Hyperthermia and protein aggregation
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GENERAL INTRODUCTION
1.1 HYPERTHERMIA

Hyperthermia can be described as any temperature above the physiological growth temperature. For humans this would be any temperature above 37°C. Exposure of cells to heat treatments above the temperature of 40°C has a sterilizing or "killing" effect. Hyperthermia has been recognized as a possible adjuvant in cancer therapy [Overgaard 1985]. Already the Egyptians described the medical use of hyperthermia in a study case of a patient with breast cancer some 5000 years ago [Overgaard 1985]. Also the ancient Greek used hyperthermia, by the induction of fever, to treat many diseases, including cancer [Hahn 1982]. In the nineteenth century tumor regression was observed after bacterial infections accompanied by fever [Busch 1866, Coley 1893, reviewed by Nauts 1981]. Westermark used a coil containing hot water as a controlled localized source of heat in the treatment of cervical cancer and found that with the use of higher temperatures the time of treatment for release of symptoms became shorter [Westermark 1898]. Westermark's son continued the studies on the use of hyperthermia in cancer therapy and described the effects of heat on normal and malignant tissues in the rat [Westermark 1927]. In the last decades hyperthermia is extensively investigated, the biological effects as well as its use as adjuvant in cancer therapy. The hyperthermic temperatures of interest for cancer therapy are limited to the temperature range of 40 - 46°C, based on effectiveness and damage to the surrounding normal tissue. Beside the cell killing effect of heat alone, it was found that heat acts synergistically with ionizing radiation [Müller 1910, 1912, Dewey et al. 1977, 1980] and with a number of anticancer drugs [reviewed by Field and Hand 1990, Klostergaard and Tomasovic 1992].

1.1.1 Cell survival curves

The killing or sterilizing effect on proliferating cells (reproductive capacity) of a hyperthermic treatment on mammalian cells can be represented as survival curves (figure 1.1). The surviving cell fraction (cell fraction capable of reproduction) is plotted on a logarithmic scale versus the duration of the heating on a linear scale. Cell killing by hyperthermia depends on both the temperature used as well as on the duration of the exposure to that temperature.

Several mathematical models have been postulated to describe heat-induced cell killing. The most uncomplicated model is assuming a one-step reaction

\[ S = e^{-kt} \]

where S is survival, t is the treatment time and k the inactivation rate at the treatment temperature [Landry and Marceau 1978]. k is often replaced by 1/D₀ as used in radiation biology, where D₀ is the time resulting in 1/e cell survival [Hahn 1982].

model, however, does not describe all survival curves since it does not account for the shoulder part of the curve.

Figure 1.1 Cell survival after heat treatments. (a) The percentage of cells surviving a heat treatment is plotted on a logarithmic scale versus the treatment time on a linear scale; (b) representation of a typical survival curve using the multi-target single-hit model (see text for further details).

The most used model to describe these survival curves is the "multi target-single hit" model

\[
S = 1-(1-e^{-D/D_0})^n
\]

where \( n \) is the number of targets to be inactivated before the cell is killed [Alper 1979, Hahn 1982]. The survival curves in this model are characterized by the parameters \( n \), \( D_0 \) and \( D_q \) (quasi threshold dose) (figure 1.1). \( D_q \) can be calculated by the expression \( D_q = \ln n^*D_0 \). Using this model, one can construct Arrhenius plots based on the \( D_q \) of the survival curves. These plots show an inflection point around 42.5°C [Dewey et al. 1977]. This inflection point seems to be associated with the induction of thermotolerance i.e. the transient resistance against heat killing (see 1.2.4) [Lepock and Kruuv 1980, Li et al. 1982]. The Arrhenius plots indicate that the activation enthalpy for hyperthermic cell killing above 42.5°C is around 600 kJ/mol [Westra and Dewey 1971, Hahn 1982], which is about the same as known for protein denaturation [Johnson et al. 1954], suggesting that protein denaturation is the main cause for hyperthermic cell killing.
Using the activation enthalpy and entropy for hyperthermic cell killing (determined from Arrhenius plots based on $D_0$'s) Lepock et al. [1988, 1990a] introduced a thermodynamic model for hyperthermic cell killing based on the existence of a "critical target". The rate of cell killing is thought to correspond with the rate of inactivation/denaturation of this "hypothetical critical target". The calculated transition temperature of this critical target appears to be 46.0°C for V79-WNRE cells, when the temperature is raised by 1°C/min [Lepock et al. 1990a]. Using the scan rates 0.1°C/min and 10°C/min for Differential Scanning Calorimetry (see 1.1.2.1), the calculated transition temperatures are 43.5°C and 48.5°C respectively. Thus, transition temperatures are only meaningful when the scan rate used in the measurements is specified [Lepock et al. 1990a]. Cellular fractionation to localize this "hypothetical critical target" showed that in all subcellular fractions protein denaturation occurs at 46°C.

A fourth model, which nicely describes heat killing under various conditions (single heating, thermotolerance, step-down heating) was proposed by Jung [1986, 1991]. This model assumes a two step process. The first step is the production of nonlethal lesions, that can be converted into lethal lesions upon further heating in the second step. After heating the cells for a time period (t) at a certain temperature, the surviving fraction (S) is given by the equation:

\[
S = \exp[(p/c)^*(1-c^t-\exp(-c^t))]
\]

where $p$ is the rate constant for the production of nonlethal lesions per cell per unit of time, and $c$ is the rate constant for the conversion of a nonlethal lesion into a lethal lesion per unit of time. One lethal lesion is sufficient to kill a cell. Jung assumed that the production and conversion of nonlethal lesions are random and the rate constants only depend on the temperature of the heat treatment, while the number of nonlethal lesions is not influenced by repair processes during single or fractionated heat treatments. The nature of the lesions is not specified, so it may be possible to apply it to many forms of molecular damage.

1.1.2 Protein denaturation

Under physiological conditions an equilibrium between the native (N) and a denatured (D) state of proteins exists [Creighton 1984, Dill and Shortle 1991], with most proteins in the folded native state [Privalov 1979, Creighton 1990, Dill and Shortle 1991].

\[ N \xrightarrow{\text{equilibrium}} D \]

This equilibrium is determined by the free energy of the two states. The stability of the native state is at its maximum around physiological temperatures and declines upon heating or cooling [Graves et al. 1965, Tamura et al. 1991a, 1991b]. Upon heating (or
cooling) proteins undergo an order-disorder transition from the native to the unfolded or denatured state referred to as protein denaturation. Protein denaturation/unfolding has long been considered a two state phenomenon with only the completely folded and the completely unfolded state (see equation) [Creighton 1984, Privalov 1979], the conformation of the unfolded form assumed to be close to random coil [Privalov 1979]. More recent experiments concerning protein folding and protein unfolding now indicate the presence of several states of folding/unfolding [Dill and Shortle 1991, Seckler and Jaenicke 1992]. During protein folding, at least three main states can be recognized; the unfolded state, the compact intermediate or molten globule and the native state [Kim and Baldwin 1990, Creighton 1990]. Comparable states were observed during protein denaturation. Under moderate denaturing conditions the "compact denatured" state (D_c) is found, while under strongly denaturing conditions the "unfolded denatured" state (D_u) is obtained. The unfolded denatured state has a highly open conformation with almost no secondary structure (close to random coil), whereas the compact denatured state still contains hydrophobic clusters and considerable secondary structure [Dill and Shortle 1991]. So, protein denaturation (as protein folding) can be described as (at least) a two step process, with possible intermediates between the native and compact denatured state:

\[ N \leftrightarrow D_c \leftrightarrow D_u \]

Protein denaturation in cell free systems is found to be reversible, but this is limited to very diluted protein solutions [Creighton 1984, Privalov 1979] (see also molecular chaperones 1.2.1). In situ (cells in vitro) and at the higher protein concentration in vitro, denatured proteins will form aggregates. This aggregation occurs already when the proteins are in the compact denatured state. Once aggregated, protein disaggregation does not occur spontaneously, but only occurs by the aid of molecular chaperones (see 1.2.1). So, protein aggregation also appears to be a two step process, which can be modeled by the following scheme:

\[ N \rightarrow D_c \rightarrow A \]

+ hsp's and cofactors

Numerous techniques exist for measuring protein denaturation. Any parameter sensitive to protein conformation can be used such as circular dichroism, fluorescence spectroscopy, sedimentation velocity, gel filtration [Seckler and Jaenicke 1992], NMR spectroscopy [Dill and Shortle 1991] or electron spin resonance [Burgman and Konings 1992]. These measurements are based on differences in structure between the native, folded state and the denatured, unfolded state of the protein. Another approach is to measure a property of the transition from the native protein to the denatured state itself. Temperature induced transitions such as protein denaturation
are endothermic; heat is absorbed during the transition. Differential scanning calorimetry is based on the measurement of the extent of heat absorbed [Lepock et al. 1983, 1988]. Denaturation and subsequent aggregation of proteins are determined by insolubilization assays, gel mobility/filtration and protein activity measurements [Nguyen et al. 1989, Pinto et al. 1991, Burgman and Konings 1992] (see also molecular chaperones 1.2.1).

1.1.2.1 Heat-induced protein denaturation and aggregation in cells

Using DSC, Lepock and colleagues [1988, 1990a, 1992] studying protein denaturation in V79 cells and intact hepatocytes observed several irreversible peaks with transition temperatures of 45 - 98°C. These primarily represent protein denaturation with minor contributions from DNA and RNA melting. In normo, meso, and thermophilic bacteria, the onset seems to correlate with hyperthermic cell killing: the maximum growth temperatures for four tested bacteria, ranging from 32°C to 69°C, are 1 - 4°C below the onset temperatures for denaturation [Lepock et al. 1990b]. So, it appears that some protein denaturation can be tolerated in these organisms before growth ceases, but more extensive denaturation is lethal, whether it occurs at 30°C or at 70°C. In eukaryotes, the onset for protein denaturation was found to be around 40°C. Thermostolerance, cycloheximide and D₂O all increased the thermostability of proteins as determined by DSC and resulted in increased survival levels after hyperthermia [Lepock et al. 1990a, Borrelli et al. 1991, 1992]. These results are confirmed by studies of Burgman and Konings [1992], using ESR measurements and thermal gel analysis. It was shown that increasing thermostolerance ratios paralleled a shift in protein transitions to higher temperatures in the membrane fractions isolated from thermotolerant cells. Using heat sensitizers as procaine, 4-aminobenzamide or ethanol, protein denaturation temperatures were lowered; the proteins became more unstable [Burgman 1993]. So, also in eukaryotes a correlation between protein denaturation and heat killing is apparent.

Protein denaturation and subsequent aggregation has been shown to occur throughout the entire cell. Before mentioned studies [Burgman and Konings 1992, Burgman 1993, Burgman et al. 1993] pertained to denaturation of membrane proteins (see also 1.1.3.1). Bensaude and colleagues [Nguyen et al. 1989, Pinto et al. 1991, Dubois et al. 1991, Bensaude et al. 1991] found denatured and insolubilized cytoplasmic reporter enzymes after a heat treatment of cells. In these studies it is also shown that glycerol, D₂O and thermostolerance attenuated the heat-induced denaturation and aggregation of several reporter enzymes (1.1.3.2). Also, nuclear proteins have been shown to denature and aggregate upon cellular heating. Again, this was affected by glycerol, D₂O, procaine and thermostolerance (1.1.3.3).

1.1.3 Heat effects on cellular structures
1.1.3.1 Plasma membranes

Small changes in the temperature can drastically alter the structure of biomembranes and as a result of this, impair many membrane-related cellular functions [reviewed by Konings 1988, Laszlo 1992a]. Heat-induced cell surface changes are characterized by a reduction in the number of microvilli and an increased bleb formation, which correlated with cell killing when heated at 45.5°C [Borrelli et al. 1986] but not for lower heat treatments (41.5 or 43.5°C; Kapiszewska and Hopwood 1986).

Heat changes the fluidity of the lipid bilayer of the membrane. Yatvin [1977] proposed that changes in membrane fluidity may be the primary cause of heat induced cell killing. However, later reports demonstrated that changes in the membrane fluidity are probably not directly involved in heat-induced cell death [Lepock et al. 1983, Mehdi et al. 1984, Konings and Ruifrok 1985, Dynlacht and Fox 1992]. Irreversible protein transitions were found to occur in membranes at temperatures above 40°C [Lepock et al. 1983], which might be involved in the observed change in membrane fluidity. Also, Burgman and Konings [1992] using ESR, observed heat-induced conformational changes in crude membrane fractions, containing plasma membrane, mitochondria and microsomes of HeLa S3 cells. The denaturation of membrane proteins probably results in protein aggregation as was observed with freeze fracture electron microscopy [Rice et al. 1985, Arancia et al. 1986] and thermal gel analysis [Burgman and Konings 1992]. Denaturation and/or aggregation of proteins may impair several membrane protein associated functions which, by itself or in combination with other protein damage in the cell, could lead to cell death.

Membrane located receptor and histocompatibility antigen binding were shown to be reduced, due to decreased factor affinity [Magun and Fennie 1981] or a decrease in the number of receptors/antigens [Calderwood and Hahn 1983, Mehdi et al. 1984]. Glucose uptake was increased after heat [Garry and Bostick 1987, Warren et al. 1986], whereas the uptake of neutral amino acid was inhibited [Kwock et al. 1978, Lin et al. 1978]. Hyperthermia also enhances the permeability of the plasmamembrane to several compounds such as adriamycin, polyamines and several ions, although the uptake of the DNA specific dye Hoechst 33342 was inhibited [Laszlo 1992a and references therein]. Heat-induced leakage of potassium [Yi 1979, Ruifrok et al. 1985a, 1985b, 1987] was shown not to be the actual cause of hyperthermic cell killing, and was not found in all cell lines tested [Boonstra et al. 1984, Vidair and Dewey 1986]. Heat had also no effect on the post-heat levels of sodium or magnesium, but did lead to a dose dependent increase in calcium [Vidair and Dewey 1986]. The role of calcium in hyperthermic cell killing is controversial and further research is necessary to resolve it. For a detailed introduction on heat-induced changes in the level of intracellular free calcium and/or total calcium see chapter 2.
1.1.3.2 Heat effects on cytosolic structures and processes

Cytoskeleton

The cytoskeleton, a filamentous protein network in contact with the plasma membrane is composed of three types of filaments: microfilaments, microtubules and intermediate filaments. Cell type dependent hyperthermic effects on these filaments were found [Coss et al. 1982, Glass et al. 1985, Welch and Suhan 1985, Van Bergen en Henegouwen and Linnemans 1987, Wiegant et al. 1987, Laszlo 1992a]. Cytoskeletal alterations were not found to be heat-dose dependent [Van Bergen en Henegouwen et al. 1985] and seemed not related to cell killing by heat [Wiegant et al. 1985]. However, heat-induced alterations of the mitotic spindle [Coss et al. 1982] and centrosome organization can result in the formation of multinucleated, nonclonogenic cells [Vidair et al. 1993].

Mitochondria

Heat induces swelling of the mitochondria accompanied by alterations in the packing of the cristae [Cole and Armour 1988, Welch and Suhan 1985, Wheatley et al. 1989]. At high temperature (45°C), a correlation between the amount of initial mitochondrial damage and cell death was observed, whereas at 43°C no correlation was found [Cole and Armour 1988, Wheatley et al. 1989]. Morphological alterations may be involved in the heat-induced inhibition of metabolic functions as glycolysis and respiration [Dickson and Calderwood 1979]. Inhibition of respiration above 40°C is associated with mitochondrial protein denaturation in Chinese hamster V79 cells [Lepock et al. 1983]. Nevertheless, no decreased intracellular ATP level was observed immediately after hyperthermia [Henle et al. 1984, Lunec and Cresswell 1983].

Protein synthesis

Protein synthesis is transiently inhibited by hyperthermia in a dose dependent manner [Black and Subjeck 1989, Lee et al. 1990]. Several factors could have contributed to this inhibition. First, one of the consequences of a heat treatment is the destruction of polysomes, the locale of protein synthesis [Heine et al. 1971, Welch and Suhan 1985]. Secondly, heat alters the phosphorylation status of certain initiation factors for protein synthesis, such as eIF-2 and eIF4 [Duncan and Hershey 1987, 1989]. Also, the association of initiation factors and polysomes with the cytoskeleton is altered after a heat treatment, suggesting that the heat-induced collapse of the cytoskeleton may be involved in the inhibition of protein synthesis [Laszlo 1992a].

Thermotolerant CHO-HA1 cells showed protection against the inhibition of protein synthesis, although it did not correlate with the development and decay of thermotolerance [Laszlo 1988a]. Also, a heat resistant variant (overexpressing hsc70) was more resistant to this inhibition [Laszlo 1988a]. Both, these thermotolerant cells and heat resistant cells recovered more rapidly from heat-inhibited protein synthesis. Rat-1 cells transfected with human hsp70 also showed protection against heat-
induced inhibition of protein synthesis [Liu et al. 1992]. The recovery of protein synthesis back to control levels was faster in the transfected cells than the parent cells after a heat treatment of 10 or 25 min at 45°C. Whether this was due to facilitated recovery or less initial damage is not clear from these data. In contrast, CHO-10B2 cells and two heat sensitive mutants showed no correlation between the extent of inhibition or recovery time and survival after a heat treatment [Laszlo et al. 1993]. Also, no correlation was found during continuous heating at temperatures below 43°C.

1.1.3.3 Heat effects on nuclear structures and processes

Hyperthermia induces various morphological changes in the eukaryotic cell nucleus including the appearance of actin bundles [Welch and Suhan 1985, Iida et al. 1986], increased vesiculation [Heine et al. 1971], sometimes the disappearance of nucleoli [Welch and Suhan 1985], and an increase in nuclear protein content when isolated after the heat treatment. Beside these morphological changes, functional perturbations in RNA and DNA synthesis occur as a result of hyperthermia.

RNA synthesis

The cells ability to incorporate uridine nucleotides into TCA-precipitable material is inhibited by heat. The dose dependency of this process was more reflected in the recovery after the treatment than in the extent of inhibition [Henle and Leeper 1979]. Transcriptional inactivation may be due to changes in chromatin structures (see below) or/and inactivation of RNA polymerases, although the latter seems not very likely [Caizergues-Ferrer et al. 1980]. The most heat sensitive process during RNA synthesis seems the processing of pre-rRNA [Sadis et al. 1988], although also the synthesis and splicing of mRNA is suppressed as well after heating cells (see also translational control of hsp’s, 1.2.3.2). Recovery of total RNA synthesis was found to be faster in thermotolerant cells and heat resistant variants [Laszlo 1992b, Laszlo et al. 1993, Liu et al. 1992] and slower in heat sensitive variants [Laszlo et al. 1993] indicating that a correlation may exist between total RNA synthesis and heat sensitivity.

DNA synthesis

DNA synthesis is, as observed for RNA and protein synthesis, also inhibited by hyperthermia dependent on the time and temperature of heating [Henle and Leeper 1982, Wong and Dewey 1982, Warters and Stone 1984]. Elevated temperatures results in perturbations in initiation of a cluster of replicons, elongation of newly synthesized DNA, assembly into nucleosomes and ligation of cluster size DNAs into 'chromosome size' molecules [for review Roti Roti and Laszlo 1988]. Initiation of DNA synthesis appears to be the most heat sensitive process and recovers less rapidly upon reincubation at 37°C [Wong and Dewey 1982, Warters and Stone 1983].
Inhibition of DNA synthesis seems not to be due to a depletion of nucleotide precursors [Warters and Stone 1984], heat-induced inhibition of histone synthesis [Warters and Stone 1983] or inactivation of enzymes involved in DNA synthesis, for instance DNA polymerase α [Kampinga et al. 1985]. It is also unlikely that DNA damage is responsible for the inhibition of DNA synthesis, since little DNA damage is observed immediately after hyperthermia [Warters and Stone 1983]. And, if occurring it is not in a way correlated to heat sensitivity [Jorritsma and Konings 1986]. Thus, as for RNA synthesis, inhibition of DNA synthesis appears to be due to heat induced changes in chromatin structure due to nuclear protein aggregation (see below).

**Nuclear protein aggregation**


![Figure 1.2](image.png)

**Figure 1.2** The kinetics of the heat-induced increase in chromatin protein content (redrawn from Roti Roti et al. 1979). The relative protein content is plotted as a function of time at the temperatures indicated and is defined as the protein-to-DNA ratio of chromatin isolated from heated cells, divided by the same ratio determined for chromatin isolated from control cells.

The extent of the increase depends on both the hyperthermic temperature and the duration of the treatment [Roti Roti and Winward 1978, Roti Roti et al. 1979, Tomasovic et al. 1978] (figure 1.2). This heat-induced increase in nuclear protein content is not due to protein cross migration during isolation of nuclei [Roti Roti et al. 1984] and no increase was found when isolated nuclei were heated in enucleated cell homogenates or serum [Roti Roti and Winward 1980]. It is known that under cell fractionation conditions some proteins such as DNA polymerases will leak from the nucleus [Lynch et al. 1975]. Such leakage seems reduced when cells were heated before isolation of nuclei [Kampinga et al. 1985]. Also other normally TX-soluble nuclear proteins such as c-myc [Evan and Hancock 1985], RNA polymerases and DNA topoisomerase II [McConnell et al. 1987,
Fisher et al. 1989] have become insolubilized after heating cells. As it is generally accepted now that hyperthermia results in protein denaturation [Lepock et al. 1988, 1990a, 1990b, Burgman and Konings 1992] leading to aggregation [Bensaude et al. 1991, Burgman and Konings 1992; Skowyra et al. 1990, Höll-Neugebauer et al. 1991, Jakob et al. 1993], the decrease in leakage during nuclear isolation (or insolubilization) is most likely due to aggregation of (partial) heat-denatured soluble nuclear proteins with each other and with (partial) heat-denatured nuclear skeleton (insoluble) proteins. The contribution of cytoskeletal proteins to the heat-induced increase in nuclear protein content of isolated nuclei is minimal, since minor cytoplasmic contaminations are equally present in nuclei isolated from heated and control cells [Blair et al. 1979, Laszlo 1992a]. We therefore refer to this phenomenon as heat-induced nuclear protein aggregation (figure 1.3).
Yet, some cytosolic proteins do enter the nucleus upon heating. Especially, a specific group of proteins, called heat shock proteins (hsp’s), translocate from the cytosol to the nucleus [Pelham, 1984, 1985, Welch and Mizzen, 1988, Ohtsuka et al. 1986, Li et al. 1991, Hayashi et al. 1991, Kampinga et al. 1988, Welch and Feramisco 1984, Welch and Suhan 1985, 1986]. These proteins also do contribute to the increased protein content of isolated nuclei, although only marginally (<10%) [Chu et al. 1993]. It has been suggested [Pelham 1984, Welch and Suhan 1986] that this co-aggregation of hsp’s is functional and that hsp’s may prevent (further) aggregation or that they are involved in the process of recovery from nuclear protein aggregates. Since it has been shown that hsp’s can protect protein aggregation or facilitate its disaggregation \textit{in vitro} (see heat shock proteins, 1.2), such suggestions are tempting. Whether or how hsp’s are involved in processes as nuclear protein aggregation and disaggregation \textit{in situ} is subject of the study presented in this thesis.

Heat-induced nuclear protein aggregates are shown to be localized for the most part at the nuclear matrix [Wheeler and Warters 1982], which is thought to be the site of replication and transcription [Berezney and Coffey 1975, Jackson and Cook 1985, 1986]. Thus, nuclear protein aggregation at the nuclear matrix could lead to interference of the DNA-replication and transcription complex interactions and to inhibition of DNA unwinding [Kampinga et al. 1988, Wynstra et al. 1990] needed for DNA replication and transcription and maybe DNA repair.

The presence of alcohols and procaine during heating, having a heat sensitizing effect on survival, enhanced the heat-induced nuclear protein aggregation [Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a]. Glycerol, cycloheximide and D$_2$O were shown to reduce the extent of protein aggregation in the nucleus when present during the heat treatment [Henle and Warters 1982, Kampinga et al. 1989a, Borrelli et al. 1992, 1993], suggesting a relation between the extent of nuclear protein aggregation and the extent of hyperthermic cell killing. The effect of thermotolerance is less clear and is further addressed in chapters 3 - 7 of this thesis.

During post-heat incubation at physiological temperatures, cells were able to recover from these nuclear protein aggregates [Roti Roti and Winward 1978, Warters et al. 1986, Kampinga et al. 1987, 1989a]. In thermotolerant cells, disaggregation is much faster compared to non-tolerant cells. This facilitated disaggregation is also further addressed in chapters 3 - 7 of this thesis. In the experimental set up used in the studies presented in this thesis no discrimination can be made between functional disaggregation (protein reactivation) and proteolysis. Pinto et al. [1991], using cytosolic reporter enzymes showed that reactivation of enzyme activity was only observed to 50-60% of the control activity, whereas complete (100%) resolubilization (disaggregation) was observed, indicating that parts of the disaggregated proteins are irreversibly damaged. These irreversibly damaged proteins may be transported to lysosomes for proteolysis. Both processes may be involved in nuclear protein disaggregation.
1.2 HEAT SHOCK PROTEINS

Cells respond to unfavourable conditions such as heat shock by the rapid, vigorous, and transient acceleration in the rate of expression of a small number of specific genes (heat shock genes) [see Morimoto et al. 1990 for review]. The product of these genes (heat shock or stress proteins) consequently increase and accumulate in the cell. The first observations of a heat shock or stress response was made by Ritossa in the early 60’s in giant salivary gland chromosomes of Drosophila larvae [Ritossa 1962, 1963, 1964]. Heat shock treatment (or treatments with DNP, sodium salicylate or sodium azide) resulted in the appearance of new puffs on the chromosomes, while the puffs active prior to the heat treatment regressed or disappeared. Experiments with radioactive precursors showed that the heat-induced puffs were the sites of intense RNA transcription of active, induced genes.

Figure 1.4 Overview of the conditions that induce heat shock protein expression (redrawn after Morimoto and Milarski 1990).

In parallel with the new puffs, also newly synthesized proteins appeared [Tissières et al. 1974]. Later on, it was shown that purified fractions of heat shock mRNA hybridized in situ to specific heat shock puffs [Lindquist-McKenzie et al. 1975,
Spradling et al. 1975] and that these mRNA’s were translated into specific heat shock proteins, using in vitro translation systems [Lindquist-McKenzie and Meselson 1977, Spradling et al. 1977, Mirault et al. 1978]. The observations of a heat shock response in chicken embryo fibroblasts [Kelley and Schlesinger 1978], E. coli [Lemaux et al. 1978, Yamamori et al. 1978], yeast [McAllister and Finkelstein 1980], plants [Barnett et al. 1980] and other organisms indicated that this response was a universal one and not strictly associated with Drosophila. The heat shock response takes place in all cells of organisms with only a few exceptions, such as during early embryonic development [Dura 1981, Wittig et al. 1983, Banerji et al. 1984, 1987]. As already shown by Ritossa [1962, 1964], heat shock proteins are induced in response to a wide range of physiological or chemically induced stress conditions. This list of known inducers has greatly increased and many other stimuli including those related to cell growth and differentiation were shown to evoke hsp gene transcription [Morimoto and Milarski 1990] (figure 1.4).

The major heat shock proteins have been classified into four protein families: the hsp90 family, ranging from 83 to 90 kD, the hsp70 family, ranging from 66 to 78 KD, the hsp60 family, and the small heat shock proteins, ranging from 15 to 30 kD [Morimoto et al. 1990]. Beside these families, several other heat shock proteins have been characterized, e.g. hsp110 [Subjeck et al. 1983], hsp40, the cohort of hsp70 [Hattori et al. 1992, 1993], hsp47 [Nagata et al. 1986], hsp10, the cohort of hsp60 [Hartman et al. 1992] to name a few (table 1.1). The heat shock proteins are among the most highly conserved proteins in nature [Lindquist and Craig 1988]. They appear to carry out a number of similar functions, such as binding to polypeptides to assist in e.g. folding and transport, which are known as chaperone functions.

1.2.1 Molecular chaperones

The successful in vitro (cell free system) refolding of purified ribonuclease A by Anfinsen [1973] led to the suggestion that all information necessary for a polypeptide to fold was an intrinsic feature of its primary structure, independent of other factors [self assembly, Ellis and Hemmingsen 1989]. Most of these refolding experiments were performed in cell free systems by first denaturing a purified polypeptide with chemical agents like urea or guanidinium-chloride (GdmCl), and then removing the denaturant.
Table 1.1 Intracellular localization of the main mammalian heat shock proteins under physiological conditions and after a heat treatment.

<table>
<thead>
<tr>
<th>HSP</th>
<th>Normal</th>
<th>After HT</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>mitochondria</td>
<td>mitochondria</td>
<td>cohort of hsp60 homology with GroES</td>
</tr>
<tr>
<td>27</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>increased phosphorylation after stress aggregates in nucleus upon heat</td>
</tr>
<tr>
<td>40</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>co-localizes with hsc70 homology with DnaJ</td>
</tr>
<tr>
<td>47</td>
<td>ER</td>
<td>ER</td>
<td>(pro)collagen binding protein</td>
</tr>
<tr>
<td>56</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>found in complexes with hsp/c70, hsp90 and steroid receptors</td>
</tr>
<tr>
<td>60</td>
<td>mitochondria</td>
<td>mitochondria</td>
<td>involved in protein folding homology with GroEL homolog in cytoplasm (TRiC)</td>
</tr>
<tr>
<td>70</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>hsp70 highly stress inducible constitutively expressed in primates hsc70 constitutively expressed homology with DnaK involved in protein folding and translocation</td>
</tr>
<tr>
<td>90</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>associated with steroid receptors dissociates after heat shock</td>
</tr>
<tr>
<td>110</td>
<td>nucleus</td>
<td>nucleus</td>
<td>possible involvement in ribosomal protection during heat shock</td>
</tr>
</tbody>
</table>

The probability that such a polypeptide will fold correctly after removing the denaturant, increases at low protein concentration (which limits inter-polypeptide interaction) and low temperatures (which attenuates hydrophobic interactions). The high protein concentrations and temperatures within the cell lead to premature interactions of newly synthesized polypeptides, often accompanied by misfolding and aggregation [Jaenicke 1991, 1993]. To assist polypeptide folding (assisted self assembly) in vivo, a set of proteins, called molecular chaperones, exists whose function is to ensure that polypeptides will either fold or be transported properly. The
term 'molecular chaperone' was first used by Laskey and colleagues [1987] to describe the role of nucleoplasmin in nucleosome assembly. The word chaperone is normally used to describe a particular and largely outdated form of social behaviour by humans. It is defined in the Oxford English Dictionary (2nd ed) as "person, usually a married or elderly woman who, for the sake of propriety, accompanies a young unmarried lady in public as a guide and protector." In biochemical terms, a molecular chaperone is a "protein that prevents improper interactions between potentially complementary surfaces and disrupts any improper liaisons that may occur" [Ellis and Hemmingsen 1989]. The molecular chaperones are defined as a family of unrelated classes of proteins that mediate the folding of other proteins and, in some cases, mediate their correct assembly into oligomeric structures without being components of the final functional structures themselves [Ellis and Hemmingsen 1989, Ellis and Van der Vies 1991, Hightower 1991, Georgopoulos 1992, Hendrick and Hartl 1993, Parsell and Lindquist 1993]. The proposed function of chaperones is to assist in self assembly of proteins by inhibiting alternative assembly pathways that produce nonfunctional structures. During a number of fundamental cellular processes (protein synthesis, protein transport (to organelles), protein functioning (e.g. in subunit-subunit interactions, and organelle biosynthesis)) interactive protein surfaces are transiently exposed to the intracellular environment. This means that improper interactions may occur against which a cell has to defend itself. Given the fact that stress (in particular heat) can cause denaturation of proteins and (subsequent) formation of protein aggregates, it is conceivable that those hsp's for which chaperone activities have been described are likely candidates for being protectors against thermal protein damage, leading to heat resistance of cells (clonogenic ability). Their induced synthesis upon stress can be viewed as an amplification of their basic chaperone function [Ellis and Van der Vies 1991].

In the search for molecular chaperones, refolding experiments of chemically denatured polypeptides in the absence and presence of proposed chaperones are used [Buchner et al. 1991, Martin et al. 1991, Wiech et al. 1992, Jakob et al. 1993] (figure 1.5). In such 'cell free' approaches, purified polypeptides are first denatured by urea or guanidinium-chloride. After removal of the denaturant (by dilution), the unfolded peptides aggregate or are capable of spontaneous folding to the native state (step 1). The balance between these processes is dependent on the polypeptide concentration and temperature at which the refolding occurs [Buchner et al. 1991]. Aggregation of the polypeptides can be measured by light scattering, and refolding in to the native state by measuring the activity of the polypeptides. The addition of a chaperone usually decreases the extent of aggregation, thereby increasing the amount of spontaneous refolding and protein activity (step 2). Still a large amount of the substrate protein remains inactive, probably bound to the chaperone.

**Figure 1.5** Schematic representation of the role of molecular chaperones on protein aggregation and refolding of chemically denatured proteins. Top, see text for detailed discussion; bottom, extent of aggregation and activity of purified proteins during chemical
denaturation and renaturation, Native, native proteins; A, chemically denatured proteins; B, removal of the denaturant by dilution (step 1); C, same as B in the presence of hsp’s (step 2); D, same as B in the presence of hsp’s and cofactors (step 3 and 4).

The increase in activity is often stated as renaturation of the substrate protein, suggesting an active folding process. In most of the studies, however, these chaperones just prevent aggregation, resulting in more spontaneous refolding, without involvement in the actual folding process. Addition of cofactors, such as helper chaperones, nucleotides, and K⁺ or Mg²⁺ ions may lead to ‘active’ folding cycles resulting in high efficiently refolding and a high percentage of protein activity (step 3). Addition of the chaperone (+ cofactors) after aggregation already occurred, may give
insight in whether chaperones are capable in reactivation these polypeptides from their aggregated state (disaggregation; step 4). Similar set ups are used during heat denaturation of purified folded proteins to get insight in the capability of chaperones to protect against thermal aggregation and thermal inactivation, and whether heat-induced aggregates can be disaggregated and the polypeptides reactivated by a certain chaperone or chaperone complexes [Jakob et al. 1993, Knauf et al. 1994, Skowyra et al. 1990].

1.2.2 Heat shock proteins as molecular chaperones

1.2.2.1 Small heat shock proteins

Functions under physiological conditions

Hsp27 is constitutively expressed in unstressed cells at low levels. Although the family of the small hsp’s (shsp’s) are ubiquitous and highly conserved in eukaryotes [Ignolia and Craig 1982, Hickey et al. 1986, De Jong et al. 1993], they are the least conserved family of the major hsp families [for review see Parsell and Lindquist, 1993]. They share sequence homology with the central portion of α-crystallin [Ignolia and Craig 1982, Hickey et al. 1986, de Jong et al. 1993] and both can be phosphorylated. Hsp27 is localized in the cytoplasm around the Golgi cisternae [Arrigo et al. 1988] and exists in multimeric aggregates (200-400 kD) in its native form [Arrigo and Welch 1987]. The function of hsp27 (and other shsp’s) under physiological conditions is still elusive. It is speculated that it is involved in the signalling pathways of cellular proliferation [Landry et al. 1992, Moretti-Rojas et al. 1988, Knauf et al. 1992, Oesterreich et al. 1993]. The shsp’s also seem to play a role in development and in drug-resistance [Arrigo 1987, Edwards et al. 1981, Cohen et al. 1985, Ciocca et al. 1993, Huot et al. 1991, Oesterreich et al. 1991, 1993] and their expression is hormone responsive [Moretti-Rojas et al. 1988].

Functions under stress conditions

During and after a heat treatment, hsp27 levels increase and the protein is phosphorylated [Crête and Landry 1990, Landry et al. 1989, Lee et al. 1992, Landry et al. 1991]. It is translocated to the nucleus, although low levels are also found dispersed through the cytoplasm [Arrigo et al. 1988], probably related to the heat-induced fragmentation of the Golgi complex [Pelham et al. 1985]. During/after stress when high levels of hsp27 are present, they form large aggregates (> 2000 kD), so called heat-shock granules [Arrigo and Welch 1987]. It is not clear whether these granules are functional or are merely aberrant aggregates [Arrigo and Tanguay 1991, Nover 1991].

Selective induction of small hsp’s, including hsp27, by hormones was shown to induce thermotolerance [Berger and Woodward 1983]. Studies in mammalian cell

As molecular chaperone

Recently, a chaperone-like activity for the shsp's has been demonstrated: shsp's were found to be able to prevent heat-induced aggregation of unfolded proteins and thereby facilitate refolding of chemically-denatured proteins in cell free systems (figure 1.5, step 2) [Knauf et al. 1992, Horwitz 1992, Jakob et al. 1993, Merck et al. 1993]. In one study, mouse hsp25 and α-crystallin completely suppressed the heat-induced aggregation of βl-crystallin at 58°C. Similar results were obtained with hsp25, that could completely suppress the aggregation of native α-glucosidase at 49°C [Merck et al. 1993]. In another study, mouse hsp25, human hsp27, and α-crystallin all prevented the heat-induced aggregation of citrate synthase and α-glucosidase and promoted the renaturation of these proteins after denaturation in urea (figure 1.5, step 2) [Jakob et al. 1993]. It is not clear how these chaperone activities can be accomplished by the shsp's, since no complex between the shsp's and the substrate protein was reported [Knauf et al. 1992, Horwitz 1992, Jakob et al. 1993, Merck et al. 1993].

Using a cell free system, Miron et al. [1991] showed that the avian homologue of mammalian hsp27 acts as an inhibitor of actin polymerization and disassembled previously assembled F-actin. Also, in situ experiments have revealed that hsp27 can protect against actin depolymerization induced by heat [Lavoie et al. 1993b]. These studies suggest a role for hsp27 in the stabilization of the microfilament organization. Protection of the cytoskeleton against heat-induced alterations may then result in thermotolerance or heat-resistance as was found for hsp27 overexpressing cells [Landry et al. 1989, Lavoie et al. 1993a]. However, disruption of the cytoskeleton by dihydrocytochalasin B and colchicine had no influence on the heat-induced synthesis of heat shock proteins and these agents themselves did not cause hsp synthesis [van Bergen en Henegouwen et al. 1985]. Also the absence of any type of a heat-dose
dependency on the extent of cytoskeletal alterations [van Bergen en Henegouwen et al. 1985] and the marginal (if any) differences in heat sensitivity between monolayer (extensive cytoskeleton) and suspension (minimal cytoskeleton) grown cells [Kamplinga unpublished data] argue against a major role for the cytoskeleton in heat sensitivity. Therefore, although involved in microfilament (re)organization, hsp27 mediated protection against heat killing may have to be through another pathway. It is suggestive from the cell free experiments that hsp27 acting as a chaperone mediates heat resistance via reduction of heat-induced protein aggregates, but in situ experiments are needed to elucidate the role of hsp27 in heat resistance.

Under stress conditions, hsp27 overexpressing cells [Landry et al. 1989] showed no protection against heat-induced inhibition of RNA and protein synthesis, but these cells showed a faster recovery from heat-inhibited RNA synthesis, but not from protein synthesis, than the parent non-transfected cells [Laszlo et al. 1993]. Whether this better recovery is an indirect result from the protection against actin depolymerization (cells are less damaged elsewhere and more attention can be paid to repair of other damage (not necessarily by hsp27)) or whether hsp27 is functionally involved in these recovery processes is yet unclear. Faster recovery due to hsp27 mediated protection of the cytoskeleton (actin depolymerization), may be unlikely since the heat effects on protein synthesis (and post-heat recovery) were the same in hsp27 transfected and non-transfected cells. Since especially a role for cytoskeletal organization in protein synthesis has been proposed [Cervera et al. 1981, Ben Ze’evo 1985] hsp27 mediated protection at this level should have lead to protection against heat effects on the protein synthesis machinery.

Phosphorylation of hsp27 is suggested to be necessary for its protective action against thermal killing [Landry et al. 1991, 1992, Créte and Landry 1990]. Such phosphorylation of hsp27 is e.g. reported to be induced by A23187 in the absence of enhanced hsp27 (or any other hsp) synthesis; the A23187 treatment alone was able to induce thermotolerance [Crête and Landry 1990]. Recently, the necessity of hsp27 phosphorylation to confer heat-resistance has become controversial [Knauf et al. 1992, 1994, Landry et al. 1994, Lavoie et al. 1995]. Overexpression of the intact human hsp27 gene (4 ng hsp27/µg total cellular protein) resulted in a 100-fold increase in survival after 60 min at 44°C, whereas overexpression of the non-phosphorylatable form (4 ng/µg) only gave a 5-fold increase [Landry et al. 1994]. The non-phosphorylatable mutant was also not capable to regulate the actin filament dynamics [Lavoie et al. 1993b], again suggesting that intermediate filament stabilization might be an important step in heat-resistance (see above). Yet, in contrast, recent data on mouse hsp25 [Knauf et al. 1992, 1994] have shown that phosphorylation may be essential for proliferation related functions, but not for chaperone and thermoprotective functions of hsp25. In cell free experiments, the non-phosphorylatable mutant still protects α-glucosidase against thermal aggregation and an
increase in activity of urea denatured α-glucosidase (figure 1.5, step 2) independent of phosphorylation of hsp25 was observed [Knauf et al. 1994].

The reason for the controversy is yet unclear. The differences might be explained by the different origin of the proteins (human versus mouse). Human hsp27 contains three phosphorylation sites, whereas mouse hsp25 only has two phosphorylation sites [de Jong et al. 1993]. An other point of discussion is the replacement of the serines of the human hsp27 by glycines [Lavoie et al. 1993b]; this may result in thermal protein instability [Imanaka et al. 1986, Matthews 1987, Sauer et al. 1990, Eijsink 1991], thereby losing its function, especially during a heat shock. However, the non-phosphorylatable form of hsp27 is also still capable to assemble into large multimeric aggregates and to localize in nuclei during/after heat shock, suggesting that the non-phosphorylatable form is still functional [Lavoie et al. 1993b]. The serines in the mouse hsp25 were replaced by alanine [Knauf et al. 1992] known to increase the thermostability of a protein [Argos et al. 1979, Imanaka et al. 1986, Matthews 1987, Sauer et al. 1990, Eijsink 1991]. The role of phosphorylation of shsp’s in heat resistance is also not supported by the findings that thermotolerant EAT cells surviving a second heat treatment show an increased amount of dephosphorylated hsp25 [Oesterreich et al. 1990]. Furthermore, the state of phosphorylation of hamster hsp27 does not correlate with the extent of thermotolerance [Landry et al. 1991]. At 5 and 7 h after the thermotolerance trigger, the relative level of phosphorylated hsp27 is similar to that found in control, unheated cells, and heat-induced phosphorylation is completely inhibited. It was suggested that the ratio of unphosphorylated over phosphorylated hsp27 was important for heat resistance, as this ratio paralleled the level of thermotolerance [Landry et al. 1991]. However, the ratio of unphosphorylated over phosphorylated hsp27 is the same in parental and human hsp27 transfected O23 cells, at 37°C as well as at 44°C, whereas the heat sensitivity differs 200 to 10,000-fold after 4 h at 44°C. So, although it remains yet unclear whether the total amount of unphosphorylated hsp27 or the ratio of phosphorylation is important, most studies suggest that phosphorylation is not an important step in shsp’s mediated heat resistance.

1.2.2.2 Hsp60 family

Most of the investigations on the 60 kD protein have been done on the prokaryotic analogue of hsp60: GroEL [Zeilstra-Rylls et al. 1991]. Since there is a remarkable homology in amino acid sequence and since there also seems to be a high conservation functionally [Zeilstra-Rylls et al. 1991], the mammalian hsp60 and GroEL gene product are e.g. interchangeable in refolding chemically denatured ribulose-1,5-biphosphate carboxylase (Rubisco) [Viitanen et al. 1990], the functions of this heat shock protein will be discussed without making further distinction between pro- and eukaryotic studies.
Functions under physiological conditions

Hsp60 is a constitutively expressed protein that is found in the cytosol of bacteria (GroEL), in the matrix compartment of mitochondria and in the stromal compartment of chloroplasts (chaperonin-60). The oligomeric structure is a double toroid, each consisting of 7 subunits of hsp60 [Viitanen et al. 1990]. In the matrix compartment of mammalian mitochondria hsp60 seems to form a single seven-membered ring [Viitanen et al. 1992]. The protein has a weak ATPase activity that seems K+ dependent [Zeilstra-Ryalls et al. 1991, Viitanen et al. 1990]. Besides its involvement in bacteriophage assembly in E. coli, the protein is involved in interaction with new or imported proteins [Zeilstra-Ryalls et al. 1991, Osterman et al. 1989] and in promoting protein assembly [Wynn et al. 1992]. Hsp60 can bind to unfolded polypeptides [Zeilstra-Ryalls et al. 1991], e.g. after chemical denaturation, and inhibit/retard aggregation of those polypeptides (figure 1.5, step 2): such include β-lactamase precursor [Laminet et al. 1990], ribulose-1,5-biphosphate carboxylase (Rubisco) [Viitanen et al. 1990], citrate synthase [Buchner et al. 1991], rhodanese [Martin et al. 1991], and trimeric ornithine transcarbamoylase (OTC) [Hartman et al. 1992]. Temperature sensitive hsp60 yeast mutants were found to be deficient in protein assembly and protein translocation [see Osterman et al. 1989 for discussion]. Although biochemical and genetic evidence revealed that many hsp60 actions critically depend on a physical interaction with hsp10 (cpn10 or GroES), hsp60 alone seems sufficient to protect against aggregation of denatured proteins (figure 1.5, step 2) [Martin et al. 1991, Zeilstra-Ryalls et al. 1991, Viitanen et al. 1990, 1992, Hartman et al. 1993, Lubben et al. 1990, Rospert et al. 1993]. Addition of hsp10 and ATP is required to release the polypeptide chain bound to hsp60 and to allow protein folding to its active state (figure 1.5, step 3) [Martin et al. 1991, Buchner et al. 1991]. Addition of hsp10, hsp60, and ATP after protein aggregation did not lead to disaggregation of citrate synthase within the tested period (5 min at 25°C): it is questionable whether this time span is long enough to allow for such a (difficult) enzymatic process (maximal reactivation of the protein takes about 60 minutes [Buchner et al. 1991]) and therefore it is yet unclear whether proteins can be reactivated by hsp60/hsp10 from the aggregated state.

Functions under stress conditions

Not much is known about the function of hsp60 under heat stress. Hsp60 (and hsp10) is essential for growth at normo- and hyperthermic temperatures in yeast and the GroE operon has a Eo^{32}-directed promoter typical for heat shock expression such that more GroE chaperonins are produced as temperature elevates [Zeilstra-Ryalls et al. 1991, and references herein]. Electron microscopy studies have indicated that heat drastically alters the subunit arrangement of hsp60 [Carazo et al. 1991]. Recently [Martin et al. 1992], it was shown that the action of hsp60 to prevent the aggregation
of dihydrofolate reductase (DHFR) during the course of thermal denaturation in cell free systems [Martin et al. 1991] is also seen in situ: Hsp60 was required to prevent heat-inactivation of DHFR localized in the mitochondria. This heat-inactivation in vivo is probably due to protection against aggregation (figure 1.5, step 2) and subsequent reactivation by Mg-ATP (and hsp10) (figure 1.5, step 3), which are also present in vivo. Hsp60 was also capable to protect RNA polymerase (RNAP) from heat-inactivation in the absence of ATP, with maximal protection at a molar ratio of 10:1 (hsp60 14-mer:RNAP) (figure 1.5, step 2) [Ziemienowicz et al. 1993]. Hsp10 in the presence of ATP did have a stimulative effect on this protective effect of hsp60 at an optimal molar ratio of 1-2 hsp10 7-mers to 1 hsp60 14-mer. For efficient reactivation of heat-inactivated RNAP hsp60, hsp10 and ATP are all three absolutely required. It is not clear from this study whether heat-inactivated (10 min at 45°C) RNAP does form heat-induced aggregates. So, it remains to be elucidated whether hsp60/hsp10 can reanimate aggregated polypeptides.

**Hsp60-hsp10 reaction cycle**

Under physiological conditions, hsp60 exists as a complex with hsp10 [see Hartl et al. 1994 for review]. The binding and release of polypeptides are regulated by an ATPase activity. Complex formation between hsp60 and hsp10 leads to the stabilization of hsp60 in the ADP-bound state (figure 1.6). Unfolded proteins (up to \( \approx 90 \text{ kD} \)) are thought to enter the hsp60 cavity at the end of the toroid that is not bound to hsp10. Upon binding of an unfolded protein, ADP and, consequently hsp10 dissociates, rendering hsp60 accessible for ATP binding (b). Binding of ATP reduces the affinity of hsp60 for the substrate protein and hsp10 can reassociate to hsp60 in the ATP-bound state (c), which is followed by ATP hydrolysis. ATP hydrolysis causes release of the protein from hsp60, allowing it to fold (d). Folding of the native state is either completed, or the protein is rebound by hsp60 in a partially unfolded form (e), re-entering the reaction cycle. For relatively large polypeptides (e.g. rhodanese) multiple rounds of interaction with hsp60 may be necessary for complete folding (hydrolysis of \( \approx 130 \) ATP molecules per molecule of rhodanese) [Martin et al. 1991].

**Figure 1.6** Reaction cycle of GroEL/GroES in protein folding. Top; see text for detailed discussion. Bottom; model for the ATP-dependent release of substrate protein from multiple attachment sites on GroEL into the central cavity for folding. The dark shaded areas represent the polypeptide-chain-binding sites of the GroEL subunits. (Redrawn after Hartl et al. 1994).
Hsp60-like chaperone in the eukaryotic cytosol

Recently, a heat shock protein of the thermophilic archaebacterium *Sulfolobus shibatae*, has been discovered with an oligomeric structure similar to that of hsp60 [Trent *et al.* 1990, 1991]. This heat inducible 55 kD protein (TF-55) forms a double, nine membered ring. It was found that the TF-55 protein has a high level of homology (40-60%) with the eukaryotic cytosolic T-complex polypeptide-1 protein (TCP-1) [Trent *et al.* 1991]. TCP-1 also shows sequence homology to hsp60 [Ellis 1990, Gupta 1990] and also forms double ring shaped particles similar to TF-55, consisting of at least four to six other structurally related subunits in the 52-65 kD range [Frydman *et al.* 1992, Lewis *et al.* 1992, Mummert *et al.* 1993]. This complex is termed TRiC (TCP-1 containing ring complex).

Disruption of the TCP-1 gene in yeast results in a temperature sensitive mutant displaying aberrant microtubule staining [Ursic and Culbertson 1991]. This suggested a role for TCP-1 in microtubule metabolism. It was shown that TRiC chaperoned the folding of (denatured) tubulin *in vitro* [Frydman *et al.* 1992, Yaffe *et al.* 1992]. TRiC does also promote the folding of actin [Gao *et al.* 1992], denatured luciferase [Frydman *et al.* 1992] and the phytochrome photoreceptor [Mummert *et al.* 1993] in a Mg-ATP dependent manner indicating that TRiC is a (hsp60-like) chaperone. However, other denatured test substrates (e.g. cyclin B, α- and β-globin, cap binding protein) could not be properly folded by TRiC. TRiC did not even form binary complexes with these substrates [Gao *et al.* 1993]. There are also differences observed between TRiC and the hsp60-multimer. TRiC was able, to reactivate denatured luciferase, whereas GroEL/ES was not [Frydman *et al.* 1992, Schröder *et al.* 1993]. Furthermore, the tubulin assembly reaction was possible with TRiC alone; GroEL depended on the cofactor GroES for this reaction. It may be that TRiC does not need a cofactor or that this cofactor is already part of the (9-ring) hetero-
oligomeric structure of TRiC, in contrast to the (7-ring) homomeric structure of hsp60. Another difference is that hsp60/hsp10 (and TF-55) are strongly heat-inducible, whereas TRiC proteins are not.

### 1.2.2.3 Hsp70 family

Proteins encoded by the hsp70 gene family are the most investigated hsp’s. They are highly conserved and found in all organisms, from bacteria to man. In the first part of this section, the emphasis will be on the prokaryotic hsp70, DnaK, and in the second part, the eukaryotic hsp70 family, in particular mammalian hsp70 and hsc70 will be discussed.

**Prokaryotes**

In prokaryotes, it was thought for several years that only one hsp70 protein (DnaK) exists. Recently [Seaton and Vickery 1994], however, identified a related protein (hs66) in *E. coli* which shares 40% amino-acid identity with DnaK. Also another protein, MreB, might be a member of the prokaryotic hsp70 family, since it shares 27% identity with the N-terminal part of DnaK [Gupta and Singh 1992]. In this part, however, focus will be only on DnaK, on which most of the work on the prokaryotic hsp70 has been done [see Gross et al. 1990, Georgopoulos et al. 1990 for review]. DnaK (together with DnaJ and GrpE) has originally been discovered as a protein involved in the replication of phage λ DNA [Friedman et al. 1984, Zyclicz et al. 1989]. DnaK is constitutively expressed and inducible by heat shock. Genetic analysis in *E. coli* has revealed that DnaK is essential for cell growth at all temperatures [Lindquist and Craig 1988]. Recently, several studies reported on the interaction of DnaK with unfolded polypeptides and a role for DnaK as a molecular chaperone has been postulated [Skowyra et al. 1990, Georgopoulos 1992, Schröder et al. 1993, Buchberger et al. 1994a, 1994b, Hendrick and Hartl 1993].

Cell free experiments have pointed to a role for DnaK in protein folding [Langer et al. 1992, Hartl et al. 1992, Martin et al. 1991, 1992]. Using an unfolded substrate protein (rhodanese in 6 M GdmCl), in a cell free system, it was shown that DnaK when present at high molar excess (10-20 fold) can stabilize this unfolded polypeptide and prevent aggregation [Langer et al. 1992] (figure 1.5, step 2). It appeared that this action of DnaK was much more efficient when also DnaJ was present. So it seemed that, as found for λ DNA replication [Zyclicz et al. 1989], DnaK and DnaJ work together in stabilizing unfolded proteins. The addition of GrpE, a small heat shock protein (=20 kD), was necessary to release the unfolded polypeptide from DnaK/DnaJ (figure 1.5, step 3), but did not result in a more efficient folding of rhodanese. It turned out that also GroEL and GroES were needed for proper folding and activation of this chemically denatured protein [Langer et al. 1992]. Based on these observations, a model for the cooperative action of DnaK/DnaJ/GrpE and GroEL/GroES in the folding of newly synthesized polypeptides in vivo has been postulated (figure 1.7). DnaK
Chapter 1

probably interacts with extended polypeptide chains early in translation, preventing premature folding and aggregation. As the polypeptide chain grows, DnaJ cooperates with DnaK in stabilizing an early folding intermediate. Folding would then occur by GrpE-dependent release of these polypeptides from DnaK/DnaJ and by additional folding by GroEL in a GroES and ATP dependent reaction.

Figure 1.7 Model for the cooperative action of DnaK/DnaJ/GrpE and GroEL/ES in the folding of newly-synthesized proteins (see text for detailed discussion). (Redrawn after Hartl et al. 1994).

Under stress conditions cells have to deal with increased amounts of unfolded, aberrant polypeptides. The stress-induced increase in the amount of DnaK suggests that this protein may be involved in polypeptide stabilization/refolding. Cell free experiments [Skowyra et al. 1990, Liberek et al. 1991a, 1991b] revealed that DnaK can indeed prevent inactivation of RNA polymerase (RNAP) by a 10 min 45°C heat shock: this protection was only seen when DnaK was present during heating and was only marginally dependent on ATP (figure 1.5, step 2). A mutant DnaK protein (DnaK754: lacking ATPase activity) was fully competent in protection as well. When DnaK was added after thermal protein inactivation, protein reactivation (disaggregation) occurred (figure 1.5, step 4). It must be realized that this activity was only seen with a 60-fold molar excess of DnaK to RNAP. The reactivation was fully dependent on functional DnaK and ATP (the mutant protein DnaK754 showed no activity) [Skowyra et al. 1990]. The necessary ATPase activity of DnaK was up to 50
fold enhanced by the two 'assistant' hsp's: DnaJ and GrpE [Liberek et al. 1991a]. This was the first direct demonstration of an hsp activity involved in reactivating a heat inactivated, aggregated protein. The authors state that similar results were also obtained for other proteins (e.g. DNA polymerase I). Another, often used substrate protein, luciferase, is rapidly inactivated when cells are shifted to 42°C, but is reactivated when cells are returned to their normal growth temperature [Nguyen et al. 1989, Pinto et al. 1991, Schröder et al. 1993]. In situ experiments with E. coli revealed that inactivation occurs to the same extent irrespective of functional DnaK, DnaJ or GrpE. However, all three proteins were found to be required for post-heat reactivation [Schröder et al. 1993]. Also, in cell free experiments, the presence of DnaK, DnaJ and GrpE in various combinations did not alter the time course of heat-inactivation [Schröder et al. 1993] (figure 1.5, step 2). In this case, even a 100-fold molar excess of DnaK did not affect the kinetics of this process, which contrasts the observations of Skowyra and colleagues that DnaK protects RNAP from heat-inactivation [Skowyra et al. 1990]. It seems that DnaK is able to associate with native RNAP [Skelly et al. 1988], which might be a prerequisite for protection against heat-inactivation by DnaK. Reactivation of heat-denatured luciferase requires the presence of DnaJ during the initial heat-denaturation step. This protein suppresses heat-induced aggregation of luciferase, but not the heat-inactivation (in vitro/in vivo). So, protection against aggregation and protection against heat-inactivation seem distinct processes for which different protection mechanisms may exist. Binding of a chaperone to a protein may prevent heat-induced aggregation, but still can lead to inactivation (inactive chaperone-protein complex, figure 1.5) [Schröder et al. 1993]. On the other hand, a chaperone may bind to a native or partially unfolded, but still active protein to prevent heat inactivation and aggregation (active chaperone-protein complex) [Skowyra et al. 1990]. For luciferase, DnaK alone did not suppress heat-induced aggregation, but together with DnaJ a synergistic effect was observed in preventing aggregation. It was speculated [Schröder et al. 1993] that DnaJ targets DnaK to the substrate: once DnaJ has bound the denatured substrate it is refolded after the addition of DnaK, GrpE and ATP with high efficiency at nearly stoichiometric concentrations of DnaK, DnaJ and GrpE (figure 1.5, step 3). DnaK alone even at large molar excess, was unable to reactivate luciferase. Also, this is in contrast to the ability of DnaK to reactivate RNAP in the absence of DnaJ and GrpE. The reason for these apparent substrate specific differences are however, yet unclear.

So, two models of DnaK-DnaJ interactions for protein stabilization and disaggregation have been proposed:

1) DnaK is the key protein that binds to unfolded polypeptides (and aggregates) and its ATP hydrolysis is stimulated by DnaJ, thereby enhancing the rate of disaggregation/rerenaturation [Skowyra et al. 1990, Liberek et al. 1991a, 1991b].
2) DnaJ has a central role and recognizes the unfolded polypeptide (aggregate), hereby targeting DnaK to the unfolded substrate (aggregate) [Schröder et al. 1993].

It might be that both models exist in vivo and that the association of DnaK or DnaJ with the denatured protein might be dependent on the substrate protein. This is suggestive from the observations of Langer et al. [1992]: DnaK and DnaJ were both able to bind unfolded casein and rhodanese, but only DnaK was able to interact with reduced carboxymethylated α-lactalbumin. Also the denatured state of the substrate (random coil versus molten globules) might be important for the recognition by DnaK and/or DnaJ.

Eukaryotes

In eukaryotic cells, the hsp70 family consists of several members. In the lower eukaryotes such as yeast, at least eight members of this protein family have been identified. Localized in the cytosol are SSA1-4 and SSB1-2, in the mitochondria SSC1 and in the endoplasmic reticulum KAR2 [Lindquist and Craig 1988, Craig 1990]. In mammalian cells, at least four members of the hsp70 family have been identified [Welch 1990] (table 1.2). Hsp70 and hsc70, and two glucose regulated proteins; GRP78 (or BiP), localized within the lumen of the endoplasmic reticulum and GRP75, localized in the mitochondria. Hsp70 is the major heat-inducible hsp70 protein, with basal expression in primates but not rodent cells; it can be serum stimulated, it is cell cycle regulated and adenovirus E1a-inducible in primates. The hsc70 is constitutively expressed and is only slightly inducible in rodent but a major inducible protein in human cells [Lindquist and Craig 1988]. In contrast to the prokaryotic cells where only one member of the hsp70 family is present at high levels, in eukaryotic cells (especially in primates) two major hsp70 family proteins exists in the cytosol. Are these proteins (hsp70 and hsc70) functionally related or are they involved in different processes? In the next paragraph, a comparison is made between the hsp70 and hsc70 genes and proteins for DNA and amino acid sequences, functional domains, and chaperone functions under physiological and stress conditions. If no distinction is made in Table 1.2 The mammalian hsp70 proteins: A family of related ATP binding proteins present within different intracellular compartments (after Welch 1990).

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<th>hsp70</th>
<th>Constitutive protein in primates and negligible levels in other mammalian cells but induced to high levels upon stress. Present within the nucleus, nucleolus and cytoplasm. Likely to function, similar to hsc70, in the stressed cell to maintain the solubility of cytosolic and nuclear proteins as well as perhaps facilitate the removal of denatured proteins.</th>
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<tbody>
<tr>
<td>hsc70</td>
<td>Abundant and constitutive protein present in the cytoplasm and nucleus. Implicated in the uncoating and/or reformation of clathrin-coated vesicles and in the maintenance of a translocation-competent state of certain proteins that are transferred across intracellular membranes.</td>
</tr>
</tbody>
</table>
General introduction

grp75  Constitutive protein localized within the mitochondria, most likely present in the matrix. Presumed to function in the proper assembly of mitochondrial monomeric proteins into larger macromolecular complexes.

grp78 or BiP  Abundant constitutive protein present within the lumen of the endoplasmic reticulum. Facilitates, similar to grp75, the proper assembly of monomeric proteins into larger macromolecular complexes.

studies between hsp70 and hsc70, it is indicated by using hsp/hsc70. Hsp70 and hsc70 are also called hsp72 and hsp73 respectively. This nomenclature has been used in some of the previously published chapters.

Hsp70 and hsc70 genes and gene products

DNA sequence analyses have demonstrated that whereas the hsp70 and hsc70 proteins are highly related proteins, they are distinct gene products [Lindquist and Craig 1988]. DNA sequence of both genes revealed that the hsp70 gene contains no introns whereas the hsc70 gene is split by 8 introns [Dworniczak and Mirault 1987, Sorger and Pelham 1987] (figure 1.8). Comparing the nucleotide sequences of both genes a homology of \(\approx 74\%\) can be observed. The DNA sequences code for polypeptides of approximately 641 and 646 amino acids for hsp70 and hsc70 respectively [O’Malley et al. 1985, Hunt and Morimoto 1985, Dworniczak and Mirault 1987]. Sequence homology between hsc70 and hsp70 at the predicted amino acid level is 81% with higher divergence at the carboxy terminal region of the proteins (figure 1.9). Comparing the amino acid sequences of both human genes with the sequence of the prokaryotic DnaK, a much lower homology was found (\(\approx 45\%\)) with very low homology in the C-terminal end (figure 1.9).
Chapter 1

TAC CTG GGC TAC CCG GTG ACC AAC GCG GTG ATC ACC GTG CCG GCC TAC TTC AAC

CTT CTT GGG AAG ACT GTT ACC GAT GCT GTG TGC ACT GTA GCC CCA GCT TAC TTT GAT

TCT CAG CGT CAG GCT ACC AAG GAT GCG GTG ATC GCG GGG CTC AAC GTG CTG CCG

TCG CAG CGC CAG GCC ACC AAG GAT GCG GTG ATC GCC GCC ATC GCC TAC GCC CTG GAC

AGA GAC AAC GAG CCC ACG GCC GCC GCC ATC GCC TAC GCC CTG GAC AGA ACG GCC

AGG CAC AAC GAC GTC CTG ATC TTC GAT GCG GGG GGC ACC TTC GAC GTG TCC ATC

GGC TGG GAG GAC TTT GAC AAG CGC CTG GTG AAG GCC ACG GCC GCC TGT GGC ACC CAC

CTG ACT ATT GAG CCA ACT GCT GCT GCT ATT GCT TAC GGC TTA GAC AAA AAG GTT GGA

GGG GAG CGC AAC GTC CTG ATC TTC GAC TTT GAC CTG GCC TAC GGC CTG GCC TAC TTT

GCA GAA AGA AAC GTG CTC ATC TTT GAC CTG GGA GGT GCC ACT TTT GAT GTG TCA ATC

CTG ACG ATC GAC GCC ATC TTT GAC CTG GCC ACC AAC GAC TTC GAC ACT AAG TCC AGC

GGT GGG GAG TTT GAC AAG ACC CTG GTG AAG GCC AGG GCC ACC GCC ACC CAC CTG

GCA CTT AGA AAC AGA AAC GAC GTC ATC AGT GAG AAC AAG GAG GCC GTG AGG CGG CTG

CTG TGG TCT GCA GAG ATC GCC CTG AGT TTT GAT GAC GCA CTG GAC CCA GTA AGA GCC

GCC AAA CTA GAC AAG TCA CAG ATT CAT GAT ATT GTC CTG GTC GGG GCC TCC ACC CGC

GCC AAA CTA GAC AAG TCA CAG ATT CAT GAT ATT GTC CTG GTC GGG GCC TCC ACC CGC

30
Figure 1.8 DNA nucleotide sequences of human hsp70 and human hsc70. Identical nucleotides are indicated (·). The sites where the introns of hsc70 are located are underlined. Hsp70, normal lettertype; hsc70, bold lettertype.
Figure 1.9 Amino acid sequences of hsp70, hsc70 and DnaK. Identical amino acids between hsp70 and hsc70 are indicated (\*). The amino acids conserved in all three proteins are underlined. Amino acids involved in ATP binding or S-S bridge formation [Buchberger et al. 1994a] are indicated (*)
Functional domains of hsc70 and hsp70

Hsc70

It has been shown that the hsc70 facilitates the uncoating and release of clathrin triskelions from clathrin-coated vesicles [Ungewickel 1985, Chappell et al. 1986, DeLuca-Flaherty et al. 1990]. This uncoating activity appears to require ATP hydrolysis to disassociate the clathrin from the vesicles. Hsc70 has a weak basal ATPase activity that can be substantially stimulated by interaction with other peptides [DeLuca-Flaherty et al. 1990, Huang et al. 1993, Wang and Lee 1993]. Chymotrypsin digestion of hsc70 yields a 44 kD fragment which is located in the highly conserved N-terminal region of the hsp70 family (figure 1.10). This fragment was still able to hydrolyse ATP at the same rate as stimulated intact hsc70 [Chappell et al. 1987]. The three dimensional structure of the 44 kD N-terminus has been solved by X-ray diffraction revealing that the ATPase domain consists of two domains with the nucleotide bound at the base of the deep cleft between them [Flaherty et al. 1990, McKay et al. 1994] (figure 1.11). Changing one amino acid in the ATPase domain of hsc70 had a dramatic effect on the ATPase activity. Huang and colleagues [1993] used site directed mutagenesis to change Asp-10 into Asn (figure 1.10). This mutated protein binds ATP and peptides comparable to the intact hsc70 protein. However, the peptide-stimulated ATPase activity as well as its basal ATPase activity were lost. So, Asp-10, as highly conserved in the proteins from the hsp70 family (see figure 1.9) is important for the hydrolysis of ATP but not for ATP binding [Huang et al. 1993].

The peptide binding domain of hsc70 has been assumed to be located at the C-terminal region, since the 44 kD fragment failed to interact with clathrin triskelions [Chappell et al. 1987]. A purified glutathione S-transferase fusion protein containing the C-terminal domain of hsc70 (figure 1.10) was able to bind synthetic peptides (RNA S-peptide, GT4, P3a) with the affinity similar to that of intact recombinant hsc70 [Wang et al. 1993]. Also an 18 kD internal fragment, located just immediately after the 44 kD ATPase domain (figure 1.10) shows the same peptide binding properties [Wang et al. 1993]. It has been shown that hsc70 binds a variety of proteins containing an amino acid sequence of KFERQ to stimulate lysosomal degradation. Peptide binding occurs by recognition and binding to this KFERQ sequence of the target peptides [Terlecky et al. 1992]. These KFERQ-like regions are also found in mammalian hsc70 and hsp70 proteins and localized in the C-terminal end (see figure 1.9). This suggests that these proteins can also be recognized by hsc70 and form homodimers or heterodimers. However, there is still no clear evidence that hsp70 and hsc70 are indeed able to form (functional) heterodimers. The yeast homolog SSA1 as well as the prokaryotic homolog DnaK were not capable of binding to RNase S-peptide containing the KFERQ-like sequence, indicating that hsc70 differs from these proteins in recognizing certain substrate proteins. Also hsp70 did not bind to the
### Figure 1.10 Functional domains of hsc70

- **N-ter**: mutant hsc70 protein only consisting of the N-terminal part
- **C-ter**: mutant hsc70 protein consisting of the C-terminal part
- **Int18**: mutant hsc70 protein consisting of a 18kD internal part
- **Asp10**: hsc70 mutant were Asp10 is changed into Asn10
- **70-Cter**: mutant hsc70 protein missing a 10kD C-terminal part

#### Table

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
<th>ATP binding</th>
<th>ATP hydrolysis</th>
<th>Clathrin/peptide binding</th>
<th>Clathrin uncoating</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsc70</td>
<td>± 70kD</td>
<td>+</td>
<td>low basal, stimulated by peptide binding</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-ter</td>
<td>± 44kD</td>
<td>+</td>
<td>high</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-ter</td>
<td>± 28kD</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Int18</td>
<td>± 18kD</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Asp10</td>
<td>± 70kD</td>
<td>+</td>
<td>none</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>70C-Cter</td>
<td>± 60kD</td>
<td>+</td>
<td>low basal, stimulated by peptide binding</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 1.11** Structure of the hsc70 ATPase domain (taken from McKay et al. 1994).
S-peptide, suggesting that also hsc70 and hsp70 differ in their interaction with certain peptides.

Besides KFERQ-like regions, also other peptide sequences can be recognized by hsc70. E.g., it has been shown that hsc70 binds to a region of clathrin light chain A (P3a) not containing such a KFERQ-like region [DeLuca-Flaherty et al. 1990, Wang et al. 1993].

Deletion of the C-terminal end (approximately 10 kD, 100 aa, figure 1.10) from hsc70 with a mild chymotrypsin digestion resulted in a 60 kD fragment that was still capable of binding clathrin triskelions. However, it was unable to facilitate the dissociation of clathrin from coated vesicles [Chappell et al. 1987]. This fragment is still able to bind other peptides (RNAase S-peptide, GT4, P3a) and the peptide stimulated ATPase activity is retained as well [Tsai and Wang 1994]. So, peptide binding and stimulated ATP activity are not sufficient for the uncoating of clathrin vesicles. The C-terminal end is essential for this function. It was speculated [Tsai and Wang 1994] that this fragment may be necessary for interaction with a helper protein as was observed for DnaK [Liberek et al. 1991a, Georgopoulos et al. 1990]. Recently [Prasad et al. 1993], such a cooperation for hsc70 has been reported. Clathrin baskets prepared from highly purified clathrin and AP2, the assembly protein associated with plasma membrane coated vesicles, could not be uncoated by hsc70 alone. A 100 kD cofactor, which was isolated from crude prepared clathrin coated vesicles, was essential for uncoating activity of hsc70. How these two proteins interact with each other is not clear yet, but it might be via interaction of the cofactor with the C-terminal end of hsc70 through recognition of the two KFERQ-like regions, which are localized in the C-terminal end (see figure 1.9).
Like hsc70, also hsp70 is able to bind to ATP-agarose which has facilitated its purification [Welch and Feramisco 1985]. The DNA and amino acid sequence showed a high homology to hsc70, especially in the N-terminal domain, suggesting that the ATP binding domain of hsp70 is conserved in the N-terminal part. To identify the functional domains of hsp70, deletion mutants have been constructed [Milarski and Morimoto 1989, Li et al. 1992] (figure 1.12). The NSC mutant protein with a deletion of the total N-terminal domain (aa 5-479) was not capable of binding to ATP-agarose. So, as suggestive from the DNA and amino acid sequence, the ATP binding domain is indeed localized in the N-terminal part of hsp70. Smaller deletions of this part of the protein revealed that there are two ATP binding domains localized between aa 122-264 (NF) and aa 351-414 (PB), since these two deletion mutants were unable to bind to ATP-agarose beads (figure 1.12). Deletions of the aa 5-122 (SN) and aa 415-479 (BC) had no effect on the ATP binding capacity of the mutant protein. Also deletions localized in the C-terminal domain of hsp70 (aa 437-617, SMA and aa 504-641, CRI) had no effect on the ATP binding capacity (figure 1.12). The latter deletion mutants (SMA, CRI) as well as the PB mutant with a central localized deletion (aa 415-479) showed an altered intracellular localization after a heat treatment. These mutants did not localize to the nucleoli whereas wt hsp70 (and the other mutant proteins) did (figure 1.12). The results of the CRI mutant protein were somewhat diffuse: some cells showed nucleolar staining whereas other did not [Milarski and Morimoto 1989]. All mutant proteins showed nuclear staining except the protein with a central localized deletion (PB mutant). This mutant is somewhat peculiar in that deletion of this region affects both the intracellular localization and ATP binding properties. Cleavage of this mutant protein with trypsin indicated that deletion of this part of the protein affects the folding of the protein giving rise to an improper folded protein [Milarski and Morimoto 1989].

Hsp70 translocates to the nucleus and associates with the nucleus and nucleolus during/after heating. Upon recovery, as nucleoli regain their structural integrity, hsp70 exits the nucleolus and accumulates in the cytoplasm [Welch and Feramisco 1984, Welch and Suhan 1986]. Constitutive expression of hsp70 from transfected genes in unstressed cells was demonstrated to accelerate the recovery of heat-induced nucleolar damage [Pelham 1984]. Based on these observations, Pelham [1984, 1986] proposed that one of the functions of hsp70 family proteins is to assist in the repair of nucleolar damage. Milarski and Morimoto [1989] used the exit of hsp70 (both endogenous and transfected immunotagged) from nucleoli and its cytoplasmic accumulation as a measure of nucleolar recovery after a heat treatment of 2 h at 43°C as has been described by Welch and Suhan [1986].
**General introduction**

![Table and diagram of functional domains of hsp70](image)

<table>
<thead>
<tr>
<th>Name</th>
<th>aa del.</th>
<th>Size (kD)</th>
<th>Localization</th>
<th>ATP binding</th>
<th>accel. nucleol</th>
<th>hsp70 exit</th>
<th>cell. heat resist.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp70</td>
<td>-</td>
<td>± 70kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CRI</td>
<td>504-641</td>
<td>± 57kD</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>n.d.</td>
</tr>
<tr>
<td>NSC</td>
<td>5-479</td>
<td>± 20kD</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>SN</td>
<td>5-122</td>
<td>± 59kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>NF</td>
<td>122-264</td>
<td>± 56kD</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>BC</td>
<td>415-479</td>
<td>± 65kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sma</td>
<td>437-617</td>
<td>± 52kD</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PB</td>
<td>351-414</td>
<td>± 66kD</td>
<td>not properly folded</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.12** Functional domains of hsp70 (see text for further details).
CV1 (monkey kidney) cells transfected with the wt immunotagged hsp70 showed no nucleolar localization of hsp70 (endogenous and immunotagged) after 4 h at 37°C, whereas in the untransfected cells the distribution of the endogenous hsp70 was still predominantly nucleolar [Milarski and Morimoto 1989]. Thus, cells expressing the transfected wt hsp70 protein showed an accelerated nucleolar exit of hsp70 after heat shock treatment, similar to the observation by Pelham [1984] that COS 7 (monkey) cells transfected with *Drosophila* hsp70 recovered faster from heat-induced nucleolar damage. Cells expressing the NF mutant, not capable of binding ATP, did not show accelerated hsp70 exit from the nucleolus suggesting that ATP binding is necessary for the nucleolar exit of hsp70 [Milarski and Morimoto 1989]. In contrast, cells expressing the NSC mutant protein did show accelerated nucleolar exit of hsp70. This 20 kD mutant protein missing its complete N-terminal domain also cannot bind ATP (figure 1.12). So, the NF and NSC mutants give apparent conflicting results. From the observations of Lewis and Pelham [1985] the interaction between hsp70 and the nucleus/nucleolus did seem to depend on ATP. As a possible explanation for the difference observed between the NF and NSC mutant Milarski and Morimoto [1989] speculated that two nucleolar localization sites might exist: one localized in the C-terminal part, independent of ATP for its release from nucleoli and another one dependent of ATP for its release. Based on their own mutants, such an explanation seems unlikely, since the NSC deletion completely covers the NF deletion. Yet, the NF mutant is incapable, whereas the NSC mutant is capable of accelerated nucleolar hsp70 exit. So, it remains unclear whether ATP is necessary for the exit of hsp70 from the nucleolus. Surprisingly, the SMA mutant protein, expressed in CV1 cells is able to facilitate accelerated nucleolar exit of endogenous hsp70, whereas this mutant itself is not able to translocate into the nucleolus upon heat shock. It is not clear how the SMA protein is involved in the nucleolar recovery process.

Li and colleagues [1992] used hsp70 deletion mutants to study the effects of hsp70 domain on cellular heat sensitivity. As discussed before, expression of the intact human hsp70 gene in Rat-1 cells conferred heat resistance [Li *et al.* 1991, 1992]. Expression of a mutant protein (Bgl) with a deletion in the N-terminal part of the protein (aa 120-428, figure 1.12), missing its ATP binding domain still conferred heat resistance [Li *et al.* 1992], indicating that ATP binding is not essential for heat protection. However, expression of the SMA mutant protein in Rat-1 cells, missing its nucleolar localization domain (figure 1.12), did not change the heat sensitivity of these cells. This indicates that translocation to the nucleolus is of major importance for protection against cell killing by heat. This finding seems to contrast the observations by Milarski and Morimot [1989]. The Sma mutant protein was capable to facilitate accelerated nucleolar hsp70 exit [Milarski and Morimoto 1989], whereas it does not confer cellular heat resistance [Li *et al.* 1991]. These observations suggest that accelerated hsp70 exit from the nucleolus is not involved in cellular heat resistance.
Functions of hsp70 and hsc70

**In situ**

In the normal unstressed situation, hsp/hsc70 is merely found in the cytoplasm of the cell, although some nuclear hsp70 can be observed in hsp70 overexpressing cells [Pelham, 1984], but not always [G.C. Li, personal communication]. The involvement of the hsp70 family in a number of cellular processes has been described [Gething and Sambrook 1992] (figure 1.13). Hsp/hsc70 interacts with newly synthesized proteins to prevent improper folding and to keep these polypeptides in a translocation competent state [Beckmann et al. 1990]. This function of hsp/hsc70 is ATP dependent [Beckmann et al. 1990, 1992]. It was also reported that constitutively synthesized proteins from the hsp70 group might play a role in protein folding and in the mechanism of protein translocation across intracellular membranes [Chirico et al. 1988, Deshaies et al. 1988]. Dice and colleagues [Chiang et al. 1989, Terlecky et al. 1992] showed that hsc70 is also involved in the intracellular lysosomal degradation pathway.

**Figure 1.13** Illustration of the proposed roles of stress-70 proteins in eukaryotic cells during the folding and membrane translocation of nascent polypeptides, during molecular rearrangements or disassembly, in protection from stress and in protein turnover (taken from Gething and Sambrook 1992).
Under stress conditions, in particular heat stress, a number of studies have shown that the level of thermotolerance shows a fairly strong correlation with the cellular levels of hsp/hsc70 [Li and Werb 1982, Li and Laszlo 1985, see also part on thermotolerance). More direct evidence for a functional role of hsp in thermal protection was obtained by microinjection of hsp/hsc70 antibodies in cells which made these cells more sensitive to thermal effects on cell morphology and membrane permeability [Riabowol et al. 1988] and cell survival [Lee et al. 1993]. Furthermore, overexpression of heat shock elements resulting in a competitive inhibition of hsp transcription after heat shock led to a reduced heat-induced increase in the level of hsp70 (and probably also for other hsp’s) and a higher cell killing effect (clonogenic ability) of heat [Johnston and Kucey 1988].

A few studies have been reported in which hsp70 expression was constitutively enhanced by gene transfection. Li and coworkers [1991] used a viral promotor to overexpress human hsp70 (cloned by Hunt and Morimoto [1985]) in Rat-1 cells and the extent of overexpression could be related to the extent of heat resistance (clonogenic ability) (figure 1.14). Angelidis et al. [1991] used the same hsp70 gene but placed under control of the ß-actin promotor. Also they found that overexpression of the gene lead to heat resistance of the CV1-cells. Others, however, were unable to overexpress this gene in Drosophila cells using constitutive promotors, or if successful, found conglomerates of hsp70 in the transfected cells without yielding heat resistant transfectants [Feder et al. 1992].

Figure 1.14 Relation between heat resistance and hsp70 level. Survival after 75 min at 45°C of various human hsp70 transfected Rat-1 cell lines is plotted against their relative level of human hsp70. (Redrawn after Li et al. 1991).
When, in the latter case, the *Drosophila* cells were transfected with hsp70 under control of the inducible promotor of the metallothionein gene no such conglomerates were found after short time induction of the transfected hsp70 by CuSO₄: this CuSO₄ treatment did lead to thermotolerance [Solomon et al. 1991]. Prolonged exposure to CuSO₄ first reduced the growth rate of the cells and hsp70 levels were distributed diffusely over the cell. Growth rates recovered subsequently in parallel with the appearance of hsp70 conglomerates [Feder et al. 1992], indicating that -at least in *Drosophila*- continuous high levels are detrimental for growth at normal temperatures and that cells may control the latter by sequestration of the protein in conglomerates. Hsp70 sequestering was not observed in Rat-1 cells transfected with hsp70 [Li et al. 1991] as is shown in figure 1.15. Interestingly, however, the HR-24 transfectants do grow slower than the parent Rat-1 cells. The reason for the differences observed between Rat-1 [Li et al. 1991], CV1 cells [Angelidis et al. 1991] on one hand, and *Drosophila* cells [Feder et al. 1992, Solomon et al. 1991] on the other hand may thus be related to the amount of hsp70 present in the cell. Cells with very high (soluble) levels of hsp70, may not be capable to grow and divide, and thus will not be picked up in stable transfection experiments [Li et al. 1991, Angelidis et al. 1991]. Milarski and Morimoto [1986] observed that expression of the human hsp70 gene in HeLa cells is tightly regulated during the cell cycle. The levels of hsp70 were low in G₁ cells, increased 3-fold upon entry into S phase and decreased gradually thereafter being low during cell division. These observations support the idea that hsp70 levels need to be low for cell division. This idea is -to some extent- confirmed by the findings of Zhang and colleagues [1992] that stimulation of acute myelogenous leukemic cells by growth factors resulted in proliferation paralleled by a decreased synthesis of hsp70, indicating that persistent 'high' levels of hsp70 may stop cells from growing. More studies are certainly necessary to test this hypothesis.

Isolation of CHO HA1 cells after several severe heat treatments resulted in stable variants (3012, 3015) only overexpressing hsc70 [Laszlo and Li 1985]. So, as for hsp70, the overexpression of endogenous hsc70 also confers heat resistance.

With regards to a putative function of hsp/hsc70 in thermal protection *in situ* many suggestions were made. Rat-1 cells expressing the human hsp70 showed accelerated recovery from protein and RNA synthesis after a heat treatment at 45°C [Liu et al. 1992]. No effect was found on the extent of heat-induced inhibition of protein or RNA synthesis, suggesting that hsp70 is only involved in recovery from heat-induced damage. Similar to Rat-1 cells, 3012 cells with elevated hsc70 levels also recover faster from heat-induced inhibition of protein and RNA synthesis [Laszlo 1992b], suggesting that these proteins have identical functions after a heat treatment.
Figure 1.15 Intracellular distribution of human hsp70 expressed in rat fibroblasts (HR-24 cells) before and immediately after a 10 min 45°C heat shock. Top, non-heated cells; bottom, heated cells. Cells were fixed and stained with anti-hsp70 mAb C92F3A-5. Photographs were kindly provided by Dr. G.C. Li.
In cells exposed to heat, both hsp70 and hsc70 are translocated to the nucleus/nucleolus (figure 1.15 for translocation of hsp70) and are found associated with the salt-insoluble nuclear matrix [Bensaude et al. 1991, Kampinga et al. 1988, 1992, Welch and Feramisco 1984, Welch and Suhan 1985, 1986, Ohtsuka et al. 1986]. During recovery from heat shock, hsp/hsc70 were observed to exit the nucleus/nucleolus e.g., in parallel with recovery from morphological nucleolar heat damage [Pelham 1984, 1986, Lewis and Pelham 1985, Welch and Mizzen 1988]. On the basis of such data, Pelham [1986] proposed that one of the functions of the hsp/hsc70 proteins is to bind to heat-denatured or otherwise damaged proteins and prevent or slow down their aggregation. In addition, a role for hsp/hsc70 proteins in "dissolving" hydrophobic protein aggregates formed under normal or stress conditions was suggested [Lewis and Pelham 1985, Pelham 1986]. The disaggregation of heat-induced nuclear protein aggregates is faster in 3012 cells, overexpressing hsc70, as compared to the parent CHO cells, suggesting that hsc70 is involved in the process of protein disaggregation [Laszlo 1992b] (see also part on nuclear protein aggregation). No effect of the overexpression of hsc70 was observed on the initial heat-induced nuclear protein aggregation [Laszlo 1992b]. Further experiments are necessary to elucidate the role of hsp70 in these processes.

Cell free studies

In contrast to hsp60, as demonstrated by Buchner and colleagues [Buchner et al. 1991], hsc70 (and BiP, the mitochondrial homologue of cytosolic hsp70) was unable to reactivate chemically denatured citrate synthase; it was, however, not shown whether or not hsc70 (or BiP) could prevent the aggregation of denatured citrate synthase. The absence of such an ability of hsc70 is even more peculiar in the light of recent data on the ability of DnaK to protect proteins against thermal denaturation/aggregation and to enable better reactivation (see prokaryotic hsp70). Buchner and colleagues, however, did not test DnaK in their experiments and thus it remains unclear whether hsc70 and DnaK are actually different. Nevertheless, experiments by Palleros and coworkers [1992] do suggest a difference between DnaK and hsc70 behaviour after heat: whereas heat-induced aggregation of hsc70 is irreversibly upon cooling, DnaK can refold after the heat treatment. Recently [Ciavarra et al. 1994], also observed a difference between hsp70 and hsc70. It was found that topoisomerase I can be co-immunoprecipitated with antibodies against hsp/hsc70 but not with antibodies against hsp70 after a heat treatment of 30 min at 45°C, suggesting that hsc70, but not hsp70 can bind 'heat-denatured' topo I. Furthermore, cell free experiments revealed that hsc70 protects topo I from thermal inactivation (figure 1.5, step 2) and that hsc70 was also able to reactivate heat-inactivated topo I, without the addition of ATP. The reactivation activity of hsc70 was more efficient in the presence of other (undefined) cytoplasmic factors, still without ATP [Ciavarra et al. 1994]. These results are almost similar to the results with DnaK protecting RNAP
against heat-inactivation [Skowyra et al. 1990], although a difference was that ATP was required for the DnaK mediated reactivation of heat-denatured RNAP. It is however not clear whether heat also induced aggregates of topo I. So, it remains unclear whether or not eukaryotic hsc70 can also reactivate proteins from the aggregated state. Also, whether or not hsp/hsc70 act similarly in situ in mammalian cells remains unclear.

Cofactors of hsp70/hsc70

DnaK has shown to be dependent on two cofactors, DnaJ and GrpE, for efficient folding or refolding proteins into their native state. In eukaryotic cells not much is known about such helper proteins. Recently, a cytosolic mammalian DnaJ analogue, hsp40 was found that was shown to co-localize with hsp/hsc70 after a heat treatment [Hattori et al. 1993]. A functional analogy to DnaJ/DnaK needs yet to be established. In yeast, a mitochondrial hsp40 analogue (MDJ1) has recently been described [Prip-Buus et al. 1994], that seems involved in protein folding in the mitochondria. The described mechanism of action of hsp70/hsp40 (DnaK/DnaJ/GrpE) seems quite similar to the ability of hsp10 to stimulate hsp60 action in enabling reactivation of chemically denatured proteins.

Similar stimulation was also found for the cytosolic yeast hsp70 (SSA1). The hsp70 ATPase activity was stimulated by YDJ1p (a yeast DnaJ homologue) [Cyr et al. 1992]. YDJ1p also stimulated the dissociation of reduced carboxymethylated α-lactalbumin (RCMLA) and hsp70, and F1β 1-52 and hsp70. The DnaJ homologue itself was not capable of binding the unfolded RCMLA, suggesting that DnaJ acts as a stimulator of hsp70 activity, confirming the model of Georgeopoulos and colleagues [Skowyra et al. 1990, Liberek et al. 1991a], but contrasting the DnaJ model of Schröder et al. [1993]. Recently, also a mitochondrial yeast homolog of GrpE was isolated by copurification with the mitochondrial hsp70 [Bolliger et al. 1994, Nakai et al. 1994]. This protein is essential for viability, and it was suggested that this protein interacts with hsp70 in a manner GrpE interacts with DnaK [Bolliger et al. 1994, Nakai et al. 1994].

Besides cofactors, also chaperone (hsp) families work together. As during protein folding of newly synthesized proteins in prokaryotes (figure 1.7), also during the translocation of proteins over intracellular membranes (mitochondria, ER), several chaperones work together [Hartl 1993, Neupert and Pfanner 1993] (figure 1.16). During protein synthesis, cytosolic members of the constitutive hsp70 family bind this polypeptide and keeps it in a translocation active state. The mitochondrial hsp70 interacts with the incoming, extended polypeptide chains. The mhsp70, together with DnaJ and GrpE homologs may partly fold the polypeptide. Hsp60 together with hsp10 completely folds the mitochondrial protein (figure 1.16).

Figure 1.16 The pathway of chaperone-mediated protein import and folding in mitochondria. (Redrawn after Hartl 1992).
**Hsc70 and hsp70 in summary**

From the DNA sequence as well as the amino acid sequence it is clear that both proteins have a high homology in the ATPase domain and are probably structurally identical with similar ATPase activity. The peptide binding domain shows less homology, suggesting that both peptides may have different protein binding properties. This difference has been shown for some proteins including topo I [Ciavarra et al. 1994] and KFERQ containing proteins [Chiang et al. 1989, Terlecky et al. 1992]. Hsp70 and hsc70 both accelerate the recovery of heat-induced inhibition of protein and RNA synthesis, whereas hsc70 overexpressing cells also show facilitated disaggregation of heat-induced nuclear protein aggregates. The role of hsp70 in this process has to be elucidated and is included as part of this thesis [see chapters 3 and 4].

Comparing the functional domains of hsc and hsp70, it seems possible to include the features of both proteins into one schematic representation (figure 1.17). The ATP binding domain is localized between aa 122-264, with Glu\textsubscript{175} as a possible ATP acceptor [Flaherty et al. 1990, Buchberger et al. 1994a]. For the hydrolysis of ATP Asp\textsubscript{10} is essential. These two amino acids are highly conserved (see figure 1.9). Also localized in the N-terminal part are two highly conserved amino acids (figure 1.9) which form a salt bridge to conserve a loop involved in the binding of GrpE to DnaK on amino acid Gly\textsubscript{32}. The salt bridge prevents opening of the nucleotide binding cleft.
and, consequently release of nucleotide. Binding of GrpE disrupts the salt bridge and facilitate cleft opening and nucleotide release. The internal part between aa 351-414 seems important for the structural integrity of the protein. The peptide binding domain is localized in the C-terminal part of both proteins and seems also the domain responsible for nucleolar localization and localized between aa 479-550. The C-terminal end of 10 kD seems responsible for binding to cofactors.

**Figure 1.17** Functional domains of hsc70 and hsp70. A, ATP binding domain; B, domain important for structural integrity of the protein; C, peptide binding/nucleolar localization domain; D, domain involved in interaction with cofactor(s); 10, Asp_{10} involved in ATP hydrolysis; 34, Gly_{34} involved in GrpE (cofactor) binding; 36, Arg_{36} forms salt bridge with Glu_{367} (367); 175, Glu_{175} involved in binding of ATP.

### 1.2.2.4 Hsp90 family

Members of the hsp90 family are present in the cytosol and nucleus of all eukaryotes examined and are also found in the ER (GRP94) of higher eukaryotes [Lindquist and Craig, 1988]. Hsp90 is the most abundant constitutive hsp in the eukaryotic cell. It is an essential protein in yeast. Two monomers have been identified: hsp89α and hsp89β (human; in mouse they are called hsp 86 and 83 or 84) which are heavily phosphorylated with at least 12 isoforms [Hardesty and Kramer 1989, Welch *et al.* 1983]. Although many hsp genes are devoid of introns, hsp89α and β genes were shown to have multiple introns [Hickey *et al.* 1988, Rebbe *et al.* 1989]. Hsp89α is expressed constitutively and not or only moderately heat-inducible; hsp89β contains six presumptive HSE sequences [Rebbe *et al.* 1989] and is the heat-inducible form of hsp90. The term hsp90 will be used in general if no distinction has been made.

**Functions under physiological conditions**

Hsp90 is primarily a cytosolic protein, participating in protein maturation [Hardesty *et al.* 1989, Hickey *et al.* 1988], steroid receptor-binding [Baulieu 1987, Hardesty *et al.*]
1989, Rose et al. 1989, Pratt 1990, Hutchinson et al. 1992, Polla et al. 1993] (figure 1.18) and transport of some protein kinases [Hardesty et al. 1989, Pratt 1990, Hutchinson et al. 1992]. It usually acts as a dimer [Pratt 1990]. Recently, the first indication for enzymatic activity of hsp90 was given: the protein was shown to possess an ATP binding site and was found to have auto-phosphorylation activity [Csermely and Kahn 1991]. In the complex of hsp90 (as a dimer) with steroid receptors and tyrosine kinase, two other proteins were identified: hsp70/hsc70 and a p56-59 protein [Pratt 1990, Hutchinson et al. 1992, Sanchez 1990, Polla et al. 1993]. The p56 protein was recently identified as a novel heat shock protein with relatively low abundance [Sanchez 1990].

Figure 1.18 Proposed model for the modulation of steroid receptor activity by heat shock proteins (hsp’s). Steroid hormone receptors within the cytosol exist in an inactive complex bound to actin. This complex includes hsp90, hsp56, 50 and 25kD proteins and probably involved either in assembly or disassembly of the complex. After steroid addition, the receptor dissociates from the complex, whereas hsp70 either remains attached to the receptor or binds it at that moment, allowing the formation of a functional steroid hormone receptor dimer. Hsp70, which possesses nuclear targeting signals and participates in protein translocation through membranes, escorts the receptor-coupled steroid to the nucleus. Hsp70 dissociates from the receptor upon ATP hydrolysis, allowing the receptor to bind and activate DNA transcription. Hsp70 is recycled and reassociates with the complex or the receptor coupled steroid (after Polla et al. 1993).

Like hsp60, aggregation of chemical denatured citrate synthase can be retarded by hsp90, leading to an increase in the percentage of protein activity [Wiech et al.
1992] (figure 1.5, step 2): this action did not depend on ATP. In addition, it was shown [Wiech et al. 1992] that the activity of a denatured Fab fragment of a monoclonal antibody (unlike citrate synthase involving interchain disulfide bonds) could be increased by hsp90, probably by preventing aggregation (figure 1.5, step 2).

**Functions under stress conditions**

How and where in the cell hsp90 might function as a stress protector is totally unclear yet. Upon heat shock, a small fraction of hsp90 translocates from the cytosol to the nucleus and/or perinuclear regions [Berbers et al. 1988, Collier and Schlesinger 1986, Kampinga et al. 1992, Akner et al. 1992]. Recently [Morcillo et al. 1993], it was shown that hsp90 associates with specific heat shock puffs (hsr∞) in polytene chromosomes of *Drosophila* and *Chironomus*. The association occurred rapidly after the onset of heat shock and disappeared during recovery, concomitant with puff regression, and seemed transcription dependent, suggestive for a role of hsp90 in the regulation of the heat shock response. Hsp90 was also found close to the plasmamembrane after heat [Carbajal et al. 1990]. Also these associations are reversible after re-incubation at 37°C [Berbers et al. 1988, Carbajal et al. 1990]. It is unclear whether this redistribution after heat is functionally related to heat protection evoked by hsp90 [Bansal et al. 1991, Yahara et al. 1986].

In yeast, hsp83 is needed for growth at physiological temperatures and higher levels seem required for growth at elevated temperatures [Borkovitch et al. 1989]. Yahara and coworkers [1986] showed that a heat resistant CHO variant isolated from MMS mutagenized cells through selection by repeated heating and hsp90 expression shows overexpression of hsp90 and none of the other known hsp’s (determined by 2-D electrophoresis) suggesting involvement of hsp90 in heat resistance. Reduction of both constitutive and inducible hsp90 synthesis by transfecting cells with a plasmid expressing hsp90 in an anti-sense orientation, was found to result in a very small increase in hyperthermic cell killing [Bansal et al. 1991]. However, it is not clear from this study whether the synthesis of other heat-shock proteins was modified by the expression of antisense hsp90.

**1.2.3 Regulation of heat shock gene expression**

The induction of heat shock protein synthesis is regulated at several levels. In all organisms investigated, transcriptional regulation plays an important role in the induction of the hsp’s. In eukaryotes, a heat-shock-activated transcription factor binds to a DNA sequence known as heat shock element that provides heat-induced transcription [Perisic et al. 1989, Sorger and Nelson 1989, Westwood et al. 1991, Sarge et al. 1993]. Beside this transcriptional regulation, regulatory mechanism at the level of RNA processing [Yost and Lindquist 1986, Yost and Lindquist 1991], translation and mRNA stabilization have also a great effect on heat shock gene expression [Lindquist 1980, for review see Yost et al. 1990a, 1990b].
1.2.3.1 Transcriptional activation of heat shock genes

Exposure of cells to heat results in the specific induction of hsp gene transcription, while the transcription of non-hsp genes is down regulated [Ritossa 1962, 1964]. The transcription of the major heat shock genes can be increased over 100-fold upon heat shock [Gilmour and Lis 1985], and is mediated by a heat shock transcription factor (HSF). This heat shock transcription factor recognizes a target sequence localized in the promoter region of heat-inducible genes, which was first described by Pelham and called "Pelham box" or "Heat-shock element (HSE)" [Pelham 1982]. HSEs consist of an array of inverted repeats of the sequence nGAAn. The arrangement and number of these units can vary to some extent [Amin et al. 1988, Xiao and Lis 1988]. In figure 1.19, the promotor region sequence of human hsp70 is shown. Beside the CCAAT and TATA sequence which are also present in non-heat shock genes, a HSE is present consisting of 5 nGAAn arrays [Abravaya et al. 1991]. The nucleotide sequence of the HSE is highly conserved from yeast to humans. The G at position 2 is absolutely conserved: substitutions of the G inactivates an HSE [Xiao and Lis 1988]. The A’s at positions 3 and 4 are also very well conserved, although base substitutions at these sites are found in functional HSE’s [Amin et al. 1988, Perisic et al. 1989, Lis et al. 1990].

\[
-120 \\
\text{GGAGCCGAAACCCCTGGAATTTCCGCACCTGGCAGCCTCATCGAGCTC} - \\
\text{GCTCGAGCTGGCAGCTG} -
\]

\[
-20 \\
\text{GTGATTGGCTCAGAAGGGAAAAGGGCTGGACCGTCGGAGTAGCTCGAGC} - \\
\text{ACTGGACGGAGCTGCCTCAGAATATTTTC}
\]

**Figure 1.19** Promotor region sequence of human hsp70. Sites with perfect or imperfect matches to consensus sites for some known transcription factors are indicated (after Abravaya et al. 1991).
With the exception of budding yeasts [Kingston et al. 1987, Zimarino and Wu 1987] the heat shock transcription factor is synthesized constitutively and present in a latent monomeric form in the cytoplasm and nucleus under normal conditions. In response to heat shock and other stresses, HSF assembles into a trimer and accumulates within the nucleus where it binds to HSE [Sorger and Nelson 1989, Westwood et al. 1991, Sarge et al. 1993]. The budding yeasts S. cerevisiae and K. lactis have constitutively trimeric HSF proteins that remain bound to HSE’s under both normal and heat shock conditions [Gross et al. 1990]. The transcriptional activity in these yeasts seems stimulated by phosphorylation of the HSF at serine and threonine residues [Sorger 1990]. Recent results [Hoj and Jakobsen 1994] however, indicate that phosphorylation of HSF in yeast serves as a regulatory mechanism to deactivate HSF, rather than being involved in its activation. Also the HSF of higher eukaryotes exhibits a stress-dependent phosphorylation that may modulate its transcriptional stimulation [Larson et al. 1988, Sarge et al. 1993], suggesting that there are multiple steps in the transcriptional activation of higher eukaryotes upon stress [Abravaya et al. 1991, Jurivich et al. 1992].

HSF binding activity can be induced in unshocked cell extracts by a variety of agents, including heat shock, that affect protein structure suggesting that the latent monomeric HSF can be activated through a simple and direct change in conformation or oligomeric state [Larson et al. 1988, Mosser et al. 1990, Zimarino et al. 1990]. However, the inability to convert the activated trimer back to the monomeric stage after restoration of the normal conditions in vitro indicates, at least for deactivation that other ‘factors’ are involved. This is further supported by the observations that expression of recombinant HSFs of higher eukaryotes in E. coli yielded a constitutively active DNA binding factor at physiological temperatures and the persistence of purified HSF in trimers in vitro [Clos et al. 1990, Rabindran et al. 1991, Nakai and Morimoto 1993]. This indicates that in situ the DNA binding ability and monomerization is controlled by an intracellular ‘factor’ that is not present in E. coli. HSF can be folded to the latent monomeric form when expressed after microinjection in frog oocytes, by DNA transfection in tissue culture cells, or by translation in reticulocyte lysates [Clos et al. 1990, Rabindran et al. 1991, Sarge et al. 1993]. It has been speculated that heat shock proteins themselves negatively regulate heat shock gene expression via an autoregulatory loop [Craig and Gross 1991, Morimoto 1993, Abravaya et al. 1991, 1992, Baler et al. 1992, Mosser et al. 1993]. As being molecular chaperones hsp’s may autoregulate the heat shock response by regulating the ratio mono - trimeric HSF. In vitro experiments suggest a role for hsp70 in HSF activation. Inactive HSF in cytoplasmic extracts from non-heat-shocked HeLa cells can be converted to the DNA binding state by exposure to heat, non-ionic detergents or low pH [Larson et al. 1988, Mosser et al. 1990]. The addition of hsp70 can block this conversion [Abravaya et al. 1992]. In vivo, the amount of activated HSF generated in response to heat shock can be reduced by experimentally manipulating the cellular
hsp level. Activation of HSF is diminished in cells that were given a previous heat shock and allowed to accumulate hsp's [Baler et al. 1992]. Constitutive overexpression of hsp70 results in a reduction of HSF activation in response to temperature elevation [Liu et al. 1993, Mosser et al. 1993], indicating that with respect to activation of the heat shock response, the cell senses temperature elevation as a decrease in the level of available hsp70 [Craig and Gross 1991]. So, activation of HSF may occur as a consequence of hsp70 being diverted away from HSF in response to an increased pool of damaged or misfolded protein substrates. Indeed, the heat shock transcriptional response was found to correlate with increased levels of denatured and misfolded proteins [Morimoto et al. 1992, Miffin and Cohen 1994]. Complexes containing hsp70 and the active trimeric form of HSF have been detected in extracts from heat-shocked human cells [Abravaya et al. 1992, Baler et al. 1992]. This suggests that the association of hsp70 with the HSF trimer may be important in the conversion of the active trimer into the inactive monomer. Activated HSF can be detected in three complexes when bound to an HSE oligonucleotide and separated in the gel mobility assay. Hsp70 was found to be present in only the two more slowly migrating complexes, probably containing HSF molecules that are being targeted for conversion to the inactive form. The rapid attenuation of HSE-binding activity in the hsp70 overexpressing cell lines suggests that hsp70 could facilitate the conversion of active HSF trimers to inactive folded monomers [Mosser et al. 1993, Liu et al. 1993]. A stable interaction between hsp70 with the inactive form of HSF has not yet been directly demonstrated, although excess exogenous hsp70 did prevent the activation of HSF \textit{in vitro} [Abravaya et al. 1992]. Also other hsp's may be involved in the regulation of HSF activation/deactivation. For instance hsp90, which negatively regulates the transcriptional activity of steroid hormone receptors, is capable of interacting with HSF [Nadeau et al. 1993]. The hsp90-HSF interaction appears to be specific for the inactive form of the factor, since antibodies to hsp90 do not interact with the active DNA bound form of HSF [Baler et al. 1992]. However, hsp90 has not been shown to regulate the activity of HSF \textit{in vivo} [Mosser et al. 1993].

A model for the regulation of HSF activity by hsp's (compiled from the data above) is shown in figure 1.20. HSF is maintained in an inactive monomeric form by the association of hsp90. This interaction does not need to be stable but may be transient. Upon heat shock, denatured and misfolded proteins compete with HSF for association with hsp90. The released HSF assembles into trimers and binds to DNA. The transcriptional activation of heat shock genes subsequently provides the cell with an amount of hsp70 that exceeds its cellular demand. Hsp70 (also hsc70 might play a role in this) then facilitates the conversion of the

\begin{figure}[h]
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\caption{Model for regulation of HSF activity by hsp70 and hsp90. HSF exists in three possible configurations. The inactive form is a monomer that either transiently interacts with or is stably associated with hsp90 (and/or hsp70 or hsc70). The active form is a trimer that is}
capable of binding to the HSE. HSF trimers associate with hsp70 during recovery from heat shock. Hsp70 and hsp90 disrupt these trimers and refold the monomers (see text for further details).

active form of HSF to inactive monomers during recovery from the heat stress, leading to repression of the heat shock response. Hsp/hsc70 may also be able to bind HSF trimers to inhibit its DNA binding. So, overexpression of hsp70 attenuates the heat shock response by inhibition of the DNA binding ability instead of inhibition of trimerization. An alternative possibility is that hsp’s are only involved in the deactivation of HSF trimers into monomers and proper refolding of these monomers. Activation may be due to stress-induced unfolding of the monomer leading to trimers in the absence of free hsp’s [Rabindran et al. 1993].

Recently, another regulatory mechanism involved in the regulation of the heat shock response has been described [Liu et al. 1993]. In addition to HSF, a constitutive HSE-binding factor (CHBF) was suggested to be involved in the regulation of hsp70 transcription. There seems to be an inverse correlation between CHBF-DNA binding and hsp70 transcription. Sodium arsenite and salicylate activated HSF-HSE binding but had little effect on CHBF binding activity, and induced a minimal amount of hsp70 mRNA. Also heat shocked M21 cells overexpressing human hsp70 had a negligible amount of HSF-HSE binding activity, but CHBF-HSE binding declined as in
the parent Rat-1 cells, which resulted in hsp70 mRNA levels comparable to Rat-1 cells. These results indicate that HSF-HSE binding may be insufficient to get hsp70 transcription.

1.2.3.2 Translational regulation of heat shock gene expression

Effect of heat shock on RNA processing

Besides the transcriptional regulation, hsp synthesis is also controlled at the level of mRNA processing. Genes of higher eukaryotes generally contain intervening sequences that must be spliced out of their initial transcripts (pre-mRNA) in order to produce a functional message. When cells are heat shocked at severe temperatures, the splicing of pre-mRNAs is disrupted and intron containing precursors accumulate [Yost and Lindquist 1986, 1991, Bond and Schlesinger 1986, Kay et al. 1987, Yost et al. 1990b]. Most heat shock genes do not contain these sequences [Holmgren et al. 1979, Ignolia et al. 1980, Hunt and Morimoto 1985], although, some hsp genes expressed at physiological temperatures do contain intervening sequences [Ignolia and Craig 1982, Hacket and Lis 1983, Dworniczak and Mirault 1987]. So, hsp mRNA is not blocked by the disruption of the splicing process and can be translated.

Translational regulation of heat shock protein synthesis

After a heat treatment, the translation of preexisting messages is blocked by blocking elongation or initiation [Ballinger and Pardue 1983] or by disruption of preexisting polysomes [Lindquist 1980b]. As newly transcribed hsp mRNAs begin to appear in the cytoplasm, polysomes are reformed on the hsp mRNAs and hsp's quickly become the major products of protein synthesis in the cell [Lindquist 1980b]. The preexisting non-hsp mRNAs extracted from heat-shocked cells are fully capable of translation in cell-free systems, indicating that it is unlikely that they undergo major modifications that would account for the lack of their translation in heat-shocked cells [Yost et al. 1990b]. So, there must be a difference between hsp mRNA and non-hsp mRNA resulting in preferential translation of the hsp mRNAs. It was found that hsp mRNAs often have long, adenine rich, untranslated leader sequences at the 5' end of the message with two regions of sequence homology [Lindquist 1981, Matthews 1986]. Fusion of the 5' end of the hsp70 gene, including the untranslated region (5'UTR) to other coding sequences resulted in translation of the chimeric transcripts at high temperatures [DiNocera and Dawid 1983, Bonner et al. 1984]. Using only the hsp70 promoter to drive transcription of the ADH gene during heat shock did not lead to translation of the message during heat shock, but only after return to physiological temperatures. Addition of the first 95 nucleotides of the 5'UTR of hsp70 mRNA resulted in translation at high temperatures [Klemenz et al. 1985]. So, it is likely that this 5'UTR plays an important role in the translational regulation of hsp mRNA.

Studies by Scott and Pardue [1981] and Sanders and colleagues [1986] indicated that the change in translational specificity during heat shock apparently results from
the inactivation or modification of a factor that is required for the translation of physiological mRNAs. Hsp mRNAs, either do not require this factor or are able to utilize it in its modified form. The S6 ribosomal protein was an early candidate for such a factor, since it was dephosphorylated immediately after heat shock [Glover 1982]. Yet, its phosphorylation state did not correlate with heat shock translation under other conditions [Olsen et al. 1986]. Also the dephosphorylation of initiation factor eIF-4b and phosphorylation of the initiation factor eIF-2a [(Duncan and Hershey 1984] may be involved in the translational regulation. Another mechanism that might be involved in this regulation is the cap binding protein [Maroto and Sierra 1988, 1989]. Preexisting mRNAs require cap binding factor for efficient translation, possibly to unwind secondary structure in the message leader. Heat shock inactivates this factor. Hsp mRNAs, containing long leader sequences with very little secondary structure are able to escape the requirement for cap binding factor and therefore can be translated at high temperatures. Yet, not all data can be explained by this mechanism [Yost et al. 1990]. Another factor that might be involved is a 25 kD protein that recognizes an element within the first 25 nucleotides of hsp70 mRNA and does not recognize the 5' end of actin mRNA. This would suggest that translation of hsp mRNA during heat shock may be facilitated by a specific (heat-activated) protein factor [Yost et al. 1990b].

In conclusion, heat shock gene expression is regulated at the transcriptional as well as at the translational level. The transcriptional activation is very rapid after a heat treatment, resulting in additional RNA transcripts. The translational regulation is very important in the discrimination between hsp mRNA and non-hsp mRNA, leading to an increased level of hsp’s during/after a heat shock.

1.2.4 Thermotolerance, thermoresistance and intrinsic heat-sensitivity; role of heat shock proteins.

Cells exposed to a (non-lethal) heat-dose (or to certain chemicals) can develop a transient increase in resistance to a subsequent heat treatment. This phenomenon is called thermotolerance [Gerner and Schneider 1975, Henle and Leeper 1976]. In contrast to this transient heat resistance, stable heat resistant cells can be obtained by isolating heat resistant variants after several cycles of severe heat treatments or treatments with mutagenizing agents or by transfection of cells with a hsp gene. This stable resistance is here defined as thermoresistance. Finally, the variety in heat sensitivity amongst species will be referred to as intrinsic heat sensitivity.

1.2.4.1 Thermotolerance

When cells are exposed to a short (3-15 min) heat treatment at or above 43°C, a transient heat resistance develops which is known as 'acute thermotolerance' [Gerner and Schneider 1975, Henle and Leeper 1976]. Continuous exposure of cells to a
relatively low hyperthermic temperature (at or below 42.5°C) results in the development of 'chronic thermotolerance'. In the latter case, cell killing by heat levels off after a few hours and the cells become resistant to longer heat treatments [Sapareto et al. 1978, Jorritsma et al. 1986, Henle 1987]. Thermotolerance can be induced in vitro and in vivo, in normal tissue as well as in tumors [Urano 1986, Li and Mivechi 1986]. Development of thermotolerance has been described for cells of different origin including bacteria McCallum and Innis 1990, Trent et al. 1990], insects [Koval and Suppes 1992], yeast [De Virgilio et al. 1991a, 1991b, Sanchez and Lindquist 1990], plants [Howarth 1990] and mammals, with the exception of embryos [Dura 1981, Wittig et al. 1983, Müller et al. 1985, Banerji et al. 1984, 1987]. These are unable to develop thermotolerance before a certain stage, probably due to a developmentally regulated inability to respond to heat shock [Morimoto and Milarski 1990]. This inability might be essential to prevent development of aberrant or mutagenic cells. A third type of thermotolerance can be induced by prior treatment with chemicals (chemical thermotolerance). Preincubation of cells with for instance alcohols or sodium arsenite followed by a drug free period leads to the development of thermotolerance [Li and Hahn 1978, Li 1983, Henle et al. 1986, Kampinga et al. 1992]. Other chemical inducers of thermotolerance are listed in table 1.3.

**Table 1.3 Inducers of thermotolerance (data from Henle 1987).**

<table>
<thead>
<tr>
<th>Inducers of thermotolerance</th>
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<tbody>
<tr>
<td>heat glucocorticoids</td>
</tr>
<tr>
<td>alcohols prostaglandins</td>
</tr>
<tr>
<td>sodium arsenite CCCP</td>
</tr>
<tr>
<td>diamide disulfram and DDC</td>
</tr>
<tr>
<td>dinitrophenol calcium related drugs</td>
</tr>
</tbody>
</table>

All inducers of thermotolerance are known to increase the expression of heat shock genes. Several reviews have been written on the involvement of hsp’s in thermotolerance [Li and Laszlo 1985, Lindquist and Craig 1988, Hahn and Li 1990, Morimoto et al. 1990, Welch 1990, to name a few]. Good correlations were observed between development and decay of thermotolerance and hsp synthesis [Li and Werb, 1982]. In particular, intracellular levels of hsp70 and hsp27 might be used as an indicator of retained thermotolerance [Li and Werb 1982, Li and Laszlo 1985, Chrétien and Landry 1988] (figure 1.21). Recently [Ohtsuka et al. 1994], also hsp40 has been
shown to be a good indicator of thermotolerance. These findings suggest a causal relation between hsp expression and thermotolerance.

Figure 1.21 Development and decay of thermotolerance in relation to the level of individual hsp’s. (a) thermotolerance and hsp27; (b) thermotolerance and hsp70. (Redrawn after Landry et al. 1991).

However, inhibition of protein synthesis during development of thermotolerance did not always or only partially inhibit the expression of thermotolerance [Henle and Leeper 1982, Laszlo 1988b, Lee and Dewey 1988, Kampinga et al. 1992]. This would imply that at least newly synthesized hsp’s are not necessary for thermotolerance expression. This was supported by the observation that cells with a mutated heat shock factor (HSF) were still able to develop thermotolerance [Smith and Yaffe, 1991]. Whether the observed thermotolerance under such conditions of inhibition of protein synthesis is totally independent on the action of hsp’s is yet unclear. Most stress treatments that induce thermotolerance, inhibit physiological cellular functions, including protein synthesis. Inhibition of these functions may release (constitutive) hsp’s from their physiological functions (see part on heat shock proteins) resulting in an increased pool of free constitutive hsp’s. These hsp’s may now function in stress protection as suggested previously [Beckman et al. 1992, Burgman et al. 1993], and as such explain the development of thermotolerance in the absence of newly synthesized hsp’s. Interestingly, heat shock induces both hsp synthesis dependent and independent states of thermotolerance [Boon-Niermeijer et al. 1986, Laszlo 1988b], whereas a sodium-arsenite treatment only induces protein synthesis
dependent thermotolerance [Laszlo 1988b, Lee and Dewey 1987, 1988, Lee et al. 1990, Kampinga et al. 1992]. Due to its mode of action on for instance protein synthesis, it can be assumed that arsenite does not increase the levels of free constitutive hsp’s [see Burgman et al. 1993].

Thus, it is likely that hsp’s are involved in thermotolerance, both in protein synthesis dependent as well as in protein synthesis independent. Rather than total levels per se, however, increases in the level of “free” hsp’s may be the most important determinant of thermotolerance.

1.2.4.2 Thermoresistance

Heat resistance cells can be obtained by introducing exogenous hsp genes into cultured mammalian cells by for instance transfection or by selection of heat resistant variants after a treatment with mutagenizing agents or after several cycles of severe heat treatments. Several methods exist to introduce exogenous hsp genes (or other genes) into cultured mammalian cells. Most well known are microinjection, calcium phosphate transfection technique and viral infection.

Microinjection

An often used technique to introduce exogenous DNA into mammalian cells is microinjection [Graessmann and Graessmann 1976, Stacey and Alfrey 1976, Capecchi 1980]. A disadvantage of this technique is the low number of cells that can be microinjected within a reasonable time. Nevertheless, a high percentage (50-100%) of the microinjected cells express the injected exogenous DNA [Capecchi 1980]. With this method one can directly inject in the nucleus with a higher chance of integration in the nuclear genome. A variation of the standard microinjection technique is ‘pricking’ which mechanically introduces DNA into the nuclei of cultured cells [Yamamoto et al. 1982]. Cells are overlayed with donor DNA and are pricked in the nuclear domain (figure 1.22). This method can also be used for protein injection. With regard to hsp’s microinjection has been used to introduce hsp70 into CHO cells [Li et al. 1989] resulting in heat resistant cells. It was also used to introduce hsp70 antibodies in rat fibroblasts [Riabowol et al. 1988] leading to increased heat sensitivity. Disadvantages of microinjection of proteins is that it is not possible to determine surviving fractions beneath 10% due to the low number of cells that can be injected (very time consuming). Furthermore, microinjection may induce a stress response and results may be a-specific: e.g. injection with BSA did result in increased heat resistance [Li et al. 1989].

Figure 1.22 Schematic representation of microinjection (1 and 2) and ‘pricking’ (3 and 4). DNA molecules are shown by the circles. In microinjection the micropipette is filled with the DNA solution whereas in ‘pricking’ the DNA is present in the external medium. (Redrawn after Spandidos and Wilkie 1984).
Calcium phosphate transfection technique

The calcium phosphate technique is the most widely used method and was first described by Graham and van der Eb [1973]. It provides a general method for introducing any DNA into mammalian cells for either transient expression assays or stable long-term transformation. The successful DNA transfer is dependent upon the formation of a co-precipitate of the exogenous DNA with calcium phosphate. After addition to the cells, the calcium phosphate granules are phagocytosed by the cells. The advantage of this transfection procedure is that it is an easy one and can be used for a large number of cells. This method is limited by the fact that a variable and rather low (1-2%) proportion of cells that take up exogenous DNA. Only in a subfraction of these cells the exogenous DNA becomes stably integrated into the nuclear genome [Old and Primrose 1985]. The uptake and expression of exogenous DNA varies with different cell lines used as recipient. Several procedures have been designed to increase this fraction with a maximum increase to about 20% of cells that take up exogenous DNA [Chu and Sharp 1981]. Especially the use of so-called 'facilitators' of transformation as DMSO, glycerol, colchicine and cytochalasin D have been described to increase the expression of transferred donor DNA in recipient cells [Farber and Eberle 1976, Stow and Wilkie 1976, Fraley et al. 1980, Spandidos and Paul 1982]. The use of these facilitators is questionable since they also exhibit toxic effects in some cell lines.

Tk- cells transfected to a Tk+ phenotype can be easily selected in HAT medium [Wigler et al. 1977]. However, the isolation of cells transfected with nonselectable genes remained problematic. Therefore, the technique of co-transfection was developed [Wigler et al. 1979]. With this technique, recipient cells are exposed to the donor DNA along with another (not physically linked) DNA sequence which encodes a selectable marker. Cells take up both DNA sequences and transformants can be selected. The frequency of co-transfection is very high: over 90% of the transformants contain both DNA sequences. Selectable markers that are often used are for instance the dihydrofolate reductase gene which leads to methotrexate resistance or the neomycin phosphotransferase which confers resistance to antibiotics as kanamycin, neomycin and G418.
The method of co-transfection has been used to (over)express human hsp27 into Chinese hamster fibroblasts [Landry et al. 1989], human hsp70 into rat fibroblasts [Li et al. 1991] and monkey cells [Angelidis et al. 1991] and mouse hsp 25 into mouse cells [Knauf et al. 1992, 1994]. Overexpression of the distinct hsp’s did not result in a stress response in these studies and generally resulted in increased heat resistance. Overexpression of hsp70 did not always result in heat resistance, but may lead to sequestration of hsp70 paralleled by growth inhibition [Feder et al. 1992]. The differences observed after transfection of hsp70 seem related to the level of hsp70 gene expression (see 1.2.2.3), but further studies are necessary for clarity.

In some studies [Knauf et al. 1992, 1994], transient transfectants were used versus stable transfectants in other studies [Landry et al. 1989, Li et al. 1991, 1992, Angelidis 1991]. The mechanisms involved in the uptake and expression of donor DNA in recipient cells are poorly understood. Shortly after the introduction of DNA there is a transient phase of gene expression. It is likely that during this phase the newly introduced genes are converted into minichromosomes [Gilmour et al. 1982]. Using the calcium phosphate technique, the transient phase of expression lasts between 2 and 3 days. This transient expression provides a rapid method of testing the effects of transferred gene products. A disadvantage of the transient expression is the impossibility of selection. One has to pool all cells, with only up to a maximum of 20% transfected cells (often lower) to test the effect of the gene products. Under selective conditions, transformed colonies appear after 1-3 weeks. Further culturing and selection are necessary to obtain individual clones. Due to the low transfection frequency, selection for co-transfected antibiotic resistance may not be sufficient. Additional heat selection may be needed to select the heat resistant hsp expressing cells [Li et al. 1991]. Often pooled populations of several colonies are used in experiments instead of individual clones.

**Viral infection**

A third method of introducing exogenous DNA into mammalian cells is via infection with viral vectors [Spandidos and Wilkie 1984 for review]. There are two classes of viral vectors: the lytic viruses such as polyoma and SV40 and the so-called 'shuttle vectors' based on retroviruses and papillomaviruses. For lytic and retroviral based vectors, the exogenous DNA needs to packaged into infectious viruses. After infection with the viruses, the virus vectors were correctly expressed. The disadvantage of the use of lytic viruses is the cell killing effect. Retroviruses are ideally to produce stable cell lines. They replicate via a circular DNA provirus intermediate, which integrates efficiently into the host cell chromosome and are often non-toxic. Retrovirus-packaging mutants are available which can be used to produce helper-virus-free recombinant viruses [Mann et al. 1983]. Also papillomavirus-based vectors can be used for the production of a stable cell line which continuously
expresses and replicates the donor DNA. This method has the advantage that, in contrast to retroviruses, it is not subjected to packaging constraints. This viral vector with donor DNA replicates as an episome. This means that the papillomavirus-transformed cells do not contain integrated viral DNA [Law et al. 1981, Moar et al. 1981]. Instead they contain 50-300 copies of circular unintegrated DNA and correctly express eukaryotic genes which have been ligated into the recombinant vector [Zinn et al. 1982, DiMaio et al. 1982, Segikuchi et al. 1982].

The advantage of the viral infection over the calcium phosphate transfection is the higher expression frequency of the transferred genes. Li and colleagues [1992] used the retroviral based procedure to infect Rat-1 cells with the human hsp70 gene. Also, for transient expression studies this infection procedure is preferential due to the higher infection/transfection frequency.

**Selection of heat resistance phenotypes**

Several studies report the isolation of heat resistant variants, which resistance seems to be based on the overexpression of hsp’s. In the heat-resistant CHO-HA1 mutants, selected after several severe heat cycles, the constitutive hsc70 was expressed in increased amounts [Laszlo and Li 1985]. Heat resistant lines selected from V79 cells after EMS treatment, expressed elevated levels of hsp27 [Chrétien and Landry 1988] and selected resistant mutants from CHO cells (EMS treatment) showed an increased expression of hsp90 [Yahara et al. 1986]. Cells selected from the murine fibrosarcoma RIF-1 after repeated cycles of heat exposure, overexpressed most major hsp’s, but especially hsp70/hsc70 [Anderson et al. 1989]. In contrast with these mutants, heat resistant variants derived from B16 melanoma cells have been selected without elevated levels of known hsp’s [Anderson et al. 1986]. Recently, also Lee and coworkers [1992] selected heat-resistant variants of CHO cells with altered cellular structures and elevated levels of vimentin, but normal hsp expression. Heat resistance may also be evoked by amino acid substitutions in heat sensitive proteins leading to thermostable proteins as has been found in thermophilic bacteria [Argos et al. 1979].

In general, heat resistance increases with increasing levels of hsp’s. In some cases other mechanism than hsp’s are responsible for the observed increase in heat resistance.

**1.2.4.3 Intrinsic heat-sensitivity**

Mammalian cells vary in their intrinsic sensitivity to heat. Raaphorst and colleagues [1979] showed that cells derived from different species varied in their heat sensitivities in a manner correlated to the normal body temperature of the animal that was the source of the particular cell line. However, also in cells from identical origin differences in the intrinsic heat sensitivity can be observed [Anderson et al. 1993]. Despite a relation with thermotolerance (1.2.4.1) and thermoresistance (1.2.4.2), the fact that competitive inhibition of hsp expression [Johnston and Kucey, 1988] and
microinjection with hsp70 antibodies [Riabowol et al. 1988, Lee et al. 1993] lead to an increased heat sensitivity, and microinjection with hsp70 resulted in heat resistance [Li et al. 1989, Lee et al. 1993] no relation could be detected between the intrinsic heat sensitivity and the level of hsp70/hsc70 expression [Anderson et al. 1993] (table 1.4). Analysis of three human (FME, HT1080 and A549) and two murine (RIF-1 and CH1) cell lines indicated that cells with less hsp70/hsc70 even have a somewhat greater resistance to heat.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$D_0$ at 44°C (min)</th>
<th>$T_{0.01}$ at 44°C (min)</th>
<th>ELISA (arbitrary units)</th>
<th>Radioscanning (% total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsc/p70</td>
<td>hsc70</td>
</tr>
<tr>
<td>RIF-1</td>
<td>3.5</td>
<td>63</td>
<td>6.3±1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>CH1</td>
<td>5</td>
<td>17</td>
<td>5.9±0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>FME</td>
<td>18</td>
<td>88</td>
<td>4.3±1.1</td>
<td>ND</td>
</tr>
<tr>
<td>HT1080</td>
<td>26.5</td>
<td>142</td>
<td>2.4±0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>A549</td>
<td>27</td>
<td>137</td>
<td>3.0±0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

In summary, there seems to be a correlation between the expression of hsp's and the extent of thermotolerance. Not the level of newly synthesized hsp's seems to be the major determinant; also the availability (free pool) of constitutive hsp's has to be considered. Heat shock proteins seem also involved in thermoresistance. In general, increasing the amount of hsp's either by transfection or by treatments with severe heat or mutagenizing agents, results in heat resistance showing that hsp's (free level) can protect from thermal killing. However, stable thermoresistance can also be based on other features such as increased (protein) stability of (heat-sensitive) structures by for instance amino acid substitutions [Anderson et al. 1986, Lee et al. 1992].

As sensitivity to heat is a multifactorial process, where protein (structure) stability, metabolic processes, cell cycle progression all may (in part) depend on the (chaperone) action of hsp's, intrinsic heat sensitivity is unlikely to be reflected by the total cellular levels of hsp's. So, although they may play a role in intrinsic heat sensitivity, levels of hsp's are unlikely to "predict" it.
1.3 HEAT AND RADIATION

1.3.1 The synergism of heat and radiation

Besides its direct cell killing potential, heat was also demonstrated to act synergistically with radiation (and several drugs). This synergistic interaction of heat and radiation is interpreted as a heat-induced sensitization of cells to radiation. Heat radiosensitization can be quantified as thermal enhancement ratios (TER) defined as:

$$\text{TER} = \frac{\text{effect of radiation}}{\text{effect of radiation plus heat (corrected for effect of heat alone)}}$$

These TERs can be expressed at the level of isodose, isosurvival or as a ratio of the $D_0$’s of the radiation survival curves. Usually, TER increases with increasing heat dose. The best synergism is obtained when heat and radiation are given simultaneously. When the two treatments are separated in time, the TER decreases with increasing time intervals. When radiation precedes hyperthermia, sensitization is no longer possible 2 to 3 hours after radiation (independent of radiation dose). When hyperthermia precedes radiation, cells can be sensitized for up to several hours, depending on the heat dose used (figure 1.23). So, at least for the highest heat doses, heat-induced damage (responsible for the interaction) in the cell is repaired at a slower rate than radiation-induced damage with which heat can interact.

**Figure 1.23** Effect of separation of heat and radiation on cell survival. Asynchronous cells received radiation before, during or after hyperthermia. The synergism is indicated by the shaded area. (Redrawn after Kampinga 1989).

1.3.2 Mechanisms of interaction

The combination of heat and radiation does not result in an enhancement of "heat lesions"; so, no radiation-induced heat sensitization takes place and the synergistic
effect must be due to radiosensitization by heat [Henle 1987]. The primary target for radiation is thought to be DNA, while protein denaturation/aggregation seems involved in thermal cell killing [Warters and Roti Roti 1982, Kampinga et al. 1987, 1989a]. DNA damage (e.g. breaks) can be detected at low lethal doses of X-rays (≤ 1 Gy), while no DNA breaks are found at low lethal heat doses [Jorritsma and Konings 1983, 1986]. Longer exposure to relatively high hyperthermic temperatures (above 43°C) may sometimes lead to a detectable amount of DNA damage [Jorritsma and Konings 1983, 1986, Warters 1985]. Yet, these are likely due to secondary reactions following (severe) heat damage to the protein component of chromatin (see 1.1.3.3). The combination of radiation and hyperthermia generally does not result in more initial DNA breaks then observed for radiation alone [Corry et al. 1977, Warters and Roti Roti 1982, Jorritsma and Konings 1983], so enhancement of radiosensitivity is not the result of an increment in initial DNA lesions.

It has repeatedly been shown that hyperthermia inhibits the rejoining of radiation-induced DNA strand breaks [Corry et al. 1977, Clark et al. 1981, Bowden and Kasunic 1981, Lunec et al. 1981, Mills and Meyn 1981, Dikomey 1982, Jorritsma and Konings 1983, Dikomey and Franzke 1992, Kampinga et al. 1993a], as well as the excision of damaged bases [Warters and Roti Roti 1978, 1979]. This inhibition of DNA repair was found to be heat dose dependent [Jorritsma and Konings 1983, Dikomey and Franzke 1992]. However, hyperthermic treatments below 42°C may cause an enhanced DNA repair, while radiosensitization is apparent [Dikomey 1982, Warters et al. 1987]. Furthermore, thermotolerance has been shown to protect against thermal inhibition of DNA repair, while no effect of thermotolerance on heat radiosensitization was observed [Jorritsma et al. 1985]. These findings indicate that hyperthermic effects on the rate of DNA repair cannot always explain heat radiosensitization. Other factors such as changes in fidelity of repair, altered fixation of damage or preferential repair of active genes might also be important. In any case, it is widely accepted that thermal radiosensitization is somehow due to an impairment of (proper) repair of damaged DNA. Such effect may be caused by 1) heat-induced reduction in the activity of DNA repair enzymes and/or by 2) alteration of the chromatin structure, due to protein denaturation/aggregation (see 1.1.3.3), causing a decreased accessibility of the damaged sites to the repair machinery.

1.3.2.1 Inactivation of repair enzymes

Among the enzymes involved in DNA repair, DNA polymerases α and β are the most extensively investigated with respect to their role in hyperthermic inhibition of repair [Dewey and Esch 1982, Spiro et al. 1982, Mivechi and Dewey, 1985, Jorritsma et al. 1985, Dikomey and Jung 1988 1993, Raaphorst et al. 1993]. Both enzymes showed a heat-dose dependent loss of activity [Dewey and Esch 1982, Spiro et al. 1982, Mivechi and Dewey 1984]. Especially DNA polymerase β was found to be very heat-sensitive [Dube et al. 1977] and was reported to correlate well with thermal
radiosensitization [Spiro et al. 1982, Jorritsma et al. 1985, Kampinga et al. 1986, Dikomey and Jung, 1988, 1993, Mivechi et al. 1990, Raaphorst et al. 1993]. However, other studies [Jorritsma et al. 1986, Kampinga and Konings 1987, Kampinga et al. 1989b] showed that inactivation of polymerases may not be taken as a general cause of thermal radiosensitization. Using non-tolerant and thermotolerant cells, Kampinga et al. [1989b] showed that in thermotolerant HeLa S3 cells the decline in TER is different from normal cells when heat and radiation are separated in time, whereas the recovery of polymerase α and β activity was similar in tolerant and non-tolerant cells [Kampinga et al. 1989b] (figure 1.24). Re-analysis of data from the literature [Mivechi and Dewey 1985, Chu and Dewey 1987, 1988] suggested a relation between DNA polymerase inactivation and heat-radiosensitization within the individual experiments. However, the variation between the slopes of these curves for the different experiments suggest no general relation between DNA polymerase inactivation and heat-radiosensitization. This is supported by recent data obtained by Dikomey and Jung [1993], showing a linear relationship between the loss of DNA polymerase β activity and the β-term, but not the α-term of the survival curves. Other enzymes involved in DNA repair such as DNA topoisomerase II and DNA glycosylases are relatively heat insensitive [Warters and Brizgys 1988, Warters and Roti Roti 1978, 1979] and thus do not seem to be responsible for the hyperthermic effects on DNA repair. Finally, recent data by Sakkers et al. [1993, 1995a] using repair of UV-induced DNA damage as a model system, revealed no effects of heat on the repair of inactive DNA sequences. These data strongly suggest that, despite sometimes dramatic loss in activities of repair enzymes after cellular heating, sufficient residual activity (including repolymerizing activity) is present in cells to repair DNA damage.

Figure 1.24 The effect of time between hyperthermia and radiation on TER (a) and polymerase β activity (b). (Redrawn after Konings 1992).

1.3.2.2 Heat-induced alteration of the chromatin structure

The activation energy for delay in repair of radiation induced damage is about 600 kJ/mol [Jorritsma and Konings 1983, Warters et al. 1985], indicating that protein denaturation/aggregation plays a crucial role in heat induced inhibition of repair. As described in section 1.1.3.3, heat induces protein denaturation which results in the formation of protein aggregates in the nucleus/chromatin. This may lead to a reduced accessibility of the damaged DNA for repair enzymes. Warters and Roti Roti [1978] showed that the excision of γ-irradiation induced 5'-6'-dihydroxydihydrothymine types of base damage (t' type) was inhibited by heat. The cell homogenate of heated or unheated cells was equally effective in excision of t' type damage from isolated irradiated DNA [Warters and Roti Roti 1979], indicating that the activity of DNA glycosylases, DNA endo-, and exonucleases the enzymes is not affected by hyperthermia in a rate limiting manner. Using irradiated chromatin isolated from
heated cells as a substrate, the rate of excision of DNA damage was remarkably lower than in chromatin isolated from unheated cells, irrespective of the cell homogenate used, suggesting that the reduced excision rate is caused by a decreased accessibility of the damaged sites. In addition, a correlation between hyperthermic inhibition of repair and protein aggregates in chromatin was reported by Mills and Meyn [1981]. More recently, studies using the fluorescent halo assay also suggested a decreased accessibility of radiation induced DNA damage related to nuclear protein aggregates [Kampinga et al. 1988, Wynstra et al. 1990]. It was shown that heat-induced nuclear protein aggregation was related to "masking" radiation damage and to repair inhibition [Kampinga et al. 1989b]. The cells' ability to repair radiation-induced DNA damage recovered to control levels within 6 h post-heating and the heat-masking effect also disappeared (figure 1.25). The recovery of these processes paralleled the removal

**Figure 1.25** The effect of post-hyperthermic incubations on the heat-induced inhibition of post-irradiation repair and masking of radiation induced damage. (a) The effects of heat and post-heat incubation at 37°C on the ability of cells to repair the radiation damage that results in inhibition of the ability to rewind DNA supercoils. The percent of initial damage remaining is plotted against post-heat incubation time. (b) The relative excess halo diameter after 10 Gy as a percent of the unheated, but irradiated cells, is plotted against post-heat incubation time. Closed circles, control; closed triangle, 30 min 45°C; open triangle, 30 min 45°C plus 3 h 37°C; open squares, 30 min 45°C plus 6 h 37°C. (Redrawn after Kampinga et al. 1988).

of nuclear protein aggregates. The before mentioned data by Sakkers et al. [1993, 1995a] also provided evidence that heat-induced structural alterations are responsible for the impairment of DNA repair: the specific effect of heat on the repair of DNA...
damage in the nuclear matrix associated active ADA gene could be explained by an altered association of that gene with the nuclear matrix. Thus, heat-induced nuclear protein aggregates may directly be involved in heat radiosensitization. The first indication that the amount of heat-induced nuclear protein aggregates correlates with the extent of thermal radiosensitization was reported by Kampinga et al. [1989b] using non-tolerant and thermotolerant HeLa S3 cells. When heat and radiation were separated in time, the decline in TER paralleled the recovery from nuclear protein aggregation (figure 1.26). Further experiments however are needed to test the generality of these correlations.

**Figure 1.26** The effect of time between hyperthermia and radiation on TER (a) and nuclear protein aggregation (b). (Redrawn after Konings 1992).

1.3.3. Heat radiosensitization and thermotolerance

For the clinical application of hyperthermia in combination with radiation it is important to know if heat radiosensitization is the same for normal and thermotolerant cells, because in radiotherapy mostly fractionated doses will be delivered to the patient. Therefore, the impact of thermotolerance on heat radiosensitization has been a topic of extensive investigation [see Konings 1987 for review]. Continuous heating at low hyperthermic temperatures (< 42.5°C) resulted in the development of chronic thermotolerance. Chronic thermotolerance did affect heat radiosensitization in some
cell lines [Freeman et al. 1979, Raaphorst and Azzam 1983, Holahan et al. 1984, Van Rijn et al. 1984, Jorritsma et al. 1986, Dikomey and Jung 1992], although for other cell lines no effect of thermotolerance was observed at the level of heat radiosensitization [Raaphorst and Azzam 1983, Streffer et al. 1984, see Konings 1987 for review].

Also the effect of acute thermotolerance on heat radiosensitization has been rather contradictory [Konings 1987]. Some studies showed less heat radiosensitization in thermotolerant cells compared to their nontolerant counterparts, whereas this was not observed by others (table 1.5). Even within cell-lines from the same origin (CHO) contradictory results were obtained [Henle et al. 1979, Holahan et al. 1986, Hartson-Eaton et al. 1984, Majima et al. 1985, Dikomey and Jung 1992]. These controversial effects of thermotolerance on heat radiosensitization need further elucidation and are subject of this thesis.

**Table 1.5 Effect of ‘acute’ thermotolerance on heat radiosensitization (after Konings 1987)**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment temperature (°C)</th>
<th>Effects of TT</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>45</td>
<td>+</td>
<td>Henle et al. (1979)</td>
</tr>
<tr>
<td>V79</td>
<td>45</td>
<td>+</td>
<td>Raaphorst and Azzam (1983)</td>
</tr>
<tr>
<td>M80/13</td>
<td>43</td>
<td>+</td>
<td>Haveman (1983a)</td>
</tr>
</tbody>
</table>
1.4 SCOPE OF THE THESIS

From the literature cited it is obvious that heat-induced protein denaturation and subsequent aggregation is involved in hyperthermic cell killing. Thermotolerant cells showed less initial formation or accelerated disaggregation of nuclear protein aggregates upon heat shock. Heat shock proteins have been suggested to play a role in these processes as they seem to be involved in thermotolerance. Most of the heat shock proteins are expressed constitutively and it has been shown that these proteins act as molecular chaperones and are involved in protein folding. Heat shock proteins have been shown to protect proteins from aggregation or are involved in disaggregation. Most of these studies were performed in cell free experiments with purified proteins. The main purpose of this thesis was to investigate the role of heat shock proteins in heat-induced protein denaturation, aggregation and disaggregation in situ, and its impact on hyperthermic cell killing and heat radiosensitization.

First [chapter 2], the effects of alterations in cytosolic free calcium on cell killing, nuclear protein aggregation and hsp synthesis were investigated, since it has been suggested that increases in the level of cytosolic free calcium is a primary event leading to hyperthermic cell killing. In chapter 3-5 the involvement of hsp70 and hsp27 in heat-induced nuclear protein aggregation was investigated using cells transfected with the individual hsp genes. Furthermore, experiments were performed to examine the relation between the different hsp’s and heat-induced nuclear protein aggregation during the development and decay of thermotolerance [chapter 6]. Besides heat, chemical agents were used to induce thermotolerance [chapter 7]. In these experiments protein aggregation was investigated in two cellular subfractions examine the relation between induced thermotolerance and induced resistance of proteins in different celfractions.

Finally [chapter 8], the effect of thermotolerance on heat radiosensitization was studied. The different levels of hsp’s present in different cell types leading to
variations in heat-induced nuclear protein aggregation may be responsible for the controversial effects of thermotolerance on heat radiosensitization.