and 4, deproteinised RNA. Lanes 2 and 5; extracts from cells grown at 25°C. Lanes 3 and 6; extracts from cells heat shocked at 42°C for one hour.

3. 2. 5 Western Immunoblot Analysis of Native Blot with Anti-Prp8.

To confirm the Northern analysis of native gels and to investigate further the composition of the U5 snRNP species in both heat shocked and non-heat shocked cells, a native gel from section 3. 3. 3, was electroblotted to Nitrocellulose membrane and immunoblotted with anti-Prp8 antibodies (A kind gift from Prof. Jean Beggs, University of Edinburgh). Prp8 is specifically associated with the U5 snRNP and with the U4/U6.U5 tri-snRNP species (Brown and Beggs, 1992, Dix, et al., 1998). Both of these species are observed in cells under normal physiological conditions (Figure 3.7, lane 1). It is interesting that the Prp8 antibody does not appear to react with any of the multiple, presumably pre-forms of U5 snRNP, observed on Northern blotted native gels in the previous section (Figure 3.6A, lane 5). However, consistent with the Northern native blot analysis, following a heat shock at 42°C the level of tri-snRNP is reduced (Figure 3.7; Compare lanes 1 and 2). Significantly, the Prp8 containing, presumably mature U5 snRNP species appears to be unaffected by the heat treatment.
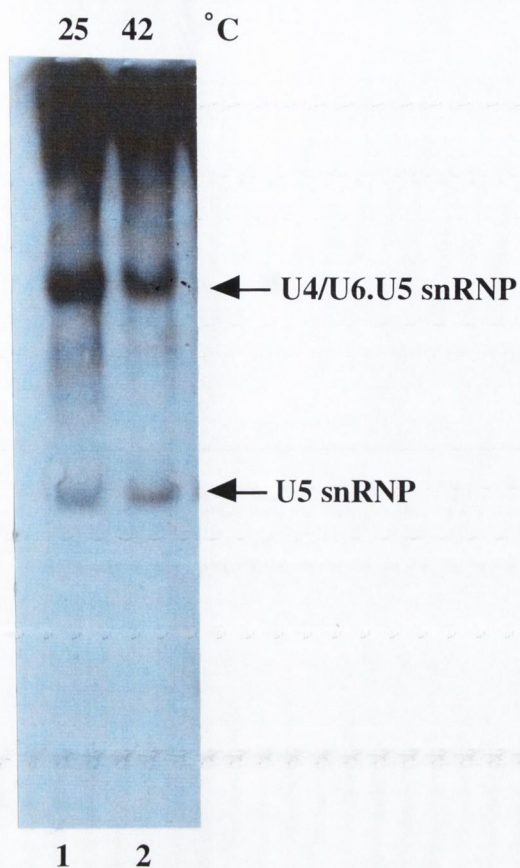
Figure 3. 7   Anti-Prp8 Western Immunoblot Analysis of Heat Shocked and Non-Heat Shocked Extracts on Native gels.

Extracts from heat shocked and non-heat shocked cells were separated on non-denaturing polyacrylamide gels as described in the Materials and Methods section and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-Prp8 antibody. The positions of the U4/U6.U5 snRNP and the U5 snRNP are indicated by arrows. Lanes 1; extract from cells grown at 25°C. Lane 2; extract from cells heat shocked at 42°C for one hour.

3. 2. 6 Splicing Inactivation is Also Correlated with Reduction in snRNA levels

It was next decided to investigate if snRNA levels are altered in heat shocked cells. RNA was extracted from splicing extracts and Northern blotted with snRNA specific probes (Figure 3.8). The levels of U1 and U2 snRNAs are unaffected by the heat shock treatment (Figure 3.8). However, the levels of both the U5S and U5L species appear to be slightly reduced upon heat shock, while the levels of both the U4 and U6 snRNA species appear to be reduced to a greater extent.
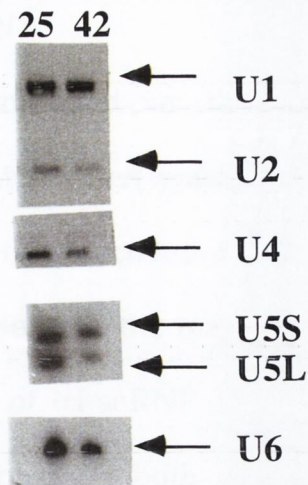
Figure 3. 8 Northern blot analysis of snRNA Levels in Heat Shocked Cells.

RNA extracted from splicing extracts prepared from heat shocked and non-heat shocked wild type yeast cells was separated on denaturing polyacrylamide/urea gels and transferred to nylon membranes. The membranes were probed with Digoxygenin-UTP labelled DNA probes specific U1, U2, U4, U5 and U6 snRNAs. Each snRNA is labelled by an arrow.

3. 3 DISCUSSION.

The data presented in this chapter confirm that splicing is completely inactivated when yeast cells are exposed to high temperatures. More significantly, this inactivation is correlated with both qualitative and quantitative alterations in the major snRNP species involved in mRNA splicing. The level of tri-snRNP is reduced significantly in heat shocked yeast cells, as observed on both glycerol gradients and native gels. The greater reduction in tri-snRNP levels observed by instantaneous as opposed to gradual heat shocking is most likely due to a degree of induced splicing thermotolerance in the latter heating regime. This phenomenon is associated with the induction of Hsps and other factors and is investigated further in Chapter 5.

Despite the reduction in tri-snRNP levels in heat shocked cells, there appears to be little if any structural alteration, as judged by both its identical sedimentation profile on glycerol gradients and electrophoretic mobility on non-denaturing gels. Significantly however, it is not clear whether the remaining tri-snRNP is in a functional state. This result differs from what has previously been shown in HeLa cells where a complete disruption of the tri-snRNP is observed in heat shocked cells (Bond, 1988, Shukla, et al., 1990, Utans, et al., 1992). This tri-snRNP protection in *S. cerevisiae* cells may be due to the presence of Hsp70 in yeast cells which may afford some degree of protection. In HeLa cells on the other hand, there is little if any Hsp70 at non heat shock temperatures. Evidence for a protective effect of Hsp70 can be seen in this chapter where

tri-snRNP levels are reduced to a greater extent in the absence of three of the four Ssa proteins (Figure 3.4, compare lanes 2 and 3 with lanes 4 and 5).

The levels of the free U6 snRNP and the Lhp1p/U6 pre-snRNP, are also drastically reduced following a heat shock at 42°C. Furthermore, alterations in the U1, U2, and U4/U6 snRNPs are also observed. Interestingly, at least two distinct forms of each of these latter snRNPs are observed under normal physiological conditions (Figure 3.6A, lanes 2 and 8 and Figure 3.6B, lanes 2 and 5). It is possible that these represent mature and pre-forms of each snRNP. In any case, the slower migrating species of each snRNP is observed to be reduced in the heat shocked cells (Figure 3.6A, lanes 3 and 9 and Figure 3.6B, lanes 3 and 6). This result is very similar to observations by Bond in heat shocked HeLa cells, where she observed a reduction in the levels of U2 snRNP coupled with an accumulation of a smaller or faster migrating form (Bond, 1988).

Multiple forms of U5 snRNA hybridising RNP species are also observed under normal physiological conditions (Figure 3.6A, lane 5). The identity of these, previously unreported, species is not at all clear at present. It is possible that they represent pre-forms of the U5 or U4/U6.U5 snRNPs or both, or alternatively represent breakdown products. The latter is less likely as similar multiple forms are not observed for other snRNPs. At least two forms of U5 snRNA, U5L and U5S are found in yeast (Chanfreau, et al., 1997, Patterson and Guthrie, 1987) (Figure 3.6A, lane 4) and maybe the various U5 hybridising species contain one or the other of these species. However, both the U5L and U5S species co-fractionate

77

together in a broad band on glycerol gradients and therefore it is not possible to distinguish if the minor U5 RNPs have different U5S or U5L incorporation (Brown and Beggs, 1992 and data not shown). Despite this, it is clear that only one species other than the tri-snRNP is observed to cross-react with anti-Prp8 antibodies on immunoblot analysis (Figure 3.7, lane 1). This raises the very interesting possibility that the U5 snRNP is assembled in a multistep process with the Prp8 protein being added at the last step. Significantly, the Prp8 containing U5 species, presumably mature U5 snRNP, does not appear to be affected by heat shock (Figure 3.7, lane 2).

Additional snRNP species are also uncovered following the heat shock treatment. Two additional faster migrating U4-hybridising snRNP species accumulate in heat shocked cells (Figure 3.6A, lane 3, see arrowheads). These species are also observed in untreated cells, but in much lower amounts which suggests that they are precursors of the U4/U6 and/or the tri-snRNP (Figure 3.6A, lane 2, see arrowheads). One possibility is that one or both of these bands represents the core U4 RNP, i.e. a U4 snRNA with the Sm proteins and no snRNP specific proteins assembled on yet. Protein import into the nucleus is blocked during heat shock (Liu, et al., 1996). It could be possible that precursor or core snRNPs are blocked from nuclear import and thus accumulate in the cytoplasm. This would ultimately mean that heat shock treatments would cause a block the assembly of snRNPs in the nucleus, consistent with the data in this chapter. Alternatively, heat shock may disassemble snRNPs into *bona fide* precursors species. Pulse chase experiments will be required to

differentiate between these two possibilities.

These results demonstrate that the majority of snRNPs are sensitive to exposure of cells to higher temperatures and alterations in each of the snRNPs may contribute to the deactivation of mRNA splicing. In general, while snRNP profiles are altered, the levels of snRNAs remain relatively constant following a heat shock, with the exception of the U4 and U6 snRNAs which decreases significantly in heat shocked cells (Figure 3.8). It has previously been reported that in a number of temperature sensitive mutants of snRNP proteins, for example *prp-4, prp-6*, *lsm-4* and *lsm-8*, under non permissive conditions, the U6 snRNA is particularly unstable while the remainder of the snRNAs remain unaffected (Cooper, et al., 1995, Galisson and Legrain, 1993, Pannone, et al., 1998). Thus the effects of heat shock are similar to the phenotype observed in many snRNP protein mutants suggesting that specific snRNP proteins may be the targets of thermal stress.

As mentioned briefly in Chapter 1, previous studies have reported redistribution's of hnRNPs and alterations in hnRNP particles in heat shocked Drosophila cells (Buchenau, et al., 1997, de Graaf, et al., 1992, Mayrand and Pederson, 1983). In HeLa cells, redistribution of the hnRNP proteins, M and 2H9, from hnRNP particles to the nuclear matrix have been observed upon heat shock (Lutz, et al., 1988, Mähl, et al., 1989). Furthermore, antibodies to both these proteins, partially inactivate mRNA splicing and both proteins are absent from splicing extracts prepared from heat shocked cells (Gattoni, et al., 1996, Mahé, et al., 1997). Taken together,

these results indicate that the inactivation of mRNA splicing in heat shocked cells is multi-faceted but specifically involves alterations in RNP complexes.

Chapter 4:

Correlation of Splicing Reactivation with

Reassembly of snRNPs.

4. 1   INTRODUCTION.

In the previous chapter, several alterations in snRNP profiles were observed in heat shocked cells. One explanation for these alterations is that snRNPs are disassembled in heat shocked cells. If this is the case, snRNPs must have to be reassembled during recovery at normal temperatures for splicing activity to return. Therefore, in this chapter the recovery of mRNA splicing, in cells recovering at normal growing temperatures after heat shock, is monitored in parallel with an analysis of snRNP profiles.

Hsp104 and members of the Ssa subfamily of Hsp70 proteins have been shown to be vital for the recovery of mRNA splicing activity after heat shock (Vogel et al., 1995). Therefore, in addition to monitoring the state of snRNPs in wild type cells during recovery, several mutants lacking specific *HSP* genes were also analysed. These included the strain SL324-1B which lacks the genes coding for the three heat inducible Ssa proteins; *SSA1, SSA3,* and *SSA4* (Table 2.1). To maintain viability in this strain, the *SSA2* gene is required. The strain SL304A lacks the single copy *HSP104* gene while the strain SL325-1B lacks *SSA1, SSA3, SSA4* and *HSP104* (Table 2.1).

4. 2   RESULTS.

4. 2. 1 Reactivation of mRNA Splicing During Recovery After Heat Shock Requires Both Hsp70 and Hsp104.

The recovery of actin mRNA splicing after heat shock was monitored for wild type and mutant strains lacking Hsp104 and the heat inducible Ssa members of the Hsp70 family (Figure 4.1). Wild type (SL303-1A) and mutant strains were heat shocked at 42°C for one hour and immediately returned to 25°C to recover. Aliquots of cells were taken at intervals following return of cells to the normal growing temperatures. Analysis of actin mRNA splicing recovery was performed for all four strains (Figure 4.1).

In the wild type strain (SL303-1A), mRNA splicing is completely inactivated by a heat shock at 42°C but recovers rapidly during recovery at 25°C, with levels of mRNA returning to normal within 60 min (Figure 4.1, lanes 1-5). Strain SL324-1B (*ssa1, ssa3, ssa4*) shows a slower rate of recovery of splicing with mRNA levels not returning to normal until 90 min (Figure 4.1, lanes 6-10). In the *hsp104* mutant, SL304A, recovery of mRNA splicing is considerably slower (Figure 4.1, lanes 11-15), as has previously been reported (Vogel, et al., 1995, Yost and Lindquist, 1991). Previous workers had not reported the rate of splicing recovery in strain SL325-1B, (*hsp104, ssa1, ssa3, ssa4*). It is clear from this work that this strain is the most affected of the strains examined with minimal recovery of splicing activity (Fig 4.1, lanes 16-20). Pre- mRNA levels accumulate in this

latter strain suggesting that transcription but not mRNA splicing was restored during the recovery period. This confirms the previous work and shows that the effect is specific to splicing and not a general effect.

To determine the levels of Hsp104 in cells, before and after heat shock and during recovery at 25°C, western immunoblot analysis of yeast extracts was performed with anti-Hsp104 antibody (A kind gift from Prof. Susan Lindquist, University of Chicago) (See Figure 4.1B). From this, it can be seen that Hsp104 is present in very low amounts at 25°C in both wild type and strain SL324-1B (Figure 4.1B, lanes 1 and 16), but is present in a much greater amount immediately after heat shock and during subsequent recovery at 25°C (Lanes 2-5 and 17-20). As expected, no Hsp104 was detected in strains SL304A and SL325-1B, both of which are *hsp104* knockout mutants (Lanes 6-10 and 11-15).

A

Wild-type

| 25°C | 0' | 30' | 60' | 90' |

pre-mRNA →
mRNA →

1   2   3   4   5

ssa1, ssa3, ssa4

| 25°C | 0' | 30' | 60' | 90' |

6   7   8   9   10

hsp104

| 25°C | 0' | 30' | 60' | 90' |

11   12   13   14   15

hsp104, ssa1, ssa3, ssa4

| 25°C | 0' | 30' | 60' | 90' |

16   17   18   19   20

85

B

Wild-type

| 25°C | 0' | 30' | 60' | 90' |

Hsp104 →

1   2   3   4   5

ssa1, ssa3, ssa4

| 25°C | 0' | 30' | 60' | 90' |

6   7   8   9   10

hsp104

| 25°C | 0' | 30' | 60' | 90' |

11   12   13   14   15

hsp104, ssa1, ssa3, ssa4

| 25°C | 0' | 30' | 60' | 90' |

16   17   18   19   20
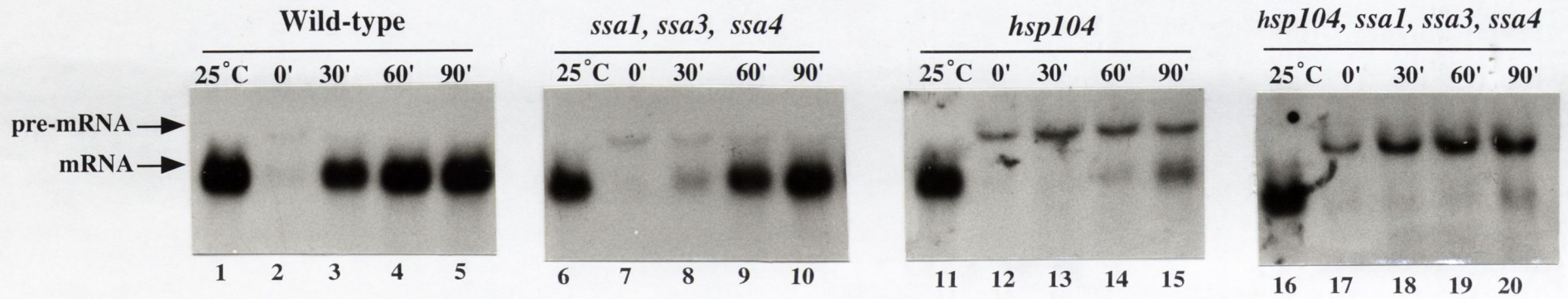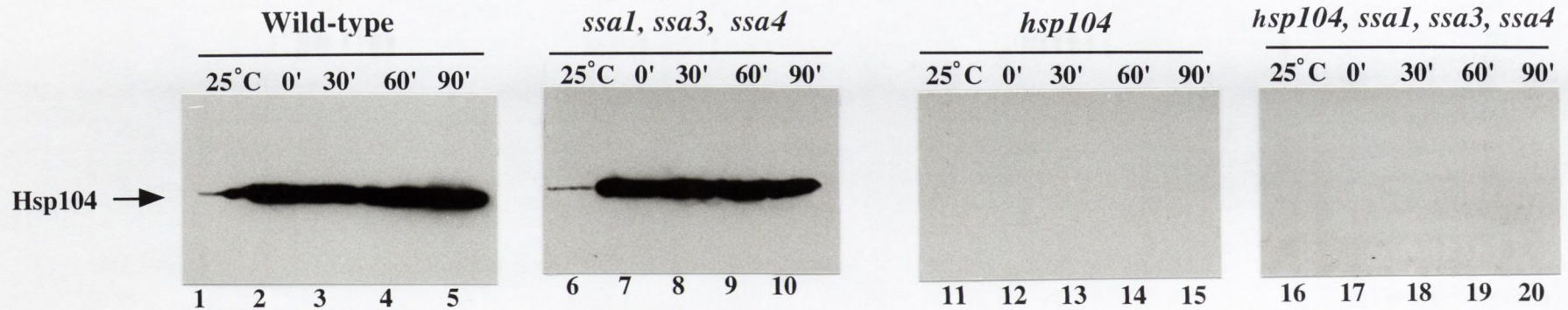
Figure 4. 1   Recovery of mRNA Splicing After Heat Shock Requires Both Hsp104 and Hsp70.

Wild type (SL303-1A) and mutant strains, SL324-1B (*ssa1, ssa3, ssa4*), SL304A (*hsp104*) and SL325-1B (*hsp104, ssa1, ssa3, ssa4*), were heat shocked for one hour at 42°C and then allowed to recover at 25°C. Cell extracts and RNA were prepared from cells prior to heat shock (25°C), immediately following the heat shock (0′) and at the time intervals indicated above the lanes. (A) Northern blots probed for actin RNA species. The positions of the pre-mRNA and mRNA are indicated by arrows. (B) Western Immunoblot analysis for Hsp104. Cell extracts were separated on SDS-PAGE gels as described in the Materials and Methods section and transferred to nitrocellulose membranes. The membranes were immunoblotted using an anti-Hsp104 antibody (A kind gift from Prof. Susan Lindquist, University of Chicago).

4. 2. 2 Reactivation of mRNA Splicing During Recovery Correlates with Hsp104 and Ssa Protein Dependant snRNP Reassembly

4.2.2.1 Hsp Dependent Reassembly of U4 containing snRNPs During Recovery After Heat Shock

The quantitative and qualitative restoration of U4 containing snRNPs correlates very well with the rate of recovery of mRNA splicing (Figure 4.2). In the wild type strain (SL303-1A), tri-snRNP levels return to normal within 30 min of recovery at 25°C (Figure 4.2, lanes 1-5). Like the recovery of splicing activity, the restoration of tri-snRNP levels is observed to be slower in the mutant strains. Little or no tri-snRNP restoration is observed in strain SL325-1B (*hsp104, ssa1, ssa3, ssa4*) reflecting the lack of mRNA splicing reactivation in the previous section (Figure 4.2, lanes 16-20). Tri-snRNP levels are restored slightly faster in strain SL324-1B (*ssa1, ssa3, ssa4*), (Figure 4.2, lanes 6-10), than in strain SL304A (*hsp104*), (Figure 4.2, Lanes 11-15), again reflecting the rate of mRNA splicing reactivation observed in the previous section for these strains (Figure 4.1).

Qualitative restoration of the U4/U6 snRNP to its normal profile is also evident during the recovery period. In the wild-type strain, the U4/U6 profile is returned to normal within 60 min of recovery (Figure 4.2, lane 4). In strain SL324-1B (*ssa1, ssa3, ssa4*), the faster migrating U4 hybridising snRNP species, previously observed in section 3. 2. 4 (Figure 3.5A, lane 3, see arrowhead), is present in a significantly greater amount immediately after the heat shock and is progressively lost as recovery proceeds (Figure

4.2, lanes 6-10, see arrowhead). Significantly, this species persists for up to 90 min into the recovery period in both strains SL304A (*hsp104*), (Figure 4.2, lanes 11-15), and SL325-1B (*hsp104, ssa1, ssa3, ssa4*), (Figure 4.2, lanes 16-20).

Wild-type | ssa1, ssa3, ssa4 | hsp104 | hsp104, ssa1, ssa3, ssa4

25°C 0' 30' 60' 90' | 25°C 0' 30' 60' 90' | 25°C 0' 30' 60' 90' | 25°C 0' 30' 60' 90'

U4/U6.U5→

U4/U6

1 2 3 4 5   6 7 8 9 10   11 12 13 14 15   16 17 18 19 20
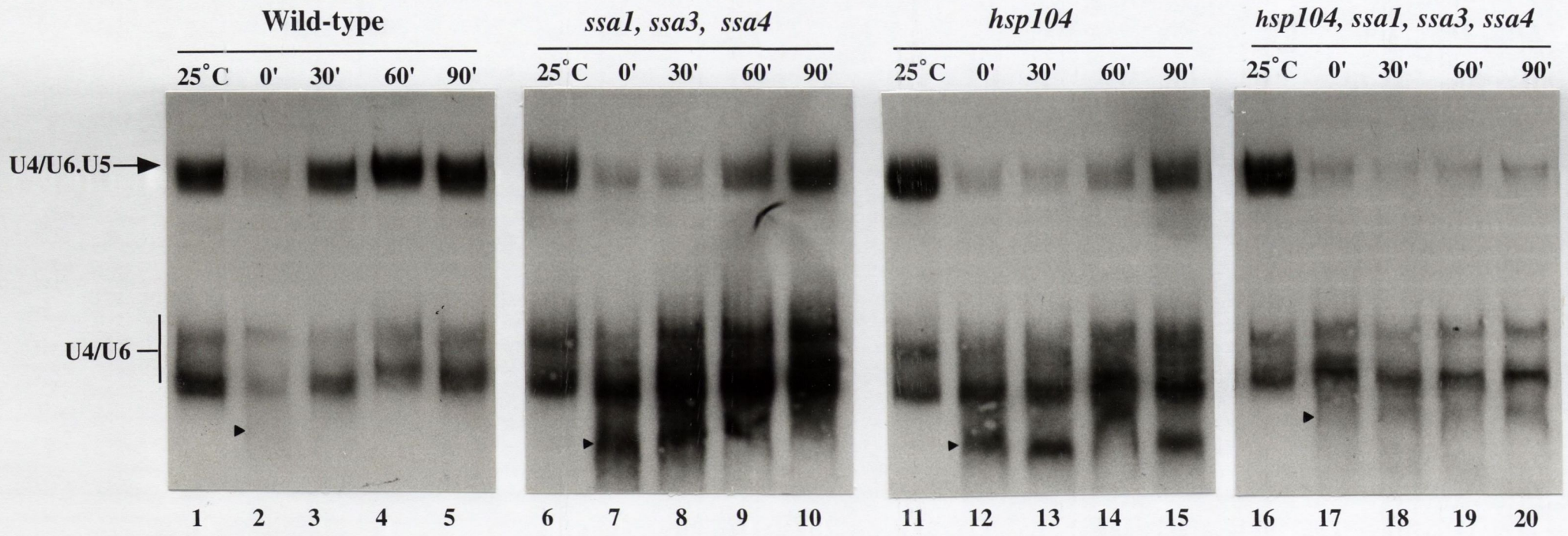
Figure 4. 2   Splicing   Recovery   After   Heat   Shock   Correlates   with Reassembly of U4 Containing snRNP by Hsps.

Wild type (SL303-1A) and mutant strains (SL304A, SL324-1B and SL325-1B) were heat shocked for one hour at 42°C and then allowed to recover at 25°C. Cell extracts were prepared before heat shock (25°C), immediately following the heat shock (0′) and during recovery at 25°C at 30 min time intervals as indicated above the lanes. Cell extracts were electrophoresed on non-denaturing gels and transferred to nylon membranes. The membranes were probed with a U4 specific DNA probe. The position of the U4/U6.U5 and the U4/U6 snRNPs are indicated. Arrowheads denote the faster migrating snRNP species which persists during recovering in the mutant strains.

4.2.2.2 *Hsp Dependent Reassembly of U6 containing snRNPs During Recovery After Heat Shock*

When native blots were prepared exactly as described in section 4.2.2.1, but probed instead with a U6 specific probe, the correlation of tri-snRNP restoration with splicing recovery in each strain is confirmed (Figure 4.3). Restoration of free U6 snRNP levels in the wild-type strain is also observed during the recovery after heat shock (Figure 4.3, lanes 1-5). This restoration is observed to be impeded in the mutant strains (Figure 4.3, lanes 6-20).

The U6 snRNA probe also detects the Lhp1p/U6, U6 snRNP precursor. The levels of this complex are reduced following a heat shock in all strains (Figure 4.3, lanes 2, 7, 12, and 17). Very significantly however, its levels are observed to accumulate during the recovery period in all the mutant strains (Figure 4.3, lanes 8-10, 13-15 and 18-20) but not in the wild-type strain (lanes 3-5).

	Wild-type					ssa1, ssa3, ssa4					hsp104					hsp104, ssa1, ssa3, ssa4				
	25°C	0'	30'	60'	90'	25°C	0'	30'	60'	90'	25°C	0'	30'	60'	90'	25°C	0'	30'	60'	90'

U4/U6.U5 →

U4/U6

U6 snRNP

Lhp1p/
U6 RNP →

1   2   3   4   5   6   7   8   9   10   11   12   13   14   15   16   17   18   19   20

Figure 4. 3   Splicing Recovery After Heat Shock Correlates with Reassembly of U6 Containing snRNP by Hsps.

Native blots were prepared exactly as described in Figure 4.2, but were instead probed with a U6 specific DNA probe. The positions of the U4/U6.U5, U4/U6, free U6 snRNPs and the Lh1p1/U6 RNP complex are indicated.

4.2.2.3 *Hsp Dependent Reassembly of U1 snRNPs During Recovery After Heat Shock*

Again, native blots were prepared exactly as described in section 4.2.2.1, but this time were probed with a U1 specific probe (Figure 4.4). A greater reduction in the level of both U1 snRNP species is observed immediately following heat shock in the mutant strains as compared to the wild type strain (Compare Figure 4.4, lanes 2 with lanes 7, 12 and 17). A greater reduction is also observed for the two principle U2 species in the mutant strains immediately following heat shock (Data not shown). However, restoration of both the normal U1 and U2 snRNP profiles is observed during the recovery period at 25°C. As with the other snRNPs, examined previously, the restorations of both U1 and U2 snRNPs were fastest in the wild type strain and slowest in strains lacking Hsp104. Thus, it appears that both Hsp104 and the Ssa proteins are essential after heat shock for both the quantitative and qualitative restoration of normal snRNP profiles. In addition, consistent with observations in Chapter 3, sections 3.2.2 - 3.2.3, it appears that one or both Hsps may actually function during the heat shock treatment to ameliorate, in this case, U1 snRNP alterations.

Wild-type | *ssa1, ssa3, ssa4* | *hsp104* | *hsp104, ssa1, ssa3, ssa4*

25°C 0' 30' 60' 90'

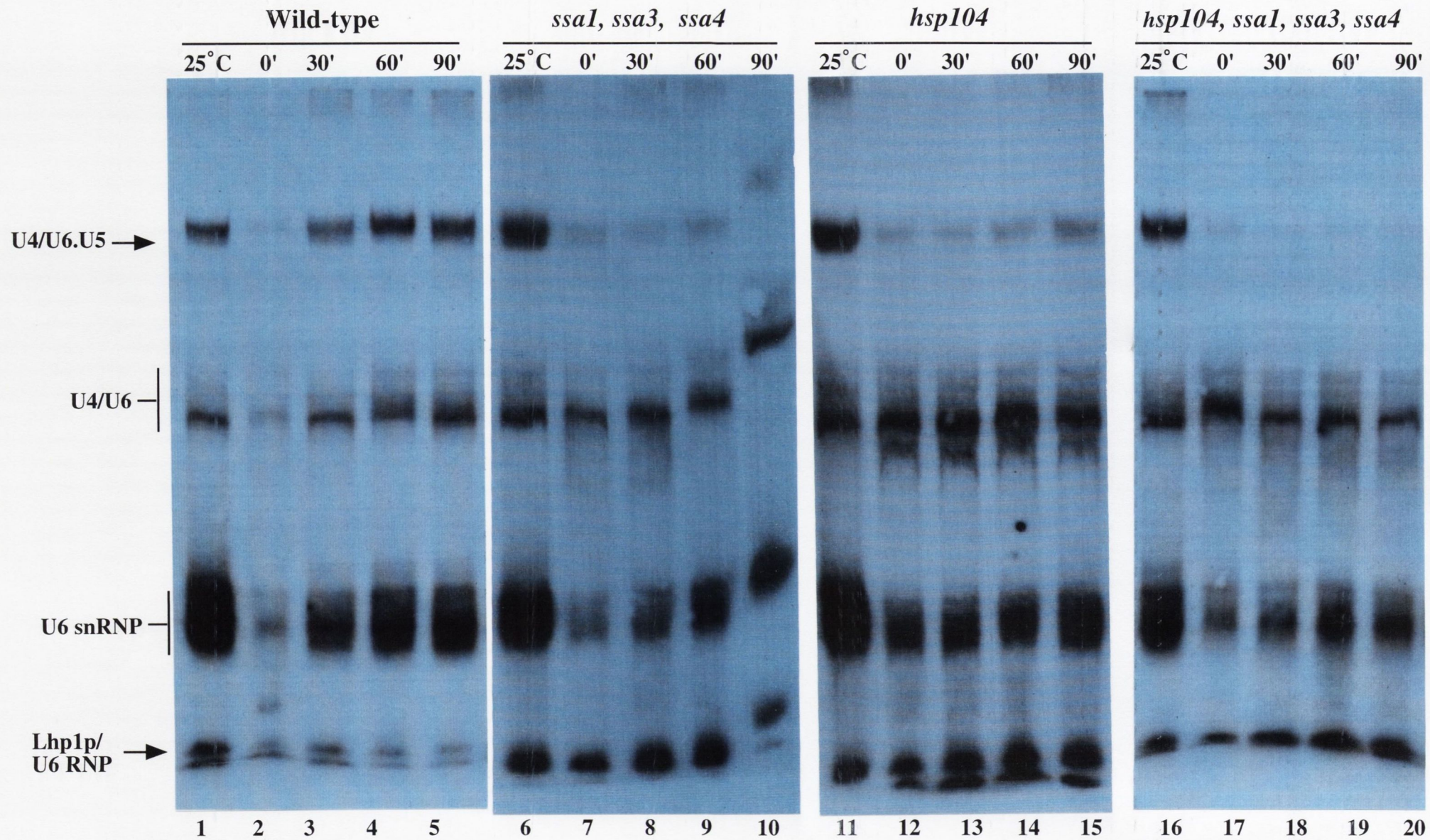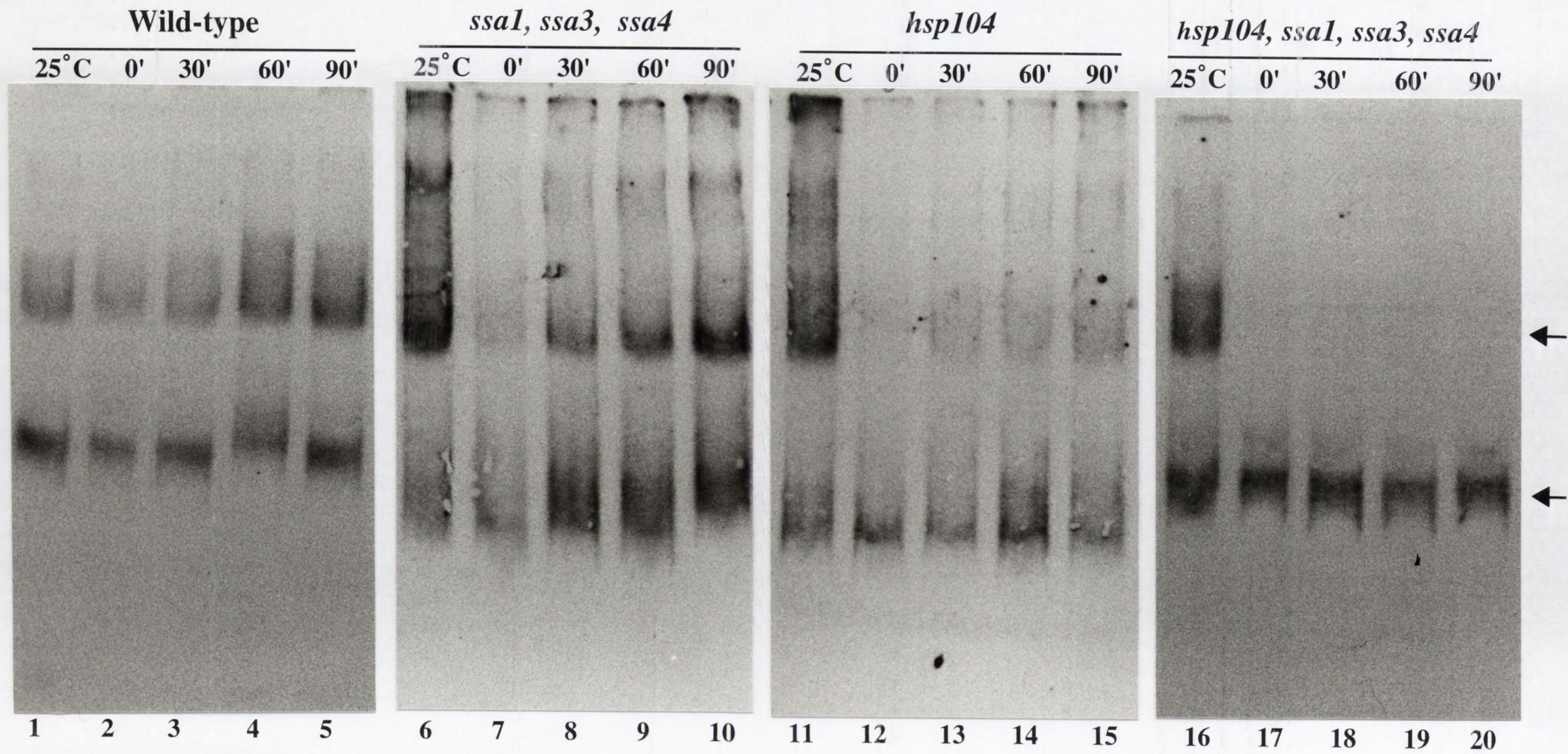1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

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Figure 4. 4   Splicing Recovery After Heat Shock Correlates with Reassembly of U1 Containing snRNP by Hsps.

Native blots were prepared exactly as described in Figure 4.2, but were instead probed with a U1 specific DNA probe. The positions of the two principle U1 hybridising species are indicated with arrows.

4.3   DISCUSSION.

A functional relationship between Hsp104 and Hsp70 has previously been demonstrated (Glover and Lindquist, 1998, Newnam, et al., 1999, Sanchez, et al., 1993). It is has been shown that Hsp70 can partially compensate for Hsp104 in the recovery from brief exposure to extreme temperatures (50°C), and Hsp104 can partially compensate for Hsp70 in growth at high temperatures (37°C) (Parsell and Lindquist, 1993, Sanchez, et al., 1993). At a molecular level, Lindquist and co-workers have demonstrated that both Hsp104 and Hsp70 contribute to the restoration of mRNA splicing activity in the cell after thermal deactivation, with Hsp104 playing the more prominent role (Vogel, et al., 1995). The data presented here expands on these earlier findings and points to the specific targets within the splicing apparatus with which Hsp70 and Hsp104 possibly interact, namely the snRNPs. These results demonstrate that the restoration of normal snRNP profiles after heat shock correlates precisely with the recovery of mRNA splicing and thus provide strong evidence that snRNP alterations likely contribute significantly to reversible splicing inactivation upon heat shock in *S. cerevisiae*.

A synergistic relationship exists between Hsp104 and the Ssa proteins, in both the restoration of normal snRNP profiles and in splicing reactivation. Hsp104 appears to play the more prominent role, as restoration of normal snRNP species is slower in a *HSP104* mutant than it is in the SL324-1B mutant (*ssa1, ssa3, ssa4*). This is consistent with the known inter-relationship between Hsp70 and Hsp104 (Glover and

Lindquist, 1998, Newnam, et al., 1999, Sanchez, et al., 1993, Vogel, et al., 1995). Furthermore, in the latter mutant, the Ssa2 protein while generally considered to be cytosolic, may relocate to the nucleus to aid in the restoration.

The data presented in this chapter also demonstrates that snRNP precursor forms can be reassembled into normal snRNP forms during the recovery period at 25°C. In addition, Hsps appear to be required for this process. This is most noticeable with the U6 snRNP precursor or the Lhp1p/U6 RNP (Figure 4.3). This complex accumulates during recovery at 25°C in all the *HSP* mutant strains (Figure 4.3, lanes 8-10, 13-15 and 18-20) but not in the wild type strain (Figure 4.3, lanes 3-5). This suggests that the Ssa proteins, in association with Hsp104, are involved directly or indirectly in the biogenesis of the U6 snRNP. In addition, the data points to the assembly of this snRNP as being a critical step in snRNP reassembly during recovery after heat shock. The lack of U6 snRNP assembly in the *HSP* mutants may affect the subsequent assembly of the U4/U6 snRNP and the tri-snRNP. In addition, the putative U4 precursor RNP, which is observed in heat shocked cells and found to be slow to clear during recovery in cells lacking Hsp104 and the Ssa proteins (Figure 4.2, arrowheads), may also be central to the lack of tri-snRNP accumulation in *HSP* mutants. Another point is that this U4 RNP does not clear in the *hsp* mutant strains. In the last chapter, I speculated that this RNP may represent a core U4 RNP which may accumulate in the cytoplasm in heat shocked cells. This and the fact that the U6 precursor also accumulates in these strains during

recovery after heat shock, leads to the likely explanation that U4/U6 and U4/U6.U5 snRNP assembly after heat shock may be due to these putative defects in U4 RNP biogenesis and import. Regardless, the blocks in snRNP assembly may consequently explain why the recovery of splicing activity is observed to be greatly hindered in the absence of Hsp104 and the Ssa proteins.

Chapter 5:

Protection of Mature snRNP Species by an Association of Ssa Proteins in Splicing Thermotolerant Cells.

5.1   INTRODUCTION.

In Chapter 3, when cells were heat shocked by the gradual heating regime, the resultant reduction in the tri-snRNP levels was observed to be greater in the strain SL325-1B (*hsp104, ssa1, ssa3, ssa4*) than in the wild type strain. This led to the hypothesis that Hsps, which are likely induced to high levels in the initial minutes of this heating regime in the wild type strain, actually protect snRNPs during heat shock. In this chapter, I have explored the induction of splicing thermotolerance in wild type and in *hsp* mutant backgrounds, to determine if splicing thermotolerance is correlated with snRNP protections and if so, are they Hsp dependant.

5. 2   RESULTS.

5. 2. 1 Correlation of Splicing Thermotolerance with snRNP Protection

The aim of this section was to determine if splicing thermotolerance is a direct consequence of the protection of snRNP integrities during heat shock in pre-treated cells. To do this, RNA and splicing extracts were prepared in parallel from cells incubated at 25°C, heat shocked at 42°C for one hour or pre-incubated at 37°C for 45 min before being immediately heat shocked at 42°C for one hour. Inactivation of mRNA splicing, as judged by the appearance of intron containing pre-mRNA species, can be seen after a heat shock at 42°C for one hour (Figure 5.1A, lane 3). As expected, no inactivation of splicing was observed when cells were heat shocked after a prior pre-treatment at 37°C. This is consistent with previous observations by Yost and Lindquist (Yost and Lindquist, 1991).

Native analysis of snRNPs was also performed by hybridising with U4, U5 and U6 specific probes (Figure 5.1B). Tri-snRNP levels are higher in cells heat shocked after a pre-treatment as compared to cells heat shocked without a prior pre-treatment (Figure 5.1B, compare lanes marked TT and 42). Thus, the reduction of tri-snRNP levels is ameliorated significantly if cells are pre-treated before heat shock at 42°C. Interestingly, both the U1 and U2 snRNPs are also protected from the effects of heat shock in thermotolerant cells (data not shown).

Very little alteration can be seen in the U5 snRNP profile in both

heat shocked thermotolerant and non-thermotolerant cells (Figure 5.1B, lanes 7 and 8). Significantly however, the levels of the U4/U6 snRNP, free U6 snRNP and the Lhp1p/U6 RNP complex are not observed to be protected in pre-treated cells (Figure 5.1B, compare lanes 3 and 4 and lanes 11 and 12). Thus, it appears that only the snRNPs that are actually required for the process of splicing, namely the U1, U2 and the U4/U6.U5 snRNPs, are protected in thermotolerant cells. Furthermore U4 and U6 snRNA levels, which were observed in chapter 3 to be reduced in heat shocked cells, are not reduced in pre-treated heat shocked cells (data not shown).

Very slight upshifts in the mobility of the U1, U2, U4/U6.U5, U4/U6 and U5 snRNP species can seen in splicing thermotolerant cells. This is investigated further, later in this chapter.
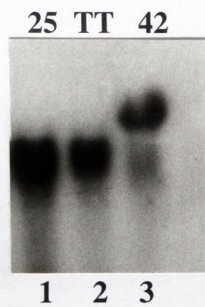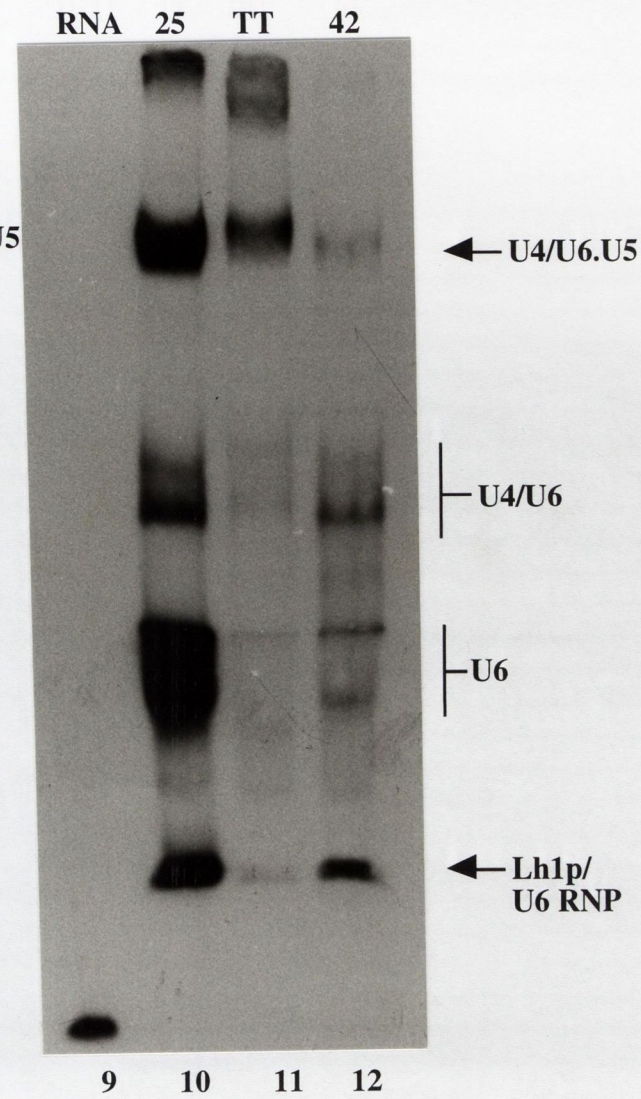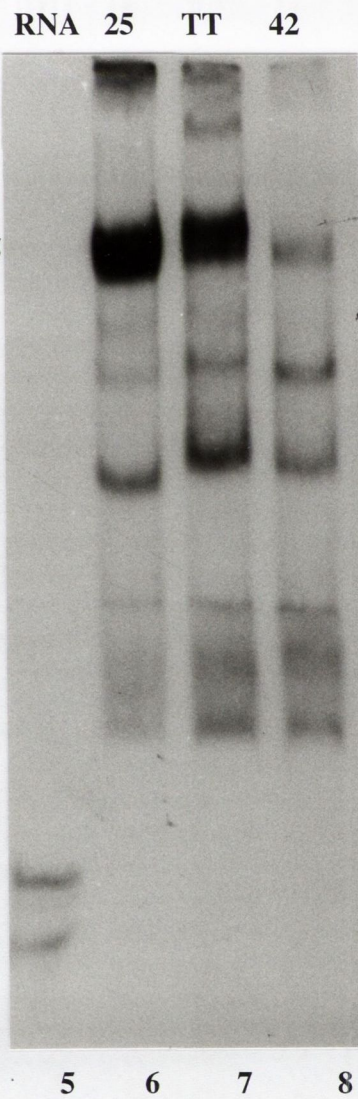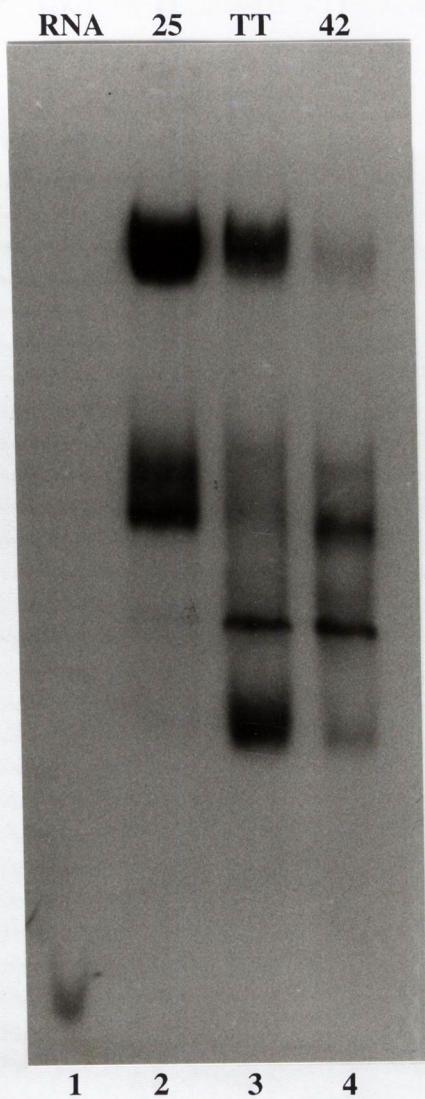
A

25 TT 42

1 2 3

B

RNA 25 TT 42

← U4/U6.U5

U4/U6

1 2 3 4

RNA 25 TT 42

← U4/U6.U5

U5 RNPs

5 6 7 8

RNA 25 TT 42

← U4/U6.U5

U4/U6

U6

← Lh1p/
U6 RNP

9 10 11 12

Figure 5. 1 Splicing Thermotolerance Correlates With Protection of Tri-snRNP Integrity

(A) Northern blot analysis of actin RNA. Lane 1; RNA from untreated cells (25°C). Lane 2; RNA from cells pre-treated at 37°C for 45 min and subsequently heat shocked at 42°C for one hour (TT). Lane 3; RNA from cells heat shocked at 42°C without a prior pre-treatment at 37°C. (B) Native blot analysis of U4, U5 and U6 containing snRNPs in splicing thermo-tolerant cells. Yeast splicing extracts were electrophoresed on non-denaturing polyacrylamide gels and transferred to nylon membranes. The membranes were probed with U4, U5 and U6 specific DNA probes. The position of the U4/U6.U5 snRNP and the Lh1p1/U6 RNP are indicated by arrows. The U6, U4/U6 and the U5 snRNPs are indicated by lines. Lanes 1, 5, 9; deproteinised RNA. Lanes 2, 6, 10; extracts from cells grown at 25°C. Lanes 3, 7, 9; extracts from cells pre-treated at 37°C for 45 min and subsequently heat shocked at 42°C for one hour (TT). Lanes 4, 8, 12; extracts from cells heat shocked at 42°C for one hour.

5 . 2 . 2   Splicing Thermotolerance is Independent of the Heat

Inducible forms of Hsp70 and Hsp104

To test the hypothesis that pre-existing Ssa proteins may be involved in conferring splicing thermotolerance, I investigated whether the mutant strain, JN519, which lacks functional genes coding for *SSA2*, *SSA3* and *SSA4* and which contains a temperature sensitive mutation in the *SSA1* gene, was capable of acquiring splicing thermotolerance. The temperature sensitive *SSA1-45BKD* allele contains a single point mutation changing P-417 to L (Becker, et al., 1996). The *URA* gene which was used for replacing the chromosomal copy of *SSA1* with the *SSA1-45BKD* gene was subsequently deleted resulting in strain JN519 (E. Craig, University of Wisconsin, personal communications). This strain is viable, but slow growing, at the permissive temperature of 25°C, while all four *SSA* gene products are absent when incubated at the non-permissive temperature of 37°C, the temperature normally used to induce thermotolerance. In addition, we examined the protection of mRNA splicing in strain SL304A (*hsp104*) and in strain JN516 (*ssa2, ssa3, ssa4*).

The wild type isogenic strain (JN55) and mutant strains were pre-incubated at 37°C for 45 min and then immediately challenged with a heat shock at 42°C for one hour as described in the previous section. Surprisingly, we find that mRNA splicing is protected from heat inactivation in pre-treated cells regardless of the mutant background, as judged by the lack of intron-containing pre-mRNAs (Figure 5.2, lanes 2, 5, 8, and 11). Splicing was also protected in the mutant strains,

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SL324-1B (*ssa1, ssa3, ssa4*) and SL325-1B (*ssa1, ssa3, ssa4, hsp104*), (data not shown). Significantly however, in strain JN519 we observe an approximately 80% reduction in spliced actin mRNA levels in pre-treated cells after the 42°C heat shock (Figure 5.2, compare lanes 4 and 5) as compared to a 50% reduction in wild type and other mutant strains (Figure 5.2, compare lanes 1 and 2; 7 and 8; 11 and 12)
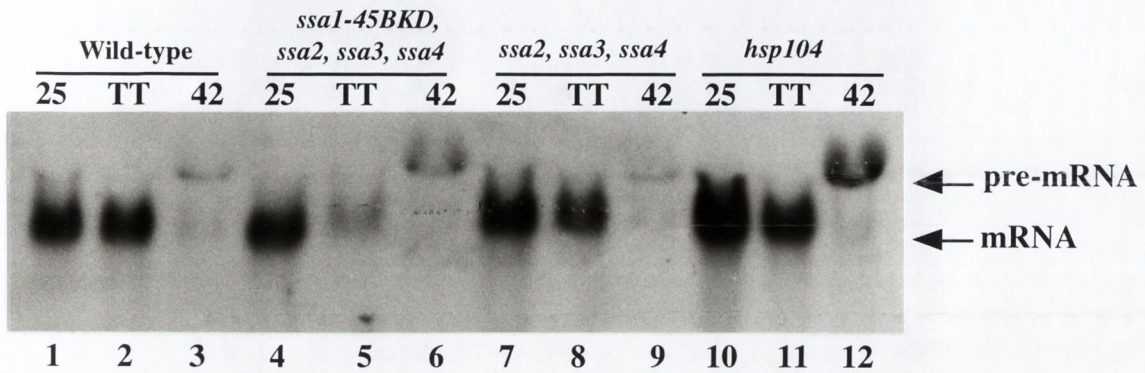
Figure 5. 2 'Splicing thermotolerance' is independent of all four Ssa proteins and Hsp104. Wild type and the mutant strains, indicated above the lanes, were subjected to a heat shock at 42°C for one hour without a prior pre-treatment at 37°C (42: Lanes 3, 6, 9, and 12) or with a prior pre-treatment at 37°C (TT: Lanes 2, 5, 8, 11). Samples from untreated cells (25°C) are shown in Lanes 1, 4, 7 and 10. RNA from these cells was separated on a denaturing agarose gel and probed with a Digoxygenin-UTP labelled actin probe. The position of the actin pre-mRNA and the mRNA are indicated by arrows.

5. 2. 3 Protection of snRNPs in Thermotolerant Cells Requires at Least One Member of the Ssa Protein Sub-Family.

Having established a correlation between splicing thermotolerance and the amelioration of the effects of heat shock on the U1, U2 and U4/U6.U5 snRNP species in wild type cells, it was next decided to determine if Hsps are required for this process. Surprisingly however, comparable protection of tri-snRNP levels was observed in pre-treated cells from the mutant strains SL304A (*hsp104*) and JN516 (*ssa2, ssa3, ssa4*) (Figure 5.3A, lanes 8 and 11) and strains SL325-1B (*ssa1, ssa3, ssa4, hsp104*) and SL324-1B (*ssa1, ssa3, ssa4*), (data not shown). However, in the strain JN519 (*ssa1-45BKD, ssa2, ssa3, ssa4*), the degree of protection of the tri-snRNP levels in heat shocked pre-treated cells is significantly less than that observed for the wild type or any of the other strains (Figure 5.3A, lanes 5). Furthermore, the amount of tri-snRNP at 25°C was markedly lower in this strain than that observed in the other strains (Figure 5.3A, compare lanes 4 with lanes 1, 7 and 10). Both of these observations in strain JN519 were reproducible.

The tri-snRNP and surprisingly the U4/U6 snRNP both exhibit upward shifts in mobility in pre-treated cells after a heat shock. This is most pronounced in the strain SL-304A (*hsp104*), (Figure 5.3A, lane 11). To emphasise the shift in this strain, cell extracts were electrophoresed for a longer time period (see Materials and Methods) (Figure 5.3B, lane 3). Under these electrophoretic conditions, the upward shifts in both U4/U6 snRNP and the tri-snRNP are more noticeable.

Amelioration of the effects of heat shock on the U1 snRNP profile is also observed in pre-treated cells (Figure 5.4). The levels of both the major U1 snRNP species, particularly the slower migrating one, are reduced upon heat shock in all strains (Figure 5.4, compare lanes 2 and 4, 5 and 7, 8 and 10). However, in pre-treated cells of all strains, the levels of both species are significantly less reduced upon heat shock (Lanes 3, 6 and 9). This protection seems to be independent of the Ssa proteins or Hsp104. Similarly, Hsp-independent protection was observed for the U2 snRNP in thermotolerant cells (Data not shown).

In addition to an amelioration of the effects of heat shock, upward shifts were also observed for the U1 (Figure 5.4) , U2 and U5 snRNPs (data not shown). These shifts do not occur if cells are immediately placed at the higher temperature of 42°C without the pre-treatment. Furthermore, as with the U4/U6 and tri-snRNP, upshifts were observed to a greater extent in strains lacking Hsp104. In addition, no upshift was observed in cells of the strain lacking all of the Ssa proteins (JN519). This led to the hypotheses that member(s) of the Ssa family, most likely Ssa1 and Ssa2, actually physically associate with certain snRNPs to confer protection. This is investigated in the next section.
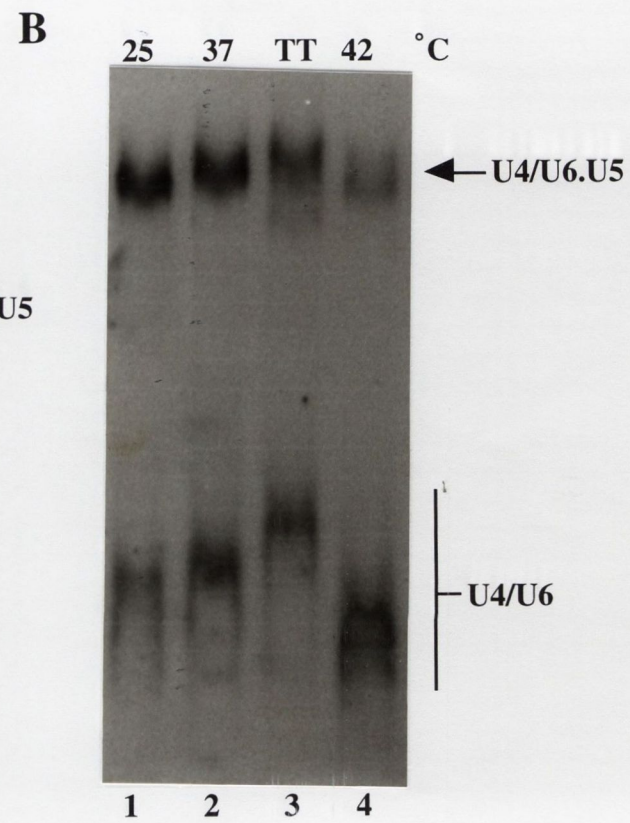
A

25 TT 42   25 TT 42   25 TT 42   25 TT 42

← U4/U6.U5

⌐ U4/U6

⌐ U6

1   2   3   4   5   6   7   8   9   10   11   12

B

25   37   TT   42   °C

← U4/U6.U5

⌐ U4/U6

1   2   3   4
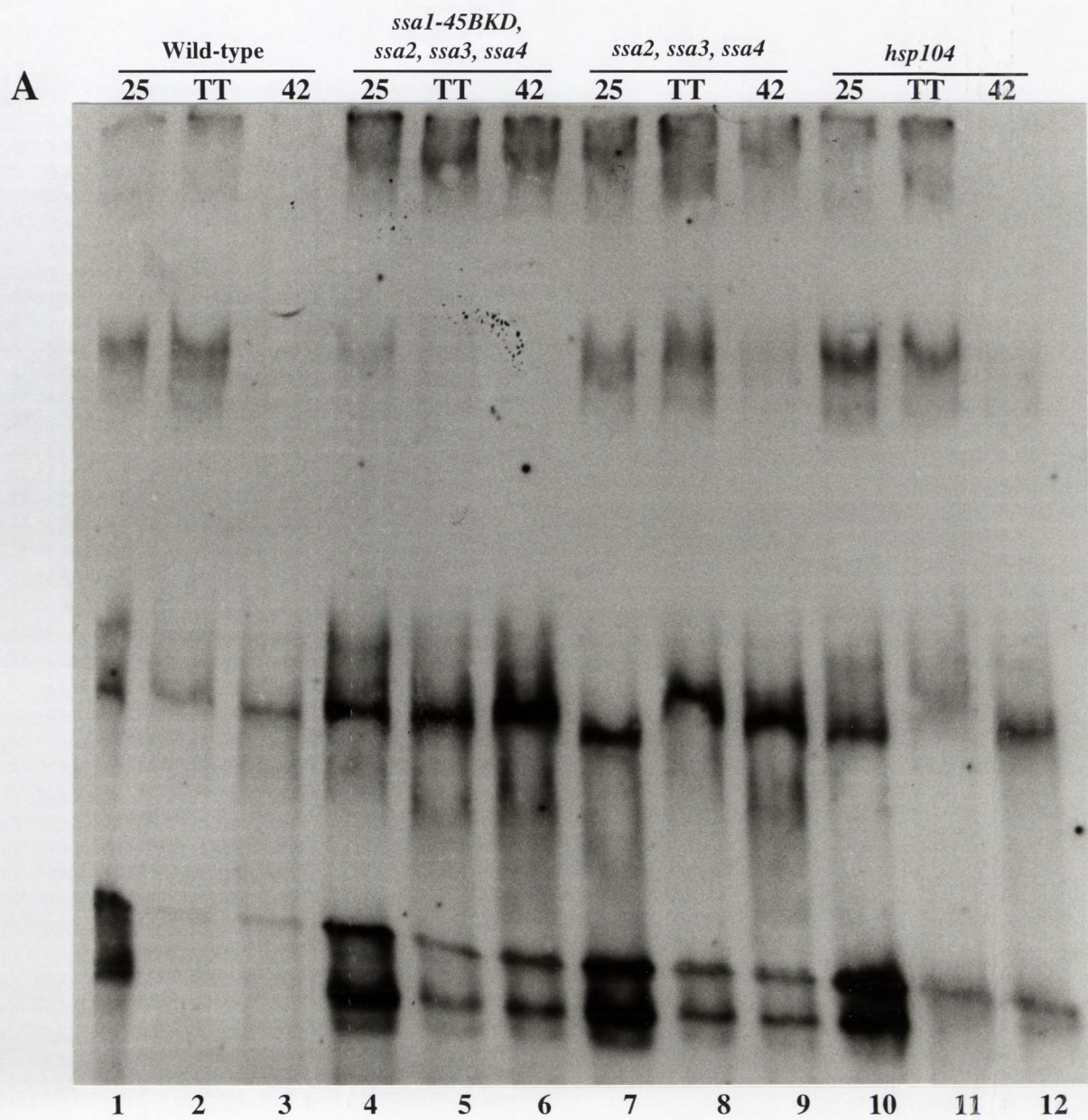
Figure 5. 3 Protection of Tri-snRNP Levels and 'Upshifts' are Observed in Splicing Thermotolerant Cells. (A) Cell extracts from wild type (JN55) and mutant strains, as indicated above the lanes, were electrophoresed on non-denaturing polyacrylamide gels and transferred to nylon membranes. The membranes were probed with a U6 specific DNA probe. The positions of the U4/U6.U5 tri-snRNP, the U4/U6 and U6 snRNPs are indicated. Lanes 1, 4, 7, and 10; extracts from untreated cells (25°C). Lanes 2, 5, 8, 11; extracts from cells pre-treated at 37°C for 45 min and subsequently heat shocked at 42°C for one hour (TT). Lanes 3, 6, 9, 12; extracts from cells heat shocked at 42°C without a prior pre-treatment at 37°C. (B) Cell extracts from strain SL304A (*HSP104*) were electophoresed for 7 hours, blotted and hybridised with a U4 specific probe. (See Materials and Methods) to illustrate the upward shift in the U4/U6.U5 and the U4/U6 snRNPs. Lane 1; untreated cells (25°C). Lane 2; cells treated at 37°C for 45 min. Lane 3; cells treated at 37°C for 45 min and then heat shocked at 42°C for one hour. Lane 4; cells heat shocked at 42°C for one hour.
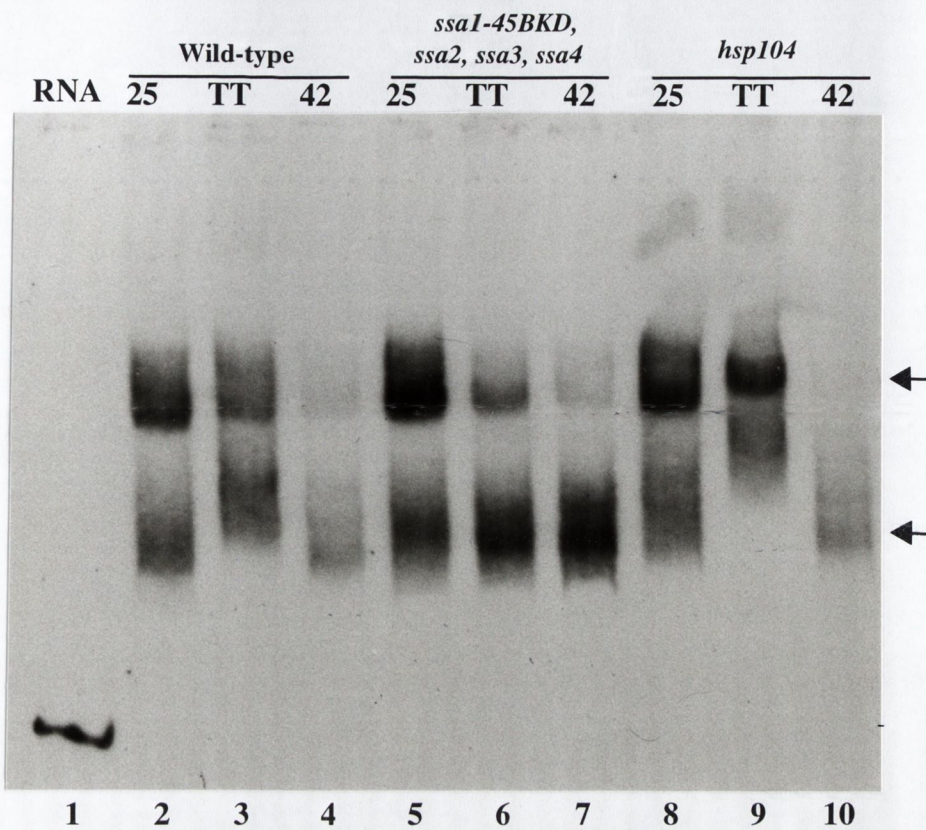
Figure 5.4   Protection of U1 snRNP Levels and 'Upshifts' are Observed in Splicing Thermotolerant Cells

Native blots were prepared exactly as described in Figure 5.3A, but were instead probed with a U1 specific DNA probe. The positions of the two principle U1 hybridising species are indicated with arrows.

5. 2. 4 Hsp70 Physically Associates with snRNPs to Confer Protection.

The data outlined above indicates that the tri-snRNP is protected if at least one member of the Ssa proteins are present in the cell. To determine if Hsp70 associates with snRNPs to confer protection, I examined if Hsp70 would co-immunoprecipitate with the tri-snRNP. The Prp4 antibody was chosen as it has been previously shown to be capable of immunoprecipitating this snRNP species (Banroques and Abelson, 1989). Normal (25°C), heat shocked (42°C) and heat shocked thermotolerant (TT), wild type (SL303-1A) cell extracts were then immunoprecipitated separately with either anti-Ssa or anti-Prp4 antibodies. Successful immunoprecipitation of snRNPs with the anti-Prp4 was confirmed by Northern blotting pellets with U4 and U6 specific probes (Figure 5.5B). All immunoprecipitated samples were then separated on a 10% SDS-PAGE gel and western immunoblotted with an anti-Ssa antibody (Figure 5.5A). From this it can be seen that, as expected, Hsp70 is efficiently immunoprecipated from all cell extracts with the anti-Ssa antibody (lanes 1, 2 and 3). Significantly, comparable levels of Hsp70 are detected in both heat shocked and non-heat shocked cells. This is because of the fact that levels of Ssa proteins are considerably high at normal temperatures and the antibody recognises both constitutive and heat inducible forms. The band below Hsp70 represents the immunoglobulin heavy chains and can also be seen in the no extract anti-Ssa immunoprecipitation control (Data not shown) and no extract anti-Prp4 immunoprecipitation control (Lane 7).

Hsp70 is not present in extracts prepared from cells grown at 25°C and immunoprecipitated with anti-Prp4 (Figure 5.5, lane 4). Very significantly however, Hsp70 is immunoprecipitated with snRNPs in heat shocked thermotolerant cells (Lane 5) and to a lesser extent in heat shocked non-thermotolerant cells (Lane 6).

From these results it is now possible to say that Hsp70, while present at 25°C, does not associate with snRNPs or at least can not be detected by this kind of analysis. However, in heat shocked cells Hsp70 is observed to associate with snRNPs. The significantly greater association of Hsp70 in thermotolerant cells, likely explains the snRNP protections observed in splicing thermotolerant cells.
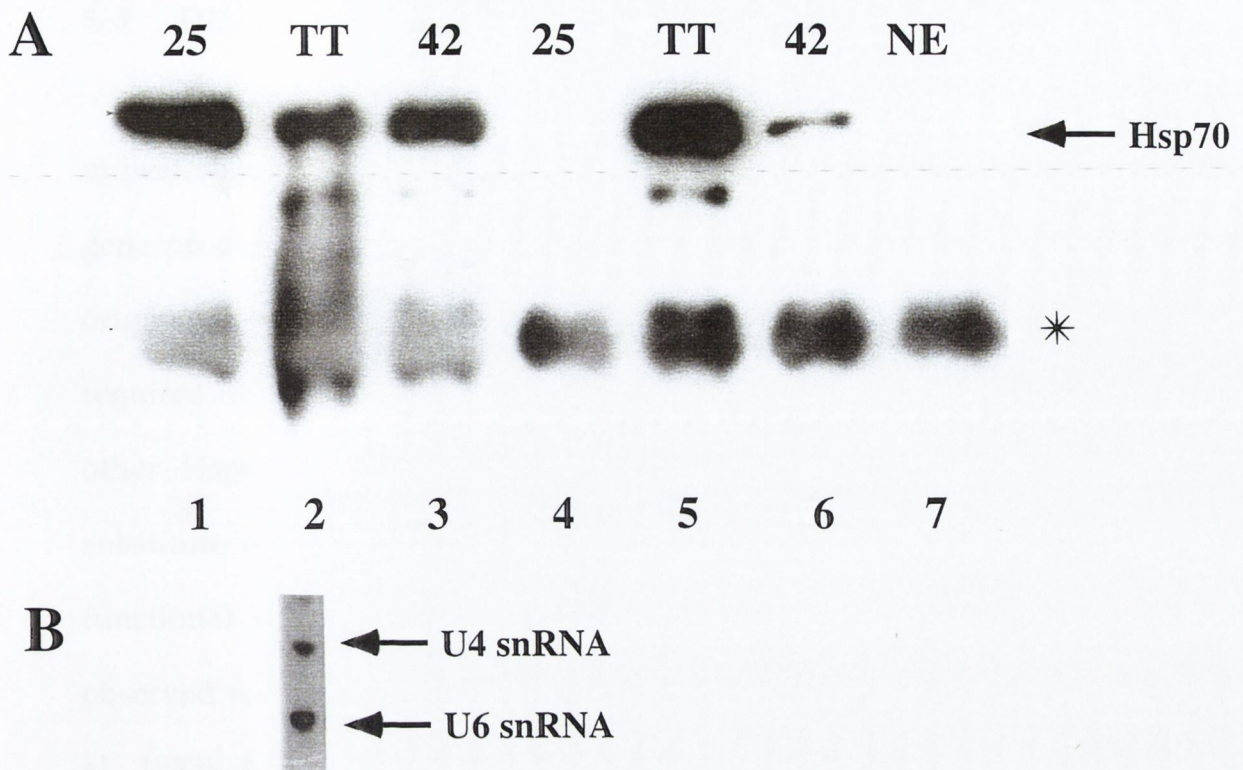
Figure 5.5   Hsp70 Associates with snRNPs in Splicing Thermotolerant Cells.

(A) Wild type (SL303-1A) splicing extracts from normal (25°C), heat shocked (42°C) and heat shocked thermotolerant cells (TT) were subjected to immunoprecipitation with an anti-Prp4 antibody (Lanes 4-6) (A kind gift from Prof. Jossette Banroques, Centre de Génétique Moléculaire du CNRS, Gif-sur-Yvette) and separately with an anti-Ssa antibody (Lanes 1-3) (A kind gift from Prof. Elizabeth Craig, University of Wisconsin). Pellets were the separated on 10% SDS-PAGE gels and western blotted with anti-Ssa antibody. The negative control of an anti-Ssa immunoprecipitation with no extract (ø; Lanes 7) is also shown. (B) Immunoprecipitation of U4 and U6 snRNAs with anti-Prp4 antibody.

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5.3   DISCUSSION.

The observation that mRNA splicing appears not to be inactivated in heat shocked pre-treated cells of strain JN519, which lacks all four *SSA* gene products, is surprising. This result suggests that, in contrast to the original hypothesis proposed by Yost, none of the *SSA* gene products are required for splicing thermotolerance at least under conditions when other Hsps are present. Thus, in the absence of Hsp70, Hsp104 may substitute to confer splicing thermotolerance and *vice versa*. A similar functional relationship between these two proteins has previously been observed with regard to their role in cellular thermotolerance (Sanchez, et al., 1993). Alternatively, trehalose accumulation may contribute to splicing thermotolerance. Analysis of splicing thermotolerance in trehalose synthase mutants may shed light on this.

While mRNA splicing appears to proceed normally in heat shocked pre-treated cells lacking all the *SSA* genes, the level of spliced mRNA was approximately 20% of the level observed in cells grown at the permissive temperature of 25°C. In the other mutant and wild type strains, spliced mRNA levels were approximately 50% reduced compared to the levels found at 25°C. This greater reduction observed in JN519 cells points to a role for at least one member of the Ssa sub-family, possibly Ssa1 or Ssa2, in maintaining spliced mRNA levels in heat shocked pre-treated cells by allowing continued transcription, stabilising pre-existing mRNA levels or both.

The major snRNPs required for spliceosomal assembly, the tri-

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snRNP (Figure 5.1B), the U1 snRNP (Figure 5.4) and the U2 snRNP (data not shown) are observed to be protected in splicing thermotolerant cells. This may reflect a protection of snRNPs in a spliceosomal context. This protection requires at least one member of the Ssa protein sub-family. The free U6 snRNP and its precursor U6/Lhp1p are not protected in thermotolerant cells. The lack of protection of the U6 snRNA-containing complexes may be a consequence of their transcription, as U6 snRNA is the only splicing snRNA transcribed by RNA polymerase III. Alternatively, the fact that the U6 snRNP is assembled in a different manner than the other snRNPs may account for the lack of protection.

Interestingly, in strain JN519 (*ssa1-45BKD, ssa2, ssa3, ssa4*), the level of the tri-snRNP is reproducibly lower even at the permissive temperature (25°C) compared to levels in the wild type or any other strain. Furthermore, the levels of free U6 and U4/U6 snRNPs, both precursors of the tri-snRNP, appear to be higher in this strain as compared to the levels in other strains. While a protein product is encoded by the *SSA1-45BKD* allele at the permissive temperature, the presence of a point mutation in the protein may reduce its efficacy. This may account for the low level of fully assembled tri-snRNP. Taken together, the results suggest that *SSA* gene products play a role in tri-snRNP assembly under normal growing conditions in addition to maintaining snRNP integrity in heat shocked thermotolerant cells.

One very interesting observation in our analysis, is that there is a noticeable upshift in the mobility of a sub-set of snRNPs in heat shocked

pre-treated cells. This is especially evident with the U4/U6 snRNP, although the U1, U2, U5 and U4/U6.U5 snRNPs also have similar upshifts (Figures 5.3, 5.4 and data not shown). These upshifts are not observed if cells are immediately shifted to 42°C without a prior pre-treatment. Significantly, the upshifts do not occur in the JN519 strain and are more pronounced in the *HSP104* strain. Activated or re-localised constitutive Hsp70, presumably *SSA1* and/or *SSA2* gene products, may bind to exposed hydrophobic surfaces of partially unfolded snRNPs upon heat shock in pre-treated cells, thus accounting for the upshift of snRNPs observed on non-denaturing gels. Due to the lack of functional Ssa proteins in the JN519 strain, no upward shift is observed.

Immunoprecipitation experiments of snRNPs from heat shocked and thermotolerant cells confirm that Ssa proteins co-immunoprecipitate with snRNPs demonstrating that Hsps exert their effect on snRNPs through a physical interaction (Figure 5.5). Future studies to investigate this direct association between snRNPs and Hsps will help to elucidate the role of Hsps in the assembly and maintenance of snRNPs during normal growth and in heat shocked cells.

Chapter 6:

General Discussion.

Splicing Inactivation by Heat Shock may be due to a Blockage in the snRNP Assembly Process

The initial aim of this project was to establish heat shocking conditions that could inactivate the process of mRNA splicing in the yeast *Saccharomyces cerevisiae*. This had previously been achieved by Vogel and co-workers by incubating cells in shaking waterbaths at 41.5°C (Vogel, et al., 1995). However in my hands, only partial inactivation was observed at this temperature. Instead, a waterbath temperature of 42°C was required to attain complete inactivation.

After this, the next goal was to thoroughly analyse the state of the snRNPs in the heat shocked *S. cerevisiae* cells. As described in Chapter 1, previous workers had correlated snRNP alterations in HeLa cells with splicing inactivation (Bond, 1988, Shukla, et al., 1990, Utans, et al., 1992). Building on the work, this thesis provides further evidence that splicing inactivation is in at least part due to a block in snRNP assembly. As discussed in Chapter 3, precursor forms of snRNPs are seen to accumulate in heat shocked cells. For example, it is clear that the U4 hybridising species observed to accumulate in heat shocked cells is a *bone fide* precursor form as it is present in very low amounts at normal temperatures (Figure 3.5A). In addition it seems that the levels of most snRNPs are reduced upon heat shock. In no case are what could be called 'thermal breakdown products' observed, i.e. bands that are not present at normal temperatures.

Despite the multiple alterations in snRNPs in heat shocked cells, it

is still very difficult to determine the precise reason for the inactivation of mRNA splicing. While levels of mature U1, U2 and U4/U6.U5 snRNPs are significantly reduced, they are not completely depleted. It may be that these forms are in an inactive state. Alternatively, the snRNPs themselves may be active, but other non snRNP factors are either inactive or not available to facilitate splicing. As described earlier, in HeLa cells, *in vitro* splicing activity can be restored to extracts from heat shocked cells by the addition of five tri-snRNP specific proteins (Utans, et al., 1992). However, this complementation experiment is limited in that it does not account for the complex compartmentalised nature of the eukaryotic cell. It is very possible that several splicing factors are not actually available to the sites of splicing within the nucleus of heat shocked cells.

snRNP Reassembly is Dependant on Hsps During Recovery of Splicing Activity After Thermal Inactivation.

The concept of a block in snRNP assembly being central to splicing inactivation in heat shocked cells is developed further in Chapter 4. In this chapter the recovery of splicing is monitored in wild type and *hsp* mutant cells. In addition the state of the snRNPs are also monitored. From this it is possible to see the 'chasing' of the U4 RNP precursor form into tri-snRNP as the recovery proceeds. It is interesting to observe that its recovery is much slower in the mutant strains lacking Ssa proteins, but even more so in the strains lacking Hsp104. So, by monitoring splicing recovery in the *hsp* mutant strains it is possible to slow down the snRNP

assembly process. For example, the previously characterised Lhp1p/RNP complex is seen to be reduced in all strains immediately after a heat shock. During recovery after heat shocking, it's levels remain about constant in the wild type strain. This is most likely because it is being chased back into the tri-snRNP as soon as it is made. Significantly, in the mutant strains, it actually accumulates during the recovery period. This suggests that there is a block in the conversion of this precursor snRNP form into the tri-snRNP in strains lacking Hsp104 or Ssa proteins, thus explaining the lack of tri-snRNP accumulation in these mutants. Thus, it is likely that this is the reason for the very slow recovery of splicing observed in these strains. Following from this logic, it seems that if tri-snRNP reassembly is required for splicing reactivation, then the small amount of tri-snRNP present immediately after heat shock is actually in an inactive state. It will be very interesting to test this hypothesis using *in vitro* complementation experiments. An alternative hypothesis is that a minimum threshold level of tri-snRNP is required for splicing to occur and that levels must be increased above this for splicing to be reactivated. However, this latter hypothesis is unlikely as early experiments in section 3.2.3, using the gradual heat shocking regime resulted in splicing inactivation even with only a slight reduction in tri-snRNP levels relative to the instantaneous heat shocking method adopted later on. Whatever, it seems very likely from this data that fresh snRNP assembly is required during recovery after heat shock to reactivate mRNA splicing. Pulse chase experiments could be employed to investigate this further.

In addition to insights into the snRNP assembly process, further light has also been shed on the *in vivo* importance of Hsp104 and Ssa proteins in stressed and recovering yeast cells. Previous workers had demonstrated that mutants which lacked either Hsp104 or three members of the heat inducible Ssa sub-family were considerably slower at recovering splicing activity after heat inactivation (Vogel, et al., 1995, Yost and Lindquist, 1991). These results are reproduced in this work. In addition this work demonstrates for the first time that a mutant lacking both Hsp104 and the heat inducible members of the Ssa sub-family, is even more restricted in recovery. It is possible that the single Ssa2 protein, still present in this latter strain, is responsible for the very slow mRNA splicing reactivation and tri-snRNP assembly observed during recovery. If this is the case, then the Hsp104 and Ssa proteins are likely absolutely essential for snRNP reassembly after heat shock and also for splicing recovery. In fact it is also likely that they co-operate synergistically together to perform this function. This would be consistent with the picture of functional cooperativity that is emerging between the Hsp100 and Hsp70 families, discussed already in Chapter 1.

Splicing Thermotolerance is Independent of The Major Hsps, yet Ssa Proteins Associate With The Tri-snRNP to Confer Protection.

So if snRNPs are blocked in assembly or are disassembled in heat shocked cells, then why is it that splicing is not inactivated in cells that have been pre-treated at 37°C for 45 min prior to heat shock? This is the

question addressed in Chaper 5. Surprisingly, splicing thermotolerance is observed in cells lacking all four Ssa proteins. It may be that other thermotolerance factors are responsible for splicing thermotolerance. One possible candidate for this is trehalose (Singer and Lindquist, 1998). This disaccharide, already mentioned in Chapter 1, is a factor that has been associated with induced cellular thermotolerance in *S. cerevisiae* (Iwahashi, et al., 1997, Piper, 1998, Singer and Lindquist, 1998). It is known to act in synergy with Hsps to contribute to cellular thermotolerance (Elliott, et al., 1996). On a molecular level it has been shown to preserve protein structure *in vivo* and *in vitro* (Singer and Lindquist, 1998). In the absence of a role for the major Hsps in splicing thermotolerance, trehalose may play a part in maintaining splicing activity in heat shocked pre-treated cells. Alternatively, while splicing thermotolerance was observed in a *HSP104* mutant strain, in the absence of *HSP70* genes, Hsp104 may substitute for Hsp70 to confer splicing thermotolerance.

Significant upshifts are observed in several snRNPs in heat shocked cells that have been pre-treated. In addition, Hsp70 is observed to be immunoprecipitated with snRNPs from these extracts. This is the first demonstration of Hsps actually physically associating with large macromolecular cell machinery, presumably to protect it from thermal damage. Intriguingly, greater upshifts are observed in mutant strains lacking the gene coding for Hsp104. Based on this data, I present a model that may explain this phenomenon and is consistent with recent results from Glover *et al.* (Glover and Lindquist, 1998). Activated or relocalised

Hsp70, presumably *SSA1* and/or *SSA2* gene products, may bind to exposed hydrophobic surfaces of partially unfolded snRNPs upon heat shock in pre-treated cells, thus accounting for the upshift of snRNPs observed on non-denaturing gels. Due to the lack of *SSA* gene products in the JN519 strain, no upward shift is observed. Furthermore, I propose a dynamic co-operation of Hsp104 with the bound Hsp70s. Hsp104 may be required to complete the repair process by removing bound Hsp70. In the absence of Hsp104, Hsp70 remains bound to the snRNPs, thereby resulting in a greater upshift observed in *HSP104* mutants.

Bibliography

1.   **Abovich, N., P. Legrain, and M. Rosbash.** 1990. The yeast PRP6 gene encodes a U4/U6 small nuclear ribonucleoprotein particle (snRNP) protein, and the PRP9 gene encodes a protein required for U2 snRNP binding. Mol. Cell. Biol. **10**:6417-25.

2.   **Abravaya, K., M. P. Myers, S. P. Murphy, and R. I. Morimoto.** 1992. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes Dev. **6**:1153-64.

3.   **Achsel, T., K. Ahrens, H. Brahms, S. Teigelkamp, and R. Lührmann.** 1998. The human U5-220kD protein (hPrp8) forms a stable RNA-free complex with several U5-specific proteins, including an RNA unwindase, a homologue of ribosomal elongation factor EF-2, and a novel WD-40 protein. Mol. Cell. Biol. **18**:6756-6766.

4.   **Achsel, T., H. Brahms, B. Kastner, A. Bachi, M. Wilm, and R. Luhrmann.** 1999. A doughnut-shaped heteromer of human Sm-like proteins binds to the 3'-end of U6 snRNA, thereby facilitating U4/U6 duplex formation *in vitro*. EMBO J. **18**:5789-5802.

5.   **Ananthan, J., A. J. Goldberg, and R. Voellmy.** 1986. Abnormal proteins serve as Eukaryotic signals and trigger the activation of heat shock genes. Science **232**:522-524.

6.   **Anthony, J. G., E. M. Weidenhammer, and J. L. Woolford.** 1997. The yeast Prp3 protein is a U4/U6 snRNP protein necessary for the integrety of the U4/U6.U5 tri-snRNP. RNA **3**:1143-1152.

7. **Arenas, J. E., and J. N. Abelson.** 1997. Prp43: An RNA helicase-like factor involved in spliceosome disassembly. Proc. Natl. Acad. Sci. **94:**11798-802.

8. **Ares, M.** 1986. U2 RNA from yeast is unexpectedly large and contains homology to vertebrate U4, U5, and U6 small nuclear RNAs. Cell **47:**49-59.

9. **Attfield, P. V.** 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. Nat. Biotechnol. **15:**1351-7.

10. **Ayadi, L., M. Miller, and J. Banroques.** 1997. Mutations within the yeast U4/U6 snRNP protein Prp4 affect a late stage of spliceosome assembly. RNA **3:**197-209.

11. **Banroques, J., and J. N. Abelson.** 1989. PRP4: a protein of the yeast U4/U6 small nuclear ribonucleoprotein particle. Mol. Cell. Biol. **9:**3710-3719.

12. **Barbe, M. F., M. Tytell, D. J. Gower, and W. J. Welch.** 1988. Hyperthermia protects against light damage in the rat retina. Science **241:**1817-20.

13. **Baxter, B. K., P. James, T. Evans, and E. A. Craig.** 1996. SSI1 encodes a novel Hsp70 of the *Saccharomyces cerevisiae* endoplasmic reticulum. Mol. Cell. Biol. **16:**6444-56.

14. **Becker, J., W. Walter, W. Yan, and E. A. Craig.** 1996. Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation *in vivo*. Mol. Cell. Biol. **16:**4378-4386.

15.   **Beggs, J. D.** 1995. Yeast splicing factors and genetic stategies for their analysis, p. 79-95. *In* A. I. Lamond (ed.), Pre-mRNA Processing. R. G. Landes Company, Texas.

16.   **Behrens, S. E., K. Tyc, B. Kastner, J. Reichelt, and R. Lührmann.** 1993. Small nuclear ribonucleoprotein (RNP) U2 contains numerous additional proteins and has a bipartite RNP structure under splicing conditions. Mol. Cell. Biol. **13:**307-19.

17.   **Bendinelli, P., R. Piccoletti, P. Maroni, and A. Bernelli-Zazzera.** 1995. The liver response to in vivo heat shock involves the activation of MAP kinases and RAF and the tyrosine phosphorylation of Shc proteins. Biochem. Biophys. Res. Commun. **216:**54-61.

18.   **Bensaude, O., S. Bellier, M. F. Dubois, F. Giannoni, and V. T. Nguyen.** 1996. Heat-shock induced protein modifications and modulation of enzyme activities. EXS **77:**199-219.

19.   **Berget, S. M., C. Moore, and P. A. Sharp.** 1977. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proc. Natl. Acad. Sci. **74:**3171-5.

20.   **Bjørn, S. P., A. Soltyk, J. D. Beggs, and J. D. Friesen.** 1989. *PRP4 (RNA4)* from *Saccharomyces cerrevisiae*: its gene product is associated with the U4/U6 small nuclear ribonucleoprotein particle. Mol. Cell. Biol. **9:**2698-3709.

21.   **Blanton, S., A. Srinivasan, and B. C. Rymond.** 1992. PRP38 encodes a yeast protein required for pre-mRNA splicing and maintenance of stable U6 small nuclear RNA levels. Mol. Cell. Biol. **12:**3939-47.

22. **Bond, U.** 1988. Heat shock but not other stress inducers leads to the disruption of a sub-set of snRNPs and inhibition of *in vitro* splicing in HeLa cells. EMBO J. **7:**3509-3518.

23. **Bordonne, R., and I. Tarassov.** 1996. The yeast SME1 gene encodes the homologue of the human E core protein. Gene **176:**111-7.

24. **Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist.** 1989. hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. **9:**3919-30.

25. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-54.

26. **Brown, J. D., and J. D. Beggs.** 1992. Roles of PRP8 protein in the assembly of splicing complexes. EMBO J. **11:**3721-3729.

27. **Buchenau, P., H. Saumweber, and D. J. Arndt-Jovin.** 1997. The dynamic nuclear redistribution of an hnRNP K-homologous protein during drosophila embryo development and heat shock. Flexibility of transcription sites *in vivo*. J. Cell. Biol. **137:**291-303.

28. **Buchner, J.** 1999. Hsp90 & Co. - a holding for folding. Trends Biochem. Sci. **24:**136-41.

29. **Buchner, J.** 1996. Supervising the fold: functional principles of molecular chaperones. FASEB J. **10:**10-19.

30. **Bukau, B.** 1993. Regulation of the *Escherichia coli* heat-shock response. Mol. Microbiol. **9:**671-680.

31. **Bukau, B., and A. L. Horwich.** 1998. The Hsp70 and Hsp60 chaperone machines. Cell **92:**351-366.

32. **Caplan, A. J.** 1999. Hsp's secrets unfold: new insights from structural and functional studies. Trends Cell. Biol. **9:**262-8.

33. **Caplan, A. J., D. M. Cyr, and M. G. Douglas.** 1993. Eukaryotic homologues of *Escherichia coli* dnaJ: a diverse protein family that functions with hsp70 stress proteins. Mol. Biol. Cell. **4:**555-63.

34. **Caplan, A. J., D. M. Cyr, and M. G. Douglas.** 1992. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell **71:**1143-55.

35. **Carratu, L., S. Franceschelli, C. L. Pardini, G. S. Kobayashi, I. Horvath, L. Vigh, and B. Maresca.** 1996. Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. Proc. Natl. Acad. Sci. **93:**3870-5.

36. **Caspary, F., and B. Seraphin.** 1998. The yeast U2A'/U2B complex is required for pre-spliceosome formation. EMBO J. **17:**6348-58.

37. **Caspary, F., A. Shevchenko, M. Wilm, and B. Seraphin.** 1999. Partial purification of the yeast U2 snRNP reveals a novel yeast pre-mRNA splicing factor required for pre-spliceosome assembly. EMBO J. **18:**3463-74.

38. **Celerin, M., A. A. Gilpin, N. J. Schisler, A. G. Ivanov, E. Miskiewicz, M. Krol, and D. E. Laudenbach.** 1998. ClpB in a cyanobacterium: predicted structure, phylogenetic relationships, and regulation by light and temperature. J. Bacteriol. **180:**5173-82.

39. **Chambers, J. C., M. G. Kurilla, and J. D. Keene.** 1983. Association between the 7 S RNA and the lupus La protein varies among cell types. J. Biol. Chem. **258**:11438-41.

40. **Chanfreau, G., S. A. Elela, M. J. Ares, and C. Guthrie.** 1997. Alternative 3'-end processing of U5 snRNA by RNase III. Genes Dev. **11**:2741-2751.

41. **Cheng, M. Y., F. U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg, and A. L. Horwich.** 1989. Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature **337**:620-5.

42. **Cheng, S. C., and J. Abelson.** 1987. Spliceosome assembly in yeast. Genes Dev. **1**:1014-1027.

43. **Chernoff, Y. O., S. L. Lindquist, B.-i. Ono, S. G. Inge-Vechtomov, and S. W. Liebman.** 1995. Role of the Chaperone Protein Hsp104 in Propagation of the Yeast Prion-Like Factor [*psi*+]. Science **268**:880-883.

44. **Chow, L. T., R. E. Gelinas, T. R. Broker, and R. J. Roberts.** 1977. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell **12**:1-8.

45. **Chung, C.** 1993. Proteases in *Escherichia coli*. Science **262**:372-4.

46. **Ciavarra, R. P., C. Goldman, K. K. Wen, B. Tedeschi, and F. J. Castora.** 1994. Heat stress induces hsc70/nuclear topoisomerase I complex formation *in vivo*: evidence for hsc70-mediated, ATP-independent reactivation *in vitro*. Proc. Natl. Acad. Sci. **91**:1751-5.

47. **Company, M., J. Arenas, and J. Abelson.** 1991. Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. Nature **349**:487-93.

48. **Cooper, M., L. H. Johnston, and J. D. Beggs.** 1995. Identification and characterisation of Uss1p (Sdb23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins. EMBO J. **14**:2066-2075.

49. **Corell, R. A., and R. H. Gross.** 1992. Splicing thermotolerance maintains Pre-mRNA transcripts in the splicing pathway during severe heat shock. Exp. Cell. Res. **202**:233-42.

50. **Craig, E. A., B. K. Baxter, J. Becker, J. Halladay, and T. Ziegelhoffer.** 1994. Cytosolic hsp70s of *Saccharomyces cerevisiae*: Roles in protein synthesis, translocation, proteolysis and regulation, p. 457-494. *In* R. Morimoto, A. Tissières, and C. Georgopoulos (ed.), The biology of the heat shock proteins and the molecular chaperones. Cold Spring Harbour Laboratory Press, New York.

51. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Micro. Rev. **57**:402-414.

52. **Craig, E. A., and K. Jacobsen.** 1985. Mutations in cognate genes of *Saccharomyces cerevisiae* hsp70 result in reduced growth rates at low temperatures. Mol. Cell. Biol. **5**:3517-24.

53. **Craig, E. A., J. S. Weissman, and A. L. Horwich.** 1994. Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell **78**:365-372.

54. **Craven, R. A., M. Egerton, and C. J. Stirling.** 1996. A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors. EMBO J. **15**:2640-50.

55. **Currie, R. W., R. M. Tanguay, and J. G. Kingma.** 1993. Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. Circulation **87**:963-71.

56. **Cutforth, T., and G. M. Rubin.** 1994. Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in *Drosophila*. Cell **77**:1027-36.

57. **Cyr, D. M., T. Langer, and M. G. Douglas.** 1994. DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. Trends Biochem. Sci. **19**:176-81.

58. **Czarnecka, E., R. T. Nagao, J. L. Key, and W. B. Gurley.** 1988. Characterization of Gmhsp26-A, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol. Cell. Biol. **8**:1113-1122.

59. **de Graaf, A., A. M. Meijne, A. J. van Renswoude, B. M. Humbel, P. M. van Bergen en Henegouwen, L. de Jong, R. van Driel, and A. J. Verkleij.** 1992. Heat shock-induced redistribution of a 160-kDa nuclear matrix protein. Exp. Cell. Res. **2**:243-251.

60. **Dillmann, W. H.** 1999. Small heat shock proteins and protection against injury. Ann. N. Y. Acad. Sci. **874:**66-8.

61. **Dix, I., C. S. Russell, R. T. O'Keefe, A. J. Newman, and J. D. Beggs.** 1998. Protein-RNA interactions in the U5 snRNP of *Saccharomyces cerevisiae*. RNA **4:**1675-1686.

62. **Duina, A. A., H. M. Kalton, and R. F. Gaber.** 1998. Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. J. Biol. Chem. **273:**18974-8.

63. **Dyck, L. v., M. Dembowski, W. Neupert, and T. Langer.** 1998. Mcx1p, a ClpX homologue in mitochondria of *Saccharomyces cerevisiae*. FEBS Lett. **438:**250-4.

64. **Ehrnsperger, M., S. Gräber, M. Gaestel, and J. Buchner.** 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. EMBO J. **16:**221-229.

65. **Elliott, B., R. S. Haltiwanger, and B. Futcher.** 1996. Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*. Genetics **144:**923-933.

66. **Ellis, J.** 1987. Proteins as molecular chaperones. Nature **328:**378-9.

67. **Eriksson, M. J., and A. K. Clarke.** 1996. The heat shock protein ClpB mediates the development of thermotolerance in the cyanobacterium *Synechococcus sp.* strain PCC 7942. J. Bacteriol. **178:**4839-46.

68. Fabrizio, P., B. Laggerbauer, J. Lauber, W. S. Lane, and R. Luhrmann.
1997. An evolutionarily conserved U5 snRNP-specific protein is a
GTP-binding factor closely related to the ribosomal translocase EF-2.
EMBO J. **16**:4092-106.

69. Faux, M. C., and J. D. Scott. 1996. Molecular glue: kinase anchoring
and scaffold proteins. Cell **85**:9-12.

70. Fernandes, M., T. O'Brien, and J. T. Lis. 1994. Structure and
regulation of heat shock gene promoters., p. 275-393. *In* R. I.
Morimoto, A. Tissieres, and C. Georgopoulos (ed.), The Biology of
Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor
Laboratory Press, Cold Spring Harbor.

71. Ferreira, P. M., V. Costa, P. Piper, and W. Mager. 1996. The
molecular defences against reactive oxygen species in yeast. Mol.
Microbiol. **19**:651-658.

72. Fetzer, S., J. Lauber, C. L. Will, and R. Lührmann. 1997. The
[U4/U6.U5] tri-snRNP-specific 27K protein that can be
phosphorylated by the snRNP-associated protein kinase. RNA **3**:344-
355.

73. Fischer, U., Q. Liu, and G. Dreyfuss. 1997. The SMN-SIP1 complex
has an essential role in spliceosomal snRNP biogenesis. Cell **90**:1023-
9.

74. **Freeman, B. C., and R. Morimoto.** 1996. The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. EMBO J. **15:**2969-79.

75. **Frydman, J., E. Nimmesgern, K. Ohtsuka, and F. U. Hartl.** 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. Nature **370:**111-7.

76. **Gaitanaris, G. A., A. Vysokanov, S. C. Hung, M. E. Gottesman, and A. Gragerov.** 1994. Successive action of *Escherichia coli* chaperones *in vivo*. Mol. Microbiol. **14:**861-9.

77. **Galisson, F., and P. Legrain.** 1993. The biochemical defects of *prp4-1* and *prp6-1* yeast splicing mutants reveal that the PRP6 protein is required for the accumulation of the [U4/U6.U5] tri-snRNP. Nucleic Acids Res. **21:**1555-1562.

78. **Garcia-Cardena, G., R. Fan, V. Shah, R. Sorrentino, G. Cirino, A. Papapetropoulos, and W. C. Sessa.** 1998. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature **392:**821-4.

79. **Gattoni, R., D. Mahé, P. Mähl, N. Fischer, M. G. Mattei, J. Stevenin, and J. P. Fuchs.** 1996. The human hnRNP-M proteins: structure and relation with early heat shock-induced splicing arrest and chromosome mapping. Nucleic Acids Res. **24:**2535-2542.

80. **Georgopoulos, C., K. Liberek, M. Zylicz, and D. Ang.** 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response., p. 233-240. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

81. **Ghetti, A., M. Company, and J. Abelson.** 1995. Specificity of Prp24 binding to RNA: A role for Prp24 in the dynamic interaction of U4 and U6 snRNAs. RNA **1**:132-145.

82. **Ghoshal, K., and S. T. Jacob.** 1996. Heat shock inhibits pre-rRNA processing at the primary site *in vitro* and alters the activity of some rRNA binding proteins. J. Cell. Biochem. **62**:506-515.

83. **Giardina, C., and J. T. Lis.** 1995. Dynamic protein-DNA architecture of a yeast heat shock promoter. Mol. Cell. Biol. **15**:2737-44.

84. **Glover, J. R., and S. Lindquist.** 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell **94**:73-82.

85. **Gottesman, S.** 1996. Proteases and their targets in *Escherichia coli*. Annu. Rev. Genet. **30**:465-506.

86. **Gottesman, S., E. Roche, Y. Zhou, and R. T. Sauer.** 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes. Dev. **12**:1338-47.

87. **Gottschalk, A., G. Neubauer, J. Banroques, M. Mann, R. Luhrmann, and P. Fabrizio.** 1999. Identification by mass spectrometry and functional analysis of novel proteins of the yeast [U4/U6.U5] tri-snRNP. EMBO J. **18:**4535-48.

88. **Gottschalk, A., J. Tang, O. Puig, J. Salgado, G. Neubauer, H. V. Colot, M. Mann, B. Séraphin, M. Rosbash, R. Lührmann, and P. Fabrizio.** 1998. A comprehensive biochemical and genetic analysis of the yeast U1 snRNP reveals five novel proteins. RNA **4:**374-393.

89. **Grandvalet, C., V. d. Crecy-Lagard, and P. Mazodier.** 1999. The ClpB ATPase of *Streptomyces albus* G belongs to the HspR heat shock regulon. Mol Microbiol **31:**521-32.

90. **Greene, M. K., K. Maskos, and S. J. Landry.** 1998. Role of the J-domain in the cooperation of Hsp40 with Hsp70. Proc. Natl. Acad. Sci. **95:**6108-13.

91. **Grimaud, R., M. Kessel, F. Beuron, A. C. Steven, and M. R. Maurizi.** 1998. Enzymatic and structural similarities between the *Escherichia coli* ATP-dependent proteases, ClpXP and ClpAP. J. Biol. Chem. **273:**12476-81.

92. **Gross, D. S., C. C. Adams, S. Lee, and B. Stentz.** 1993. A critical role for heat shock transcription factor in establishing a nucleosome-free region over the TATA-initiation site of the yeast HSP82 heat shock gene. EMBO J. **12:**3931-45.

93. **Gupta, R. S., and B. Singh.** 1992. Cloning of the HSP70 gene from *Halobacterium marismortui*: relatedness of archaebacterial HSP70 to its eubacterial homologs and a model for the evolution of the HSP70 gene. J. Bacteriol. **174:**4594-605.

94. **Hartman, D. J., N. J. Hoogenraad, R. Condron, and P. B. Hoj.** 1992. Identification of a mammalian 10-kDa heat shock protein, a mitochondrial chaperonin 10 homologue essential for assisted folding of trimeric ornithine transcarbamoylase *in vitro*. Proc. Natl. Acad. Sci. **89:**3394-8.

95. **Hendrick, J. P., and F. U. Hartl.** 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. **62:**349-84.

96. **Hendrick, J. P., S. L. Wolin, J. Rinke, M. R. Lerner, and J. A. Steitz.** 1981. Ro small cytoplasmic ribonucleoproteins are a subclass of La ribonucleoproteins: further characterization of the Ro and La small ribonucleoproteins from uninfected mammalian cells. Mol. Cell. Biol. **1:**1138-49.

97. **Hermann, H., P. Fabrizio, V. A. Raker, K. Foulaki, H. Hornig, H. Brahms, and R. Lührmann.** 1995. snRNP Sm proteins share two evolutionarily conserved sequence motifs which are involved in Sm protein-protein interactions. EMBO J. **14:**2076-88.

98. **Higashi, T., A. Nakai, Y. Uemura, H. Kikuchi, and K. Nagata.** 1995. Activation of heat shock factor in rat brain during cerebral ischemia or after heat shock. Mol. Brain Res. **34:**262-270.

99.   **Hightower, L. E.** 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell **66:**191-197.

100.   **Holt, S. E., D. L. Aisner, J. Baur, V. M. Tesmer, M. Dy, M. Ouellette, J. B. Trager, G. B. Morin, D. O. Toft, J. W. Shay, W. E. Wright, and M. A. White.** 1999. Functional requirement of p23 and Hsp90 in telomerase complexes. Genes Dev **13:**817-26.

101.   **Horowitz, D. S., and J. Abelson.** 1993. A U5 small nuclear ribonucleoprotein particle protein involved only in the second step of pre-mRNA splicing in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**2959-70.

102.   **Horwich, A. L.** 1995. Resurrection or destruction? Recent studies implicate Hsp104/Clp family chaperones in both protein disaggregation and protein degradation. How do these homologous ring-shaped complexes function in such different ways? Curr. Biol. **5:**455-458.

103.   **Horwich, A. L., K. B. Low, W. A. Fenton, I. N. Hirshfield, and K. Furtak.** 1993. Folding in vivo of bacterial cytoplasmic proteins: role of GroEL. Cell **74:**909-17.

104.   **Horwich, A. L., E. U. Weber-Ban, and D. Finley.** 1999. Chaperone rings in protein folding and degradation. Proc. Natl. Acad. Sci. **96:**11033-11040.

105. **Horwitz, J.** 1992. Alpha-crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. **89**:10449-53.

106. **Hoskins, J. R., M. Pak, M. R. Maurizi, and S. Wickner.** 1998. The role of the ClpA chaperone in proteolysis by ClpAP. Proc. Natl. Acad. Sci. **95**:12135-40.

107. **Hwang, B. J., W. J. Park, C. H. Chung, and A. L. Goldberg.** 1987. *Escherichia coli* contains a soluble ATP-dependent protease (Ti) distinct from protease La. Proc. Natl. Acad. Sci. **84**:5550-4.

108. **Ingmer, H., F. K. Vogensen, K. Hammer, and M. Kilstrup.** 1999. Disruption and analysis of the clpB, clpC, and clpE genes in Lactococcus lactis: ClpE, a new Clp family in gram-positive bacteria. J. Bacteriol. **181**:2075-83.

109. **Iwahashi, H., K. Obuchi, S. Fujii, and Y. Komatsu.** 1997. Effect of temperature on the role of Hsp104 and trehalose in barotolerance of *Saccharomyces cerevisiae*. FEBS Lett. **416**:1-5.

110. **Jakob, U., M. Gaestel, K. Engel, and J. Buchner.** 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. **268**:1517-1520.

111. **James, P., C. Pfund, and E. A. Craig.** 1997. Functional specificity among Hsp70 molecular chaperones. Science **275**:387-9.

112. **Kamada, Y., U. S. Jung, J. Piotrowski, and D. E. Levin.** 1995. The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. Genes Dev. **9**:1559-71.

113.   **Kambach, C., S. Walke, R. Young, J. M. Avis, E. d. l. Fortelle, V. A. Raker, R. Luhrmann, J. Li, and K. Nagai.** 1999. Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs. Cell **96**:375-87.

114.   **Kampinga, H. H.** 1993. Thermotolerance in mammalian cells. Protein denaturation and aggregation, and stress proteins. J. Cell. Sci. **104**:11-7.

115.   **Kao, H. Y., and P. G. Siliciano.** 1996. Identification of Prp40, a novel essential yeast splicing factor associated with the U1 small nuclear ribonucleoprotein particle. Mol. Cell. Biol. **16**:960-7.

116.   **Kao, H. Y., and P. G. Siliciano.** 1992. The yeast homolog of the U1 snRNP protein 70K is encoded by the SNP1 gene. Nucleic Acids Res. **20**:4009-13.

117.   **Kastner, B., M. Bach, and R. Luhrmann.** 1990. Electron microscopy of small nuclear ribonucleoprotein (snRNP) particles U2 and U5: evidence for a common structure-determining principle in the major U snRNP family. Proc. Natl. Acad. Sci. **87**:1710-4.

118.   **Katayama, Y., S. Gottesman, J. Pumphrey, S. Rudikoff, W. P. Clark, and M. R. Maurizi.** 1988. The two-component, ATP-dependent Clp protease of *Escherichia coli*. Purification, cloning, and mutational analysis of the ATP-binding component. J. Biol. Chem. **263**:15226-36.

119. **Kawai, R., K. Fujita, H. Iwahashi, and Y. Komatsu.** 1999. Direct evidence for the intracellular localization of Hsp104 in *Saccharomyces cerevisiae* by immunoelectron microscopy. Cell Stress Chaperones **4**:46-53.

120. **Kay, R. J., R. H. Russnakk, D. Jones, C. Mathia, and E. P. M. Candido.** 1987. Expression of intron-containing *C. elegans* heat shock genes in mouse cells demonstrates divergence of 3' splice site recognition sequences between nematodes and vertebrates, and an inhibitory effect of heat shock on the mammalian splicing apparatus. Nucleic Acids Res. **15**:3723-3741.

121. **Kessel, M., M. R. Maurizi, B. Kim, E. Kocsis, B. L. Trus, S. K. Singh, and A. C.Steven.** 1995. Homology in structural organization J. Mol. Biol. **250**:587-94.

122. **Kim, K. K., R. Kim, and S. H. Kim.** 1998. Crystal structure of a small heat-shock protein. Nature **394**:595-9.

123. **Kim, S., B. Schilke, E. A. Craig, and A. L. Horwich.** 1998. Folding in vivo of a newly translated yeast cytosolic enzyme is mediated by the SSA class of cytosolic yeast Hsp70 proteins. Proc. Natl. Acad. Sci. **95**:12860-5.

124. **Konarska, M. M., and P. A. Sharp.** 1986. Electrophoretic separation of complexes Involved in the splicing of precursors to mRNAs. Cell **46**:845-855.

125. **Kramer, A.** 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu. Rev. Biochem. **65**:367-409.

126. **Kramer, A., P. Gruter, K. Groning, and B. Kastner.** 1999. Combined biochemical and electron microscopic analyses reveal the RNA is large and has limited primary sequence homology to metazoan U1 snRNA. Cell **50**:593-602.

128. **Krieg, P. A.** 1996. A laboratory guide to RNA: isolation, analysis and synthesis, p. 44-45. Wiley-Liss Inc.

129. **Kruger, E., U. Volker, and M. Hecker.** 1994. Stress induction of clpC in *Bacillus subtilis* and its involvement in stress tolerance. J. Bacteriol. **176**:3360-7.

130. **Landry, A.-P., and J. Landry.** 1994. Expression and function of the low molecular-weight heat shock proteins, p. 335-73. *In* R. Morimoto, A. Tissières, and C. Georgopoulos (ed.), The biology of the heat shock proteins and the molecular chaperones. Cold Spring Harbour Laboratory Press, New York.

131. **Laroia, G., R. Cuesta, G. Brewer, and R. J. Schneider.** 1999. Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. Science **284**:499-502.

132. **Laszlo, A.** 1992. The effects of hyperthermia on mammalian cell structure and function. Cell Prolif. **25**:59-87.

133. **Lauber, J., P. Fabrizio, S. Teigelkamp, W. S. Lane, E. Hartmann, and R. Lührmann.** 1996. The HeLa 200 kDa U5 snRNP-specific protein and its homologue in *Saccharomyces cerevisiae* are members of the DEXH-box protein family of putative RNA helicases. EMBO J. **15**:4001-4015.

134. **Lauber, J., G. Plessel, S. Prehn, C. L. Will, P. Fabrizio, K. Gröning, W. S. Lane, and R. Lührmann.** 1997. The human U4/U6 snRNP contains 60 and 90kD proteins that are structurally homologous to the yeast splicing factors Prp4p and Prp3p. RNA **3**:926-941.

135. **Lavoie, J. N., G. Gingras-Breton, R. M. Tanguay, and J. Landry.** 1993. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. J. Biol. Chem. **268**:3420-9.

136. **Lavoie, J. N., E. Hickey, L. A. Weber, and J. Landry.** 1993. Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J. Biol. Chem. **268**:24210-4.

137. **Lavoie, J. N., H. Lambert, E. Hickey, L. A. Weber, and J. Landry.** 1995. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. Mol. Cell. Biol. **15**:505-16.

138. **Lee, G. J., A. M. Roseman, H. R. Saibil, and E. Vierling.** 1997. A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J. **16**:659-671.

139.	**Lee, Y. R., R. T. Nagao, and J. L. Key.** 1994. A soybean 101-kD heat shock protein complements a yeast HSP104 deletion mutant in acquiring thermotolerance. Plant Cell **6**:1889-97.

140.	**Lerner, M. R., and J. A. Steitz.** 1979. Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. **76**:5495-9.

141.	**Levchenko, I., S. Catherine K, N. P. Walsh, R. T. Sauer, and T. A. Baker.** 1997. PDZ-like domains mediate binding specificity in the Clp/Hsp100 family of chaperones and protease regulatory subunits. Cell **91**:939-947.

142.	**Levchenko, I., L. Luo, and T. A. Baker.** 1995. Disassembly of the Mu transposase tetramer by the ClpX chaperone. Genes Dev. **9**:2399-408.

143.	**Lin, J., and J. J. Rossi.** 1996. Identification and characterization of yeast mutants that overcome an experimentally introduced block to splicing at the 3' splice site. RNA **2**:835-48.

144.	**Lindquist, S.** 1986. The heat shock response. Ann. Rev. Biochem. **55**:1151-1191.

145.	**Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22**.

146.	**Lindquist, S., and G. Kim.** 1996. Heat shock protein 104 expression is sufficient for thermotolerance in yeast. Proc. Natl. Acad. Sci. USA **93**:5301-5306.

147. **Liu, Y., S. Liang, and A. M. Tartakoff.** 1996. Heat shock disassembles the nucleolus and inhibits nuclear protein import and poly(A)+ RNA export. EMBO J. **15**:6750-6757.

148. **Lockhart, S. R., and B. C. Rymond.** 1994. Commitment of yeast pre-mRNA to the splicing pathway requires a novel U1 small nuclear ribonucleoprotein polypeptide, Prp39p. Mol. Cell. Biol. **14**:3623-33.

149. **Loo, M. A., T. J. Jensen, L. Cui, Y. Hou, X. B. Chang, and J. R. Riordan.** 1998. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. EMBO J **17**:6879-87.

150. **Lopez-Buesa, P., C. Pfund, and E. A. Craig.** 1998. The biochemical properties of the ATPase activity of a 70-kDa heat shock protein (Hsp70) are governed by the C-terminal domains. Proc. Natl. Acad. Sci. **95**:15253-8.

151. **Lossky, M., G. J. Anderson, S. P. Jackson, and J. Beggs.** 1987. Identification of a yeast snRNP protein and detection of snRNP-snRNP interactions. Cell **51**:1019-26.

152. **Lubben, T. H., A. A. Gatenby, G. K. Donaldson, G. H. Lorimer, and P. V. Viitanen.** 1990. Identification of a groES-like chaperonin in mitochondria that facilitates protein folding. Proc. Natl. Acad. Sci. **87**:7683-7.

153. **Lutz, Y., M. Jacob, and J. P. Fuchs.** 1988. The distribution of two hnRNP-associated proteins defined by a monoclonal antibody is altered in heat-shocked HeLa cells. Exp. Cell. Res. **175**:109-124.

154. **Lybarger, S., K. Beickman, V. Brown, N. Dembla-Rajpal, K. Morey, R. Seipelt, and B. C. Rymond.** 1999. Elevated levels of a U4/U6.U5 snRNP-associated protein, Spp381p, rescue a mutant defective in spliceosome maturation. Mol. Cell. Biol. **19:**577-84.

155. **Mager, W. H., and A. J. D. Kruijff.** 1995. Stress-induced transcriptional activation. Microbiol. Rev. **59:**506-31.

156. **Mahé, D., P. Mähl, R. Gattoni, N. Fischer, M.-G. Mattei, J. Stévenin, and J.-P. Fuchs.** 1997. Cloning of human 2H9 heterogenous nuclear ribonucleoproteins relation with splicing and early heat shock-induced splicing arrest. J. Biol. Chem. **272:**1827-1836.

157. **Mähl, P., Y. Lutz, E. Puvion, and J.-P. Fuchs.** 1989. Rapid effect of heat shock on two heterogenous nuclear ribonucleoprotein-associated antigens in HeLa cells. J. Cell Biol. **109:**1921-1935.

158. **Maniatis, T., E. F. Fritsch, and J. Sambrook (ed.).** 1989. Molecular cloning: a laboratory manual, 2nd Edition, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

159. **Marber, M. S., R. Mestril, S. H. Chi, M. R. Sayen, D. M. Yellon, and W. H. Dillmann.** 1995. Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. J. Clin. Invest. **95:**1446-56.

160. **Martin, J., A. L. Horwich, and F. U. Hartl.** 1992. Prevention of protein denaturation under heat stress by the chaperonin Hsp60. Science **258:**995-8.

161. **Mathew, A., and R. I. Morimoto.** 1998. Role of the heat-shock response in the life and death of proteins. Ann. N. Y. Acad. Sci. **851**:99-111.

162. **Mattaj, I. W.** 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. Cell **46**:905-11.

163. **Mattaj, I. W., N. A. Dathan, H. D. Parry, P. Carbon, and A. Krol.** 1988. Changing the RNA polymerase specificity of snRNA gene promoters. Cell **55**:435-42.

164. **Mattaj, I. W., and E. M. D. Robertis.** 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell **40**:111-8.

165. **Mayer, M. P., and B. Bukau.** 1999. Molecular chaperones: the busy life of Hsp90. Curr. Biol. **9**:R322-5.

166. **Mayes, A. E., L. Verdone, P. Legrain, and J. D. Beggs.** 1999. Characterization of Sm-like proteins in yeast and their association with U6 snRNA. EMBO J. **18**:4321-31.

167. **Mayhew, M., A. C. d. Silva, J. Martin, H. Erdjument-Bromage, P. Tempst, and F. U. Hartl.** 1996. Protein folding in the central cavity of the GroEL-GroES chaperonin complex. Nature **379**:420-6.

168. **Mayrand, S., and T. Pederson.** 1983. Heat shock alters nuclear ribonucleoprotein assembly in Drosophila cells. Mol. Cell. Biol. **3**:161-171.

169. **McCarty, J. S., S. Rudiger, H. J. Schonfeld, J. Schneider-Mergener, K. Nakahigashi, T. Yura, and B. Bukau.** 1996. Regulatory region C of the *E. coli* heat shock transcription factor, sigma32, constitutes a DnaK binding site and is conserved among eubacteria. J. Mol. Biol. **256:**829-37.

170. **Merck, K. B., P. J. Groenen, C. E. Voorter, W. A. d. Haard-Hoekman, J. Horwitz, H. Bloemendal, and W. W. d. Jong.** 1993. Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein. A family of chaperones. J. Biol. Chem. **268:**1046-52.

171. **Mestril, R., S. H. Chi, M. R. Sayen, K. O'Reilly, and W. H. Dillmann.** 1994. Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury. J. Clin. Invest. **93:**759-67.

172. **Michels, A. A., B. Kanon, A. W. Konings, K. Ohtsuka, O. Bensaude, and H. H. Kampinga.** 1997. Hsp70 and Hsp40 chaperone activities in the cytoplasm and the nucleus of mammalian cells. J. Biol. Chem. **272:**33283-9.

173. **Minami, Y., J. Hohfeld, K. Ohtsuka, and F. U. Hartl.** 1996. Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. J. Biol. Chem. **271:**19617-24.

174. **Moczko, M., B. Schonfisch, W. Voos, N. Pfanner, and J. Rassow.** 1995. The mitochondrial ClpB homolog Hsp78 cooperates with matrix Hsp70 in maintenance of mitochondrial function. J. Mol. Biol. **254:**538-43.

175. **Moenne, A., S. Camier, G. Anderson, F. Margottin, J. Beggs, and A. Sentenac.** 1990. The U6 gene of Saccharomyces cerevisiae is transcribed by RNA polymerase C (III) in vivo and *in vitro*. EMBO J. **9:**271-7.

176. **Moore, M., J. Schaack, S. B. Baim, R. I. Morimoto, and T. Shenk.** 1987. Induced heat shock mRNAs escape the nucleocytoplasmic transport block in adenovirus-infected HeLa cells. Mol. Cell. Biol. **7:**4505-12.

177. **Morimoto, R., A. Tissières, and C. Georgopoulos.** 1994. The biology of the heat shock proteins and the molecular chaperones. Cold Spring Harbour Laboratory Press, New York.

178. **Morimoto, R. I.** 1993. Cells in stress: transcriptional activation of heat shock genes. Science **259:**1409-1410.

179. **Morimoto, R. I., M. P. Kline, D. N. Bimston, and J. J. Cotto.** 1997. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem. **32:**17-29.

180. **Morimoto, R. I., P. E. Kroeger, and J. J. Cotto.** 1996. The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. EXS **77:**139-163.

181. **Morimoto, R. I., and M. G. Santoro.** 1998. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. Nat. Biotechnol. **16**:833-838.

182. **Motohashi, K., Y. Watanabe, M. Yohda, and M. Yoshida.** 1999. Heat-inactivated proteins are rescued by the DnaK.J-GrpE set and ClpB chaperones. Proc. Natl. Acad. Sci. **96**:7184-9.

183. **Muhich, M. L., and J. C. Boothroyd.** 1988. Polycistronic transcripts in trypanosomes and their accumulation during heat shock: evidence for a precursor role in mRNA synthesis. Mol. Cell. Biol. **8**:3837-3846.

184. **Nathan, D. F., and S. Lindquist.** 1995. Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. Mol. Cell. Biol. **15**:3917-25.

185. **Nathan, D. F., M. H. Vos, and S. Lindquist.** 1997. *In vivo* functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. Proc. Natl. Acad. Sci. **94**:12949-56.

186. **Neidhardt, F. C., R. A. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18**:295-329.

187. **Nelissen, R. L., C. L. Will, W. J. v. Venrooij, and R. Luhrmann.** 1994. The association of the U1-specific 70K and C proteins with U1 snRNPs is mediated in part by common U snRNP proteins. EMBO J. **13**:4113-25.

188. **Nelson, R. J., T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne, and E. A. Craig.** 1992. The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. Cell **71**:97-105.

189. **Netzer, W. J., and F. U. Hartl.** 1998. Protein folding in the cytosol: chaperonin-dependent and -independent mechanisms. Trends Biochem. Sci. **23**:68-73.

190. **Neubauer, G., A. Gottschalk, P. Fabrizio, B. Seraphin, R. Luhrmann, and M. Mann.** 1997. Identification of the proteins of the yeast U1 small nuclear ribonucleoprotein complex by mass spectrometry. Proc. Natl. Acad. Sci. **94**:385-90.

191. **Newman, A.** 1998. RNA splicing. Curr. Biol. **8**:R903-5.

192. **Newman, A. J.** 1994. Pre-mRNA splicing. Curr. Opin. Gen. Dev. **4**:298-304.

193. **Newnam, G. P., R. D. Wegrzyn, S. L. Lindquist, and Y. O. Chernoff.** 1999. Antagonistic interactions between yeast chaperones hsp104 and hsp70 in prion curing. Mol. Cell. Biol. **19**:1325-1333.

194. **Noble, S. M., and C. Guthrie.** 1996. Identification of novel genes required for yeast pre-mRNA splicing by means of cold-sensitive mutations. Genetics **143**:67-80.

195. **Nollen, E. A., J. F. Brunsting, H. Roelofsen, L. A. Weber, and H. H. Kampinga.** 1999. *In vivo* chaperone activity of heat shock protein 70 and thermotolerance. Mol. Cell. Biol. **19**:2069-79.

196. **Nover, L.** 1991. Heat Shock Response. CRC Press, Boca Raton, Florida.

197. **Pak, M., and S. Wickner.** 1997. Mechanism of protein remodeling by ClpA chaperone. Proc. Natl. Acad. Sci. **94**:4901-6.

198. **Panaretou, B., C. Prodromou, S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl.** 1998. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone *in vivo*. EMBO J. **17**:4829-36.

199. **Pannone, B. K., D. Xue, and S. L. Wolin.** 1998. A role for the yeast La protein in U6 snRNP assembly: evidence that the La protein is a molecular chaperone for RNA polymerase III transcripts. EMBO J. **17**:7442-53.

200. **Parsell, D. A., A. S. Kowal, and S. Lindquist.** 1994. *Saccharomyces cerevisiae* Hsp104 protein. J. Biol. Chem. **269**:4480-4487.

201. **Parsell, D. A., A. S. Kowal, M. A. Singer, and S. Lindquist.** 1994. Protein disaggregation mediated by heat-shock protein Hsp104. Nature **372**:475-8.

202. **Parsell, D. A., and S. Lindquist.** 1993. The function of Heat-Shock Proteins in stress tolerance: degradation and reactivation of damaged proteins. Ann. Rev. Genet. **27**:437-496.

203. **Parsell, D. A., and S. L. Lindquist.** 1994. Heat shock proteins and stress tolerance, p. 457-494. *In* R. Morimoto, A. Tissières, and C. Georgopoulos (ed.), The biology of the heat shock proteins and the molecular chaperones. Cold Spring Harbour Laboratory Press, New York.

204. **Patterson, B., and C. Guthrie.** 1987. An essential yeast snRNA with a U5-like domain is required for splicing *in vivo*. Cell **49**:613-624.

205. **Perisic, O., H. Xiao, and J. T. Lis.** 1989. Stable binding of Drosophila heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell **59**:797-806.

206. **Pfund, C., N. Lopez-Hoyo, T. Ziegelhoffer, B. A. Schilke, P. Lopez-Buesa, W. A. Walter, M. Wiedmann, and E. A. Craig.** 1998. The molecular chaperone Ssb from *Saccharomyces cerevisiae* is a component of the ribosome-nascent chain complex. EMBO J. **17**:3981-9.

207. **Piper, P.** 1998. Differential role of Hsps and trehalose in stress tolerance. Trends Microbiol. **6**:43-44.

208. **Piper, P. W.** 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiol. Lett. **134**:121-7.

209. **Plumier, J. C., B. M. Ross, R. W. Currie, C. E. Angelidis, H. Kazlaris, G. Kollias, and G. N. Pagoulatos.** 1995. Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. J. Clin. Invest. **95:**1854-60.

210. **Preville, X., F. Salvemini, S. Giraud, S. Chaufour, C. Paul, G. Stepien, M. V. Ursini, and A. P. Arrigo.** 1999. Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. Exp. Cell. Res. **247:**61-78.

211. **Prodromou, C., S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl.** 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell **90:**65-75.

212. **Raghunathan, P. L., and C. Guthrie.** 1998. RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. Curr. Biol. **8:**847-55.

213. **Raghunathan, P. L., and C. Guthrie.** 1998. A spliceosomal recycling factor that reanneals U4 and U6 small nuclear ribonucleoprotein particles. Science **279:**857-860.

214. **Raker, V. A., K. Hartmuth, B. Kastner, and R. Luhrmann.** 1999.
Spliceosomal U snRNP Core Assembly: Sm Proteins Assemble onto
an Sm Site RNA Nonanucleotide in a Specific and
Thermodynamically Stable Manner. Mol. Cell. Biol. **19:**6554-6565.

215. **Raker, V. A., G. Plessel, and R. Luhrmann.** 1996. The snRNP core
assembly pathway: identification of stable core protein heteromeric
complexes and an snRNP subcore particle *in vitro*. EMBO J. **15:**2256-
69.

216. **Rinke, J., and J. A. Steitz.** 1985. Association of the lupus antigen La
with a subset of U6 snRNA molecules. Nucleic Acids Res. **13:**2617-
29.

217. **Rinke, J., and J. A. Steitz.** 1982. Precursor molecules of both human
5S ribosomal RNA and transfer RNAs are bound by a cellular
protein reactive with anti-La lupus antibodies. Cell **29:**149-59.

218. **Ritosa, F.** 1962. A new puffing pattern induced by temperature shock
and DNP in Drosophila. Experienta **18:**571-573.

219. **Rogalla, T., M. Ehrnsperger, X. Preville, A. Kotlyarov, G. Lutsch, C.
Ducasse, C. Paul, M. Wieske, A. P. Arrigo, J. Buchner, and M.
Gaestel.** 1999. Regulation of Hsp27 oligomerization, chaperone
function, and protective activity against oxidative stress/tumor
necrosis factor alpha by phosphorylation. J. Biol. Chem. **274:**18947-56.

220. **Roy, J., K. Kim, J. R. Maddox, J. G. Anthony, and J. John L. Woolford.** 1995. The final stages of spliceosome maturation require Spp2p that can interact with the DEAH box protein Prp2p and promote step 1 of splicing. RNA **1**:375-390.

221. **Ruby, S. W., T. H. Chang, and J. Abelson.** 1993. Four yeast spliceosomal proteins (PRP5, PRP9, PRP11, and PRP21) interact to promote U2 snRNP binding to pre-mRNA. Genes Dev. **7**:1909-25.

222. **Rüdiger, S., A. Buchberger, and B. Bukau.** 1997. Interaction of Hsp70 chaperones with substrates. Nature struct. Biol. **4**:342-349.

223. **Rüdiger, S., L. Germeroth, J. Schneider-Mergener, and B. Bukau.** 1997. Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J. **16**:1501-1507.

224. **Rymond, B. C.** 1993. Convergent transcripts of the yeast PRP38-SMD1 locus encode two essential splicing factors, including the D1 core polypeptide of small nuclear ribonucleoprotein particles. Proc. Natl. Acad. Sci. **90**:848-52.

225. **Saavedra, C., K.-S. Tung, D. C. Amberg, A. K. Hopper, and C. N. Cole.** 1996. Regulation of mRNA export in response to stress in *Saccharomyces cerrevisiae*. Genes Dev. **10**:1608-1620.

226. **Saavedra, C. A., C. M. Hammell, C. V. Heath, and C. N. Cole.** 1997. Yeast heat shock mRNAs are exported through a distinct pathway defined by Rip1p. Genes Dev. **11**:2845-2856.

227. **Sanchez, Y., and S. L. Lindquist.** 1990. Hsp104 required for induced thermotolerance. Science **248**: 679-683.

228. Sanchez, Y., D. A. Parsell, J. Taulien, J. L. Vogel, E. A. Craig, and S. Lindquist. 1993. Genetic evidence for a functional relationship between Hsp104 and Hsp70. J. Bacteriol. **175**:6484-6491.

229. Sanchez, Y., J. Taulien, K. A. Borkovich, and S. Lindquist. 1992. Hsp104 is required for tolerance to many forms of stress. EMBO J. **11**:2357-2364.

230. Santagata, S., D. Bhattacharyya, F. H. Wang, N. Singha, A. Hodtsev, and E. Spanopoulou. 1999. Molecular cloning and characterization of a mouse homolog of bacterial ClpX, a novel mammalian class II member of the Hsp100/Clp chaperone family. J. Biol. Chem. **274**:16311-9.

231. Sarge, K. D., S. P. Murphy, and R. I. Moimoto. 1993. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol. Cell. Biol. **13**:1392-407.

232. Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist. 1996. HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biotech. **21**:289-296.

233. Schirmer, E. C., S. Lindquist, and E. Vierling. 1994. An Arabidopsis heat shock protein complements a thermotolerance defect in yeast. Plant Cell **6**:1899-909.

234. **Schmitt, M., W. Neupert, and T. Langer.** 1996. The molecular chaperone Hsp78 confers compartment-specific thermotolerance to mitochondria. J. Cell. Biol. **134**:1375-86.

235. **Schoffl, F., R. Prandl, and A. Reindl.** 1998. Regulation of the heat-shock response. Plant Physiol. **117**:1135-41.

236. **Schumacher, R. J., W. J. Hansen, B. C. Freeman, E. Alnemri, G. Litwack, and D. O. Toft.** 1996. Cooperative action of Hsp70, Hsp90, and DnaJ proteins in protein renaturation. Biochemistry **35**:14889-98.

237. **Schumacher, R. J., R. Hurst, W. P. Sullivan, N. J. McMahon, D. O. Toft, and R. L. Matts.** 1994. ATP-dependent chaperoning activity of reticulocyte lysate. J. Biol. Chem. **269**:9493-9.

238. **Segal, G., and E. Z. Ron.** 1998. Regulation of heat-shock response in bacteria. Ann. N. Y. Acad. Sci. **851**:147-51.

239. **Séraphin, B.** 1995. Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. EMBO J. **14**:2089-98.

240. **Séraphin, B., and M. Rosbash.** 1989. Identification of Functional U1 snRNA-Pre-mRNA Complexes Committed to Spliceosome Assembly and Splicing. Cell **59**:349-358.

241. **Shannon, K. W., and C. Guthrie.** 1991. Supressors of a U4 snRNA mutation define a novel U6 snRNP protein with RNA-bindind motifs. Genes Dev. **5**:773-785.

242. **Shukla, R. R., Z. Dominski, T. Zwierzynski, and R. Kole.** 1990. Inactivation of splicing factors in HeLa cells subjected to heat shock. J. Biol. Chem. **265:**20377-20383.

243. **Shulga, N., P. James, E. A. Craig, and D. S. Goldfarb.** 1999. A nuclear export signal prevents *Saccharomyces cerevisiae* Hsp70 Ssb1p from stimulating nuclear localization signal-directed nuclear transport. J. Biol. Chem. **274:**16501-7.

244. **Siliciano, P. G., M. H. Jones, and C. Guthrie.** 1987. *Saccharomyces cerevisiae* has a U1-like small nuclear RNA with unexpected properties. Science **237:**1484-1487.

245. **Silverstein, A. M., N. Grammatikakis, B. H. Cochran, M. Chinkers, and W. B. Pratt.** 1998. p50(cdc37) binds directly to the catalytic domain of Raf as well as to a site on hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site. J. Biol. Chem. **273:**20090-5.

246. **Singer, M. A., and S. Lindquist.** 1998. Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. Mol. Cell. **1:**639-648.

247. **Singer, M. A., and S. Lindquist.** 1998. Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. Trends Biotechnol. **16:**460-68.

248. **Singh, S. K., and M. R. Maurizi.** 1994. Mutational analysis demonstrates different functional roles for the two ATP-binding sites in ClpAP protease from *Escherichia coli*. J. Biol. Chem. **269:**29537-45.

249. **Smith, V., and B. G. Barrell.** 1991. Cloning of a yeast U1 snRNP 70K protein homologue: functional conservation of an RNA-binding domain between humans and yeast. EMBO J. **10:**2627-34.

250. **Sorger, P. K.** 1990. Yeast heat shock factor contains separable transient and sustained response transcriptional activators. Cell **62:**793-805.

251. **Sorger, P. K., M. J. Lewis, and H. R. Pelham.** 1987. Heat shock factor is regulated differently in yeast and HeLa cells. Nature **329:**81-4.

252. **Sorger, P. K., and H. R. Pelham.** 1987. Purification and characterization of a heat-shock element binding protein from yeast. EMBO J. **6:**3035-41.

253. **Sorger, P. K., and H. R. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54:**855-64.

254. **Squires, C., and C. L. Squires.** 1992. The Clp proteins: proteolysis regulators or molecular chaperones? J. Bacteriol. **174:**1081-15.

255. **Squires, C. L., S. Pedersen, B. M. Ross, and C. Squires.** 1991. ClpB is the *Escherichia coli* heat shock protein F84.1. J. Bacteriol. **173:**4254-62.

256. **Staley, J. P., and C. Guthrie.** 1998. Mechanical devices of the spliceosome: motors, clocks, springs, and things. Cell **92:**315-326.

257. **Stege, G. J. J., J. F. Brunsting, H. H. Kampinga, and A. W. T. Konings.** 1995. Thermotolerance and nuclear protein aggregation: protection against initial damage or better recovery? J. Cell. Physiol. **164:**579-586.

258. **Stevens, S. W., and J. Abelson.** 1999. Purification of the yeast U4/U6.U5 small nuclear ribonucleoprotein particle and identification of its proteins. Proc. Natl. Acad. Sci. U S A **96:**7226-31.

259. **Stojadinovic, A., J. Kiang, R. Smallridge, R. Galloway, and T. Shea-Donohue.** 1995. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. Gastroenterology **109:**505-15.

260. **Tani, T., R. J. Derby, Y. Hiraoka, and D. L. Spector.** 1996. Nucleolar accumulation of poly (A)+ RNA in heat-shocked yeast cells: implication of nucleolar involvement in mRNA transport. Mol. Biol. Cell. **7:**173-92.

261. **Teigelkamp, S., T. Achsel, C. Mundt, S. F. Gothel, U. Cronshagen, W. S. Lane, M. Marahiel, and R. Luhrmann.** 1998. The 20kD protein of human [U4/U6.U5] tri-snRNPs is a novel cyclophilin that forms a complex with the U4/U6-specific 60kD and 90kD proteins. RNA **4:**127-41.

262. **Teter, S. A., W. A. Houry, D. Ang, T. Tradler, D. Rockabrand, G. Fischer, P. Blum, C. Georgopoulos, and F. U. Hartl.** 1999. Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. Cell **97:**755-65.

263. **Thompson, M. W., and M. R. Maurizi.** 1994. Activity and specificity of *Escherichia coli* ClpAP protease in cleaving model peptide substrates. J. Biol. Chem. **269:**18201-8.

264. **Thompson, M. W., S. K. Singh, and M. R. Maurizi.** 1994. Processive degradation of proteins by the ATP-dependent Clp protease from *Escherichia coli*. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis. J. Biol. Chem. **269**:18209-15.

265. **Tissieres, A., H. K. Mitchell, and U. M. Tracy.** 1974. Protein synthesis in salivary glands of *Drosophila melanogaster*: Relation to chromosome puffs. J. Mol. Biol. **84**:389-98.

266. **Utans, U., S.-E. Behrans, R. Lührmann, R. Kole, and A. Krämer.** 1992. A splicing factor which is inactivated during *in vivo* heat shock is functionally equivalent to the [U4/U6.U5] triple snRNP specific proteins. Genes Dev. **6**:631-641.

267. **Venetianer, A., M. F. Dubois, V. T. Nguyen, S. Bellier, S. J. Seo, and O. Bensaude.** 1995. Phosphorylation state of the RNA polymerase II C-terminal domain (CTD) in heat-shocked cells. Possible involvement of the stress-activated mitogen-activated protein (MAP) kinases. Eur. J. Biochem. **233**:83-92.

268. **Vijayraghavan, U., M. Company, and J. Abelson.** 1989. Isolation and characterization of pre-mRNA splicing mutants of *Saccharomyces cerevisiae*. Genes Dev. **3**:1206-16.

269. **Vogel, J. L., D. A. Parsell, and S. Lindquist.** 1995. Heat-shock proteins Hsp104 and Hsp70 reactivate mRNA splicing after heat inactivation. Curr. Biol. **5**:306-317.

270. **Waters, E. R., and B. A. Schaal.** 1996. Heat shock induces a loss of rRNA-encoding DNA repeats in *Brassica nigra*. Proc. Natl. Acad. Sci. **93**:1449-52.

271. **Wawrzynow, A., B. Banecki, and M. Zylicz.** 1996. The Clp ATPases define a novel class of molecular chaperones. Mol. Microbiol. **21**:895-899.

272. **Wawrzynow, A., D. Wojtkowiak, J. Marszalek, B. Banecki, M. Jonsen, B. Graves, C. Georgopoulos, and M. Zylicz.** 1995. The ClpX heat-shock protein of *Escherichia coli*, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. EMBO J. **14**:1867-77.

273. **Weber-Ban, E. U., B. G. Reid, A. D. Miranker, and A. L. Horwich.** 1999. Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. Nature **401**:90-3.

274. **Weidenhammer, E. M., M. Ruiz-Noriega, and J. L. Woolford.** 1997. Prp31p promotes the association of the U4/U6.U5 tri-snRNP with prespliceosomes to form spiceosomes in *Saccharomyces cerrevisiae*. Mol. Cell. Biol. **17**:3580-3588.

275. **Welch, W. J., and J. R. Feramisco.** 1985. Disruption of the three cytoskeletal networks in mammalian cells does not affect transcription, translation, or protein translocation changes induced by heat shock. Mol. Cell. Biol. **5**:1571-1581.

276. **Welch, W. J., and J. R. Feramisco.** 1982. Purification of the major mammalian heat shock proteins. J. Biol. Chem. **257:**14949-59.

277. **Welch, W. J., and J. P. Suhan.** 1986. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell. Biol. **103:**2035-52.

278. **Welch, W. J., and J. P. Suhan.** 1985. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. J. Cell. Biol. **101:**1198-1211.

279. **Wells, S. E., M. Neville, M. Haynes, J. Wang, H. Igel, and M. Ares.** 1996. CUS1, a suppressor of cold-sensitive U2 snRNA mutations, is a novel yeast splicing factor homologous to human SAP 145. Genes Dev. **10:**220-32.

280. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of *hsp70* genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2568-2577.

281. **Westwood, J. T., J. Clos, and C. Wu.** 1991. Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature **353:**822-7.

282. **Wickner, S., S. Gottesman, D. Skowyra, J. Hoskins, K. McKenney, and M. R. Maurizi.** 1994. A molecular chaperone, ClpA, functions like DnaK and DnaJ. Proc. Natl. Acad. Sci. **91:**12218-22.

283. **Will, C. L., and R. Lührmann.** 1997. Protein functions in pre-mRNA splicing. Curr. Opin. Cell. Biol. **9**:320-328.

284. **Wojtkowiak, D., C. Georgopoulos, and M. Z. M.** 1993. Isolation and characterization of ClpX, a new ATP-dependent Specificity component of the Clp protease of *Escherichia coli*. J. Biol. Chem. **268**:22609-17.

285. **Woo, K. M., K. I. Kim, A. L. Goldberg, D. B. Ha, and C. H. Chung.** 1992. The heat-shock protein ClpB in *Escherichia coli* is a protein-activated ATPase. J. Biol. Chem. **267**:20429-34.

286. **Xu, D., S. Nouraini, D. Field, S. J. Tang, and J. D. Friesen.** 1996. An RNA-dependent ATPase associated with U2/U6 snRNAs in pre-mRNA splicing. Nature **381**:709-13.

287. **Yan, D., R. Perriman, H. Igel, K. J. Howe, M. Neville, and M. Ares.** 1998. CUS2, a yeast homolog of human Tat-SF1, rescues function of misfolded U2 through an unusual RNA recognition motif. Mol. Cell. Biol. **18**:5000-9.

288. **Yoo, C. J., and S. L. Wolin.** 1994. La proteins from *Drosophila melanogaster* and *Saccharomyces cerevisiae*: a yeast homolog of the La autoantigen is dispensable for growth. Mol. Cell. Biol. **14**:5412-24.

289. **Yost, H. J., and S. Lindquist.** 1991. Heat shock proteins affect RNA processing during the heat shock response of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**:1062-1068.

290. **Yost, H. J., and S. Lindquist.** 1986. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. Cell **45**:185-193.

291. **Yura, T., H. Nagai, and H. Mori.** 1993. Regulation of the heat-shock response in bacteria. Annu. Rev. Microbiol. **47**:321-350.

292. **Ziemienowicz, A., M. Zylicz, C. Floth, and U. Hübscher.** 1995. Calf thymus Hsc70 protein protects and reactivates prokaryotic and eukaryotic enzymes. J. Biol. Chem. **270**:15479-84.

293. **Zieve, G. W., and R. A. Sauterer.** 1990. Cell biology of the snRNP particles. Crit. Rev. Biochem. Mol. Biol. **25**:1-46.

294. **Zou, J., Y. Guo, T. Guettouche, D. F. Smith, and R. Voellmy.** 1998. Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell **94**:471-80.