Regulation of transcription and translation

A tale of crystallins and small heat shock proteins

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Table of contents

General Introduction

Chapter 1

Introduction

The lens and crystallins

The unique feature of the lens is its transparency. This transparency is due to the special architecture of the cells and to their unusual protein content. As shown in Figure 1, the lens consists of two different cell types: the epithelial cells, which form a single layer at the anterior side of the lens, and the very densely packed, elongated fiber cells, which form the bulk of the lens. At the equator of the lens, the epithelial cells differentiate into fiber cells. They elongate, loose their membrane bounded cytoplasmic organelles and at the last step of fiber cell differentiation, they loose their nuclei, mitochondria and ribosomes in a process resembling the early steps of apoptosis (Bassnett 2002). This loss of cellular organelles is required for transparency – it removes light scattering particles - but has as a consequence that the terminally differentiated fiber cell can no longer synthesize proteins. Hence, the lens proteins located at the center of the lens and synthesized during fetal development cannot be replaced and must last the life time of the organism.

The lens is a focusing device that allows images to be formed on the retina. To serve this function, the eye lens has to fulfill two requirements; it has to be transparent and it must have a high refractive index, i.e. low light scattering and high solubility of its abundant cytoplasmic proteins, the crystallins. Three families of crystallins, the α-, β- and γ-crystallins can be found in all vertebrate lenses and are therefore known as the ubiquitous crystallins. In addition to the ubiquitous crystallins, some species contain other crystallins; these are known as the taxon specific crystallins. Examples of taxon specific crystallins are: ε-crystallin in crocodiles and some birds, δ-crystallin in most birds and reptiles, λ-crystallin in rabbits and hares, ρ-crystallin in frogs (*Rana*) and τ- crystallins in lamprey (for an overview of vertebrate crystallins see Table 1;Wistow 1993; Piatigorsky 1998).

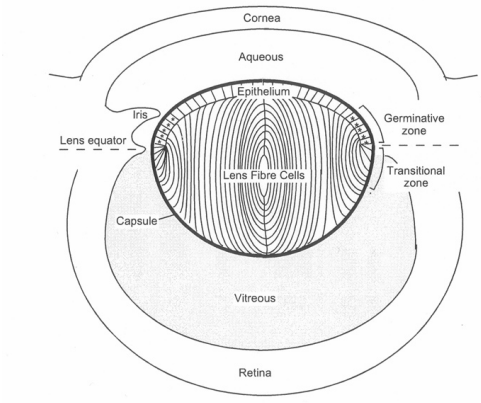

Figure 1: Schematic overview of the mammalian eye showing the fully formed lens.
The epithelial cells and fiber cells are indicated. Differentiation of the epithelial cells starts at the equator. The transitional zone contains early fiber cells; fiber cells in the nucleus are fully differentiated. Picture taken from Schulz et al. (1993).

	subunits	species
α	αA, αB	All vertebrates
β	βA3/A1, βA2, βA4 and βB1, βB2, βB3	All vertebrates
γ	γA, γB, γC, γD, γE, γF and γS	All vertebrates
δ	δ1, δ2	Birds, reptiles
ε		Birds, crocodiles
λ		Rabbit, hare
τ		Lamprey, fish, reptiles, birds
ρ		Frogs (Rana)
ζ		Guinea pig, camel, degu, llama, rock cavy
μ		Australian marsupials
η		Elephant shrews
π		Geckos

Table 1: Overview of crystallins present in vertebrates

During differentiation of the fiber cells, the different classes of crystallin genes are expressed in a strict temporal and spatial order. Furthermore, the expression of the crystallin genes is regulated in a developmental manner. The αA-crystallin gene is the first crystallin gene to be transcribed in rat lenses: its mRNA can already be found in the lens vesicle, before the formation of the primary fiber cells. αB-crystallin mRNA can be found at a later stage, namely after the formation of the primary fiber cells. The mRNA levels encoding these two crystallins stay high after birth and reach their maximum at about 4.5 months. These mRNAs can still be detected in adult rat lenses (van Leen et al. 1987; Aarts et al. 1989). In mammals, the β-crystallins are fiber cell specific. Their genes are, however, expressed at different times during development. For example, the highest amounts of βB3-crystallin mRNA can be found in pre-natal lenses and the βB3-crystallin mRNA level decreases rapidly after birth. βA3/A1-crystallin mRNA, however, reaches its maximum level at about 2 months after birth. Finally, only very low amounts of the transcript of the βB2-crystallin gene can be found in pre-natal rat lenses, but this gene is highly expressed in postnatal lenses (Aarts et al. 1989). The γ-crystallin genes are also fiber cell specific. They are the last crystallin genes to be expressed during fiber cell differentiation, being preceded by first the α-crystallins and then the β-crystallin genes (McAvoy 1978; van Leen et al. 1987; Peek et al. 1992). In rat, expression of the different γ-crystallins starts at the same time point in the developing embryonic lens. Differential expression of these γ-crystallins is mediated by differential shutdown of transcription of the corresponding genes. In the rat lens, the highest amount of total γ-crystallin mRNA can be found at approximately 2 months after birth. γA-, γE- and γF-crystallin mRNAs however decrease rapidly 1 month after birth and are absent after 3.5 months (van Leen et al. 1987). In contrast to the rapid decrease in γE- and γF-crystallin mRNA, Ueda et al. (2002) found high amounts of γE- and γF-crystallin protein in lenses of six weeks old mice. γB-, γC- and γD-crystallin mRNAs are still present after 6 months: γC- and γD-crystallin mRNA levels however have dropped significantly after 3.5

months (van Leen et al. 1987). During the in vitro differentiation of explanted newborn rat lens epithelial cells to lens fiber cells in the presence of FGF-2, this differential expression pattern of the crystallin mRNAs is seen as well (first αA-, then βB2- and then γ-crystallin mRNA; Peek et al. 1992). Data from Voorter et al. (1990) on de novo protein synthesis in rat lenses are in good agreement with these mRNA data, indicating that differential regulation at the translational level does not play a major role in crystallin gene expression.

Expression of crystallins is often not restricted to the lens. Most taxon specific crystallins are known to function as enzymes; for example ε-crystallin is the glycolytic enzyme lactate dehydrogenase B (LDHB) and τ-crystallin is α-enolase (for review, see Piatigorsky and Wistow 1989). Not only the taxon specific crystallin genes are expressed outside the lens, some of the ubiquitous crystallin genes are active in other tissues as well; for example βB2-crystallin mRNA is found in rat brain and testis and in human and bovine retina (Dirks et al. 1998; Magabo et al. 2000). Many of the α-, β- and γ- crystallin mRNAs have been found in mouse retina using RT-PCR and their protein products have been detected in retina using immunofluorescence analysis with antibodies against these proteins (Xi et al. 2003). γS-crystallin has been found in cornea as well (Sinha et al. 1998) and αB-crystallin is known to be active as a small heat shock protein (sHsp) in many tissues (Van Montfort et al. 2001; Davidson et al. 2002; Kato et al. 2002).

Recruitment of crystallins to the lens

Generally it is thought that the development of tissues in an organism recapitulates the events during evolution ('ontogeny follows phylogeny'): organs developed early during evolution will be formed early during embryonic development. However, development of the lens does not follow this rule; even though the vertebrate lens has originated late in evolution, it is induced early during the development of the organism (Grainger et al. 1992). During the evolution of the lens, structural proteins must have been recruited to the lens from existing gene products. Genes that could be recruited as primordial lens genes are likely to have been genes that were at least potentially transcriptionally active, i.e. either housekeeping genes or stress inducible genes. This evolutionary origin is still seen in the αB-crystallin promoter, which contains heat shock elements and of which expression is increased after heat shock (Srinivasan and Bhat 1994) or by oxidative stress in a lens derived cell line (Carper et al. 2001). In addition this promoter as well as other crystallin promoters, has acquired a complex set of regulatory elements which direct expression in the lens (Gopal-Srivastava and Piatigorsky, 1993; Dirks et al. 1996; Iwaki et al. 1997; Gopal-Srivastava et al. 1998; Klok et al. 1998 and chapters 2 and 3 of this thesis). How the cognate transcription factors were recruited for regulation of lens-specific expression remains obscure.

Regulation of transcription of the recruited crystallin genes

There are many transcription factors, both ubiquitous and lens restricted that play a role in regulating crystallin expression. Most is known about regulation of crystallin gene expression in the lens by members of the Pax-, Sox- and Maf-families of transcription factors, as these factors appear to regulate the developmental and

differentiation specific expression of the crystallin genes. Only these factors will be discussed below.

Pax-6 is a highly conserved member of a family of transcription factors that contain paired and homeobox DNA-binding domains. Pax-6 is essential for cell proliferation, migration and differentiation in diverse neural and non-neural tissues and more importantly it is a key regulator of eye development and it is important for lens differentiation (Simpson and Price 2002). Loss-of-function mutations of Pax-6 are semi-dominant and lead to the *Smalleye (Sey)* phenotype in mouse and rat. The *Sey* phenotype is characterized by cataractogenesis, iris hypoplasia and microphthalmia. Homozygous mouse or rat *Sey* mutants are anophthalmic and die at birth. In these mice optic vesicles are present and contact the surface ectoderm, but lens placode thickening does not occur (Chow and Lang 2001). For the initiation of lens development the interaction of Pax-6 with Sox transcription factors is crucial; Pax-6 is expressed in the whole head ectoderm, but only the future lens cells also express Sox2/Sox3 (Kamachi et al. 2001). Pax-6 is not only important in induction of lens development, but is also known to be important in the regulation of expression of many of the crystallin genes. Binding sites for this transcription factor have been found in several crystallin promoters, for example in those of the δ1-, βB1-, αB- and αA-crystallin genes (Cvekl et al. 1994; Cvekl et al. 1995a; Cvekl et al. 1995b; Srivastava et al. 1996; Haynes et al. 1996; Robinson and Overbeek 1996; Duncan et al. 1998). Pax-6 is known to dimerize with different transcription factors. For example, a direct interaction of Pax-6 with Sox2 has been detected and this complex can then bind to the DC5 enhancer element of the δ1-crystallin promoter in chicken (Kamachi et al. 2001). Not only is Pax-6 able to regulate crystallin expression in lens directly, it also does so indirectly as it is important for the activation of the expression of the Maf transcription factor in the lens (Reza et al. 2002; Simpson and Price 2002).

Sox proteins (Sox1-3) belong to a family of transcription factors that contain DNA binding HMG domains and are related to the testis determining factor Sry. These factors are expressed in different tissues and are thought to regulate differentiation of various cell types through cooperation with partner factors unique to the cell type. To activate transcription Sox proteins are thought to require a partner that binds nearby in the same enhancer (Kamachi et al. 1998). As mentioned above, Sox2 in complex with Pax-6 is important for lens induction and regulation of the δ1-crystallin gene expression. Sox2 expression is down regulated after the lens vesicle has formed and is then replaced by Sox1 (Collignon et al. 1996). Nishiguchi et al. (1998) showed that Sox1 binds to a promoter element conserved in all γ- and δ-crystallin genes. Furthermore, γ-crystallin expression is severely down regulated in Sox1 knockout mice, showing that Sox1 is essential for γ-crystallin expression in mouse.

The last family of transcription factors that will be discussed here is that of the Maf transcription factors. This family can be divided into two groups, the small Maf proteins (MafF, MafG and MafK) and the large Maf proteins (c-Maf, MafB, NRL and L-Maf); they all contain a b-Zip domain that mediates DNA binding and dimer formation. The small Maf proteins do not contain a transcriptional activation domain, and as homodimers they can thus only function as repressors. To activate transcription, the small Maf proteins need

to dimerize with other factors. The large Maf proteins contain an acidic domain that probably enables transcriptional activation (Motohashi et al. 1997; Yoshida and Yasuda 2002). Maf proteins can homodimerize or heterodimerize either with other Maf proteins or with other factors like AP-1 family members, Bach- or CNC-proteins. These interactions make Maf proteins very diverse in their effects in transcriptional regulation (Motohashi et al. 1997). In the mammalian lens several Maf transcription factors are known to be active: c-Maf, MafB and NRL. Disruption of the c-Maf gene in mice causes a failure in lens fiber formation which is accompanied by a major decrease in crystallin gene expression (Kawauchi et al. 1999; Kim et al. 1999; Ring et al. 2000). c-Maf is thus essential in mice for normal lens development (Kawauchi et al. 1999). In chicken and *Xenopus* the lens specific L-Maf has been found (Ogino and Yasuda 1998; Ishibashi and Yasuda 2001). L-Maf is essential for lens differentiation in chicken. When L-Maf is overexpressed in chick neural retina cultures, expression of several crystallin genes is induced which indicates induction of lens formation (Ogino and Yasuda 1998; Ogino and Yasuda 2000). In addition, injections of XL-Maf or X-MafB mRNA into *Xenopus* embryos induce the ectopic expression of the γ-crystallin genes. A real lens however was only formed when the injected mRNA was localized in the head region near the eye tissue, indicating that some other factors are needed as well for lens formation to take place (Ishibashi and Yasuda 2001). Mammalian species do not have a lens specific Maf protein; the other Maf family members are thus sufficient for lens formation and crystallin expression in mammals. Many of the crystallin promoters contain (putative) Maf responsive elements (MARE; Ring et al. 2000; Civil et al. 2002; Yoshida and Yasuda 2002; Shimada et al. 2003). The importance of the MARE in regulating the expression of the rat βB2-crystallin promoter is explored in chapter 2 of this thesis.

Usage of transgenic animals versus transfection experiments in lens cell lines or explanted lens epithelial cells.

Different experimental procedures have been used to determine the effect of different transcription factors on lens development and expression of crystallins, all with advantages and disadvantages. In some studies transgenic animals have been used (for example: Haynes et al. 1996; Nishiguchi et al. 1998; Kawauchi et al. 1999; Kim et al. 1999; Ring et al. 2000; Chow and Lang 2001; Reza et al. 2002; Shimada et al. 2003), in others transfections have been done in lens cell lines (for example: Ogino and Yasuda 1998; Yoshida and Yasuda 2002) or explanted lens epithelial cells (for example: Duncan et al. 1998; Doerwald et al. 2001; Civil et al. 2002). For both transgenic animals and transfection studies, overexpression of factors may lead to differences in regulation pattern: not only the mere presence but also the amount of a factor could be critical. Use of transgenic animals is closest to the normal circumstances as the most natural environment is sustained. A disadvantage of using transgenic animals is that when lens development or differentiation is blocked or arrested, as for example in Pax-6 null mutants, the effects of the transgenesis at a later developmental/differentiation stage cannot be studied. For example the effect of Pax-6 on crystallin expression during fiber cell differentiation cannot be tested in this system. Thus studies using transgenes have contributed significantly to

the understanding of the early differentiation of the lens, the formation of the primary lens fiber cells (the cells formed from differentiation of the posterior part of the lens vesicle), but add little to our knowledge of later development and the differentiation of secondary fiber cells (the cells formed from differentiation of epithelial cells at the equator).

Use of lens cell lines or lens explants makes it easier and quicker to test the effect of several factors by overexpressing them or their dominant negative mutants in transfection studies. However, differences in regulation might be caused by differences in environment as compared to the endogenous situation. Extracellular components important for the lens cell growth or differentiation need to be added to the culture medium (for example FGF-2 must be added to explanted epithelial cells to differentiate into fiber cells; Chamberlain and McAvoy 1987), the time of addition and the amount of these components may differ from endogenous situations and thus may influence the development or differentiation of the lens cells. With both lens cell lines and lens explants there are some additional problems. Lens cell lines are epithelial cell lines and these cells no longer differentiate into fiber cells. This is likely to affect the spectrum of transcription factors present and thus to influence activity of the crystallin gene promoters and their response to transcription factors. Lens explants are closer to the *in vivo* situation, and behave according to their developmental age, but are limited in the amount of experimental material and ease of handling.

This shows that there are considerable experimental difficulties in dissecting the regulation of crystallin gene expression and that caution should be taken when drawing conclusions from transfection experiments using lens cell lines or explanted lens epithelial cells or from transgenic animals.

Gene sharing and gene duplications

As described above, during the evolution of the lens, crystallin genes were recruited from existing genes, which could be adapted to high level expression. A consequence of sharing or recruitment of a gene is that during evolution its different functions may be exposed to widely differing selective constraints so that adaptive changes favourable for one function may be deleterious for another. If duplication of such a gene occurs, then the two copies of the parental gene can evolve separately, one encoding a lens protein and the other encoding the protein containing the enzymatic activity. This process of gene duplication followed by specialization has played a major role in the evolution of the crystallin genes. In the case of the α-crystallin or taxon-specific crystallin genes the housekeeping relative can still be identified, while in the case of the β- and γ-crystallins a housekeeping relative is not easily discerned.

The α-crystallin genes belong to the family of the genes encoding for the small heat shock proteins. A duplication of one of these genes resulted in the αA/αB-crystallin gene pair, where expression of the αA-crystallin gene has become lens-specific. Another example of gene duplication is that of the δ-crystallin gene in birds. Birds have two active δ-crystallin genes; the δ2-crystallin encodes the enzyme arginino-succinate lyase (ASL); the product of the δ1-crystallin gene does not have an enzymatic activity but is highly similar in sequence to δ2-crystallin. An adaptive conflict in an early ancestor of birds and reptiles may have provided the selective pressure that, after

gene duplication, resulted in one gene with enzymatic function and one with subtle sequence modifications necessary to enhance lens function. In chicken δ2-crystallin expression decreased in lens, while in duck the δ2-crystallin is still highly expressed in lens. In chicken an adaptive conflict may have resulted in loss of the lens expression of the enzyme δ2-crystallin but it is not clear why such a conflict does not occur in the duck (Wistow 1993).

Apparently for some other crystallin genes gene duplication did not occur and one gene can be used for both lens and enzymatic functions. The activity of the enzyme-crystallin is either not deleterious or even advantageous for the lens and the promoter region of these genes supports both high lens expression and expression in other tissues. An example of this gene sharing is ε-crystallin in birds and crocodiles; this crystallin is identical to the glycolytic enzyme lactate dehydrogenase B (LDH-B; Piatigorsky and Wistow 1989; Piatigorsky and Wistow 1991; Wistow 1993).

Functions of crystallins in the lens.

Crystallins are proteins that are highly soluble and stable and they provide the lens with its refractive index, but have crystallins solely been recruited for those properties or does the lens benefit in other ways from the expression of at least some crystallins? One major problem encountered by the lens is ageing. During ageing, in the lens, as in other tissues, proteins start to denature. In the lens these proteins cannot be replaced as de novo protein synthesis cannot occur in terminally differentiated lens fiber cells. Denatured proteins are prone to aggregation and usually invoke the heat shock response. The protein chaperones encoded by the heat shock genes then act to clear the aggregates (Horwitz 2003). Aggregated proteins are a particular danger to the lens as these will make the lens opaque, but the mature lens fiber cell cannot increase its protein chaperone content when non-native protein starts aggregating. The mature lens cell does however start with a high concentration of the small heat shock proteins αA- and αB-crystallin. These sHsps are able to act as molecular chaperones, and αA- and αB-crystallin thus might be important in keeping unfolded proteins in the lens from aggregating (Horwitz 2003). Indeed soluble complexes of αA-, αB- and β- or γ-crystallins have been found (Boyle and Takemoto 1994; Takemoto and Boyle 1994; Piatigorsky 1998). In addition, high amounts of α-crystallin protect the lens cells against stress such as heat shock or oxidative stress.

Other crystallins, like ε-crystallin (or LDH-B) sequester NAD(P)H in the lens; this could protect against oxidation and filter UV radiation (Wistow 1993). Furthermore, fiber cell elongation makes use of fundamental processes like osmoregulation and reorganization of the cytoskeleton. Several crystallins have a plausible connection to these processes. For example ρ-crystallin of Rana is related to rat aldose reductase, an enzyme implicated in osmoregulation and αB-crystallin can be induced by osmotic stress (Wistow 1993). Furthermore, αB-crystallin has been shown to interact with the cytoskeleton and thus might aid the elongation of fiber cells by influencing the cytoskeleton (Wistow 1993; Davidson et al. 2002; Quinlan 2002).

In chapter 5 and 6 of this thesis a possible role for αB-crystallin (and another sHsp called Hsp27 [1]) in protection of initiation of translation after stress, in

[1] This sHsp is called Hsp27 in human and Hsp25 in mouse; for clarity's sake both will be called Hsp27 here.

particular after heat shock, is investigated. Therefore first the properties and functions of the sHsps and later the mechanism of initiation of translation and its regulation will be discussed in more detail in this introduction.

Heat shock proteins

Cytoplasmic proteotoxic stress induces the synthesis of a set of proteins that is required for a cell to deal with and survive that stress. As this system was first discovered after a heat stress, this system is commonly known as the heat shock system and its constituent proteins as the heat shock proteins (Hsps).

Heat shock proteins are a complex group of proteins, ranging in size between ~110 and ~16 kD. This group can be divided into two classes, dependent on size and ATP dependency: the ATP-dependent large Hsps (for example Hsp110, 90, and 70) and their co-chaperones such as Hsp40, and the ATP-independent small Hsps (sHsps; for example αB-crystallin, Hsp27, Hsp20 and HspB2. For reviews: Van Montfort et al. 2001; Narberhaus 2002).

All Hsps (large and small) are thought to function as chaperones, preventing unfolded proteins from aggregating into insoluble complexes in the cells or facilitating the folding of nascent proteins. For example when cells are heat shocked, proteins start to unfold. Hsps bind these unfolding proteins and keep them in solution. After the stress these unfolded proteins can either be refolded again or they can be targeted for proteolysis. The sHsps αB-crystallin and Hsp27 can keep their substrates in solution, but they are not able to refold them (Van Montfort et al. 2001; Narberhaus 2002). These sHsps might deliver their substrates to the large Hsps and these can then refold the proteins with their ATP-dependent refoldase activity (Ehrnsperger et al. 1997).

In non-stressed cells, protein chaperones are present as well. In some cases, these are also the proteins that are upregulated with stress. For example, the sHsp αB-crystallin is constitutively expressed in lens, heart, brain and muscle. In the case of Hsp70, a different protein is used in non-stressed and in stressed cells. Hsc70 is the constitutively expressed Hsp70 family member and is known to function as a molecular chaperone in non-stressed cells. Hsc70 has been implicated in many processes, such as folding of newly synthesized polypeptides, translocation of proteins into the nucleus, the endoplasmic reticulum and the mitochondrion, stabilizing proteins under stress conditions, antigen presentation, uncoating of clathrine coated endocytosis vesicles and neurotransmitter exocytosis (Shi and Thomas 1992; Brodsky 1996; Bukau and Horwich 1998; Bronk et al. 2001; Chang et al. 2002). *Drosophila melanogaster* contains five constitutively expressed members of the Hsp70 family; Hsc70-1 to Hsc70-5. Of these five, Hsc70-4 shows the highest sequence similarity with mammalian Hsc70; it is constitutively expressed throughout development in neurons and non-neuronal cells. *Drosophila* larvae lacking Hsc70-4 die at the first instar larval stage, showing that expression of Hsc70-4 is essential for normal development (Bronk et al. 2001). The sequence similarity of Hsc70-4 with mammalian Hsc70 suggests that Hsc70 might also be important in vertebrate embryogenesis.

In the work presented in this thesis the focus was on the stress-related function of the sHsps. These proteins are characterized

by a conserved sequence called the α-crystallin domain (de Jong et al. 1993). In man, there are ten different sHsps (Kappé et al. 2003), of these only Hsp27 and αB-crystallin are known to be traditional heat shock proteins in the sense that their synthesis is induced by a heat shock (Kato et al. 2002). The function of the sHsps in non-stressed and stressed cells is obscure.

Some sHsps are known to interact with cellular proteins. For example, αB-crystallin and Hsp27 interact with cytoskeletal structures like actin, tubulin and intermediate filaments (for reviews: Davidson et al. 2002; Quinlan 2002). The binding of sHsp to actin may have a role in stabilizing the actin network and some sHsps are thought to be involved in the contraction of smooth muscle and cellular migration through interactions with actin (Davidson et al. 2002). Interaction of sHsps with actin can directly regulate the actin cytoskeleton. The different sHsps can have different effects; Hsp27 seems to prevent filament growth by binding to the barbed end of the actin filaments and preventing actin monomers to bind. αB-crystallin, however, appears to increase actin assembly. These interactions of both Hsp27 and αB-crystallin with actin filaments are known to be phosphorylation dependent (Davidson et al. 2002; Quinlan 2002). In contrast to Hsp27 and αB-crystallin that can bind only to polymerised actin, Hsp20 can also bind to actin monomers (Davidson et al. 2002; Quinlan 2002). sHsps not only interact directly with actin filaments or monomers, they also interact with actin binding proteins, such as myotonic protein kinase, PKB and Src family protein kinases, that can modulate the actin cytoskeleton. Interaction of the sHsps with these actin binding proteins could regulate their protein kinase activity and thus induce changes in the actin cytoskeleton (Quinlan 2002).

sHsps can also interact with both soluble complexes of vimentin and GFAP and with their filaments. Interaction of sHsps with soluble intermediate filament subunits might directly regulate assembly of intermediate filaments either by controlling the availability of assembly competent subunits or by driving filament disassembly through sequestration of the subunits (Quinlan 2002). During heat shock, when sHsps are upregulated, the soluble pool of intermediate filament proteins is indeed increased and an excessive rearrangement of the filament network takes place. Interactions of the sHsps with the filaments are thought to prevent inappropriate interactions by intermediate filaments and thus maintain an efficient cytoskeleton (Quinlan 2002). In general, sHsps are thought to be involved in maintaining the assembly and stability of cytoskeletal structures and link the different systems together to make sure that the cytoskeleton functions as a whole in both stressed and non-stressed cells. An advantage of the association of sHsps with the cytoskeleton is that chaperones are more efficient when bound to a support (Quinlan 2002). However, not all cell types contain these sHsps in non-stress conditions, thus whether these sHsps have a general function under normal conditions is still not clear.

Thermotolerance

During heat stress cells shift their resources to combat the stress. DNA replication and general transcription as well as splicing and general translation are inhibited upon a heat shock. In addition, transcription of Hsp genes is activated and translation of the mRNA for protecting proteins (the Hsps) escapes from general

inhibition of translation. When heat shocked cells are allowed to recover and then subjected to a subsequent heat shock, they are more resistant to this second heat shock; this phenomenon is called thermotolerance. As thermotolerance can also be conferred by overexpression of Hsp70 or a sHsp, it is thought to be caused by the presence of Hsps of which the synthesis was induced by the first heat shock (Laszlo 1988; van den IJssel et al. 1994; Gabai and Sherman 2002). Overexpression of Hsps results in protection not only against heat but also against other stresses and thus Hsps are general cytoprotective agents.

Hsp70 family members appear to provide an important contribution to thermotolerance in mammalian cells (Beck and De Maio 1994; Gabai et al. 1997; Nollen et al. 1999; Gabai and Sherman 2002) but also sHsps are known to provide thermotolerance/ cytoprotection. When Hsp27 is constitutively expressed, actin filaments are stabilized and recovery of transcription and translation after heat shock is stimulated (Lavoie et al. 1993; Carper et al. 1997). Overexpression of Hsp27 has also been shown to inhibit apoptosis mediated by Fas/APO-1 (Mehlen et al. 1996b), it prevents activation of pro-caspase-9 and -3 (Garrido et al. 1999) possibly by interaction with cytochrome c (Bruey et al. 2000), and like αB-crystallin, Hsp27 inhibits TNFα induced cell death (Mehlen et al. 1996a). Another sHsp, HspB2, is associated with mitochondria and can protect cells from heat-induced cell death (Nakagawa et al. 2001). This shows that apoptosis can be inhibited at different points by overexpression of sHsps.

Little is known about the mechanism of thermotolerance, whether provided by overexpression of a large or a small Hsp. In this thesis the effect of overexpression of αB-crystallin and Hsp27 on translational thermotolerance will be described. As this effect is at the level of initiation of translation, the process of initiation of translation and the regulation of translation after stress will be described in more detail in the next section.

Translation

For translation to take place, the ribosomal complex needs to be assembled on the mRNA. In general, initiation of translation consists of several linked stages mediated by initiation factors (eIF's; see Figure 2). The charged initiator tRNA is bound by eIF2 in a complex with GTP (eIF2/ GTP/ tRNAiMet). This complex binds to the 40S ribosomal subunit together with other eIF's to form the 43S pre-initiation complex. The 43S complex binds to the mRNA; this can occur either via cap-dependent or cap-independent (for example internal ribosome entry site (IRES)-dependent) mechanisms. After binding to the mRNA the 48S complex scans to find the initiation codon, usually an AUG, after which base pairing of the initiation codon with the anticodon of the initiator tRNA positions the 48S complex. After displacement of several initiation factors and binding of the 60S ribosomal subunit, the 80S ribosome can start translation (Pestova et al. 2001; Dever 2002).

Under normal conditions initiation of translation usually takes place via the cap-dependent mechanism. In this mechanism the 5' cap structure (5' m^7GpppX) is used to recruit the small ribosomal subunit to the mRNA. In some cases however IRES-dependent initiation of translation is used (Pain 1996; Holcik et al. 2000; Pestova et al. 2001; Vagner et al. 2001). This type of initiation of translation has first been identified in viruses. Lately also some

cellular mRNAs have been found to contain IRES's (Gingras et al. 1999; Pestova et al. 2001; Rubtsova et al. 2003). During heat shock, cap-dependent translation is dramatically inhibited, but translation via other mechanisms not depending on the cap-structure, such as IRES-dependent translation can still take place (Kim and Jang 2002). In thermotolerant cells protein synthesis is still rapidly inhibited by heat stress but protein synthesis recovers faster than in naive heat shocked cells, a phenomenon known as translational thermotolerance (De Maio et al. 1993a; De Maio et al. 1993b; Beck and De Maio 1994; Hallberg and Hallberg 1996).

If during heat shock general translation does not take place anymore but translation of Hsp mRNAs is still possible, then translation of the Hsps during heat shock must occur via some cap-independent mechanism. Mechanisms postulated to be used for translation of Hsp 70 mRNA are IRES-dependent translation (Gingras et al. 1996; Brostrom and Brostrom 1998; Rubtsova et al. 2003) and ribosome shunting or jumping (Yueh and Schneider 1996; Yueh and Schneider 2000). In this latter mechanism, the 40S ribosomal subunit would bind to the cap and then jump, rather then scan, to the initiation codon. How translation of Hsp mRNAs after heat shock is regulated is still not clear. The best studied case is that of the Hsp70 mRNA. In *Drosophila melanogaster,* translation of this mRNA is cap-dependent while in mammalian cells translation initiation via either ribosome shunting or an IRES has been suggested (see below; Song et al. 1995; Rubtsova et al. 2003).

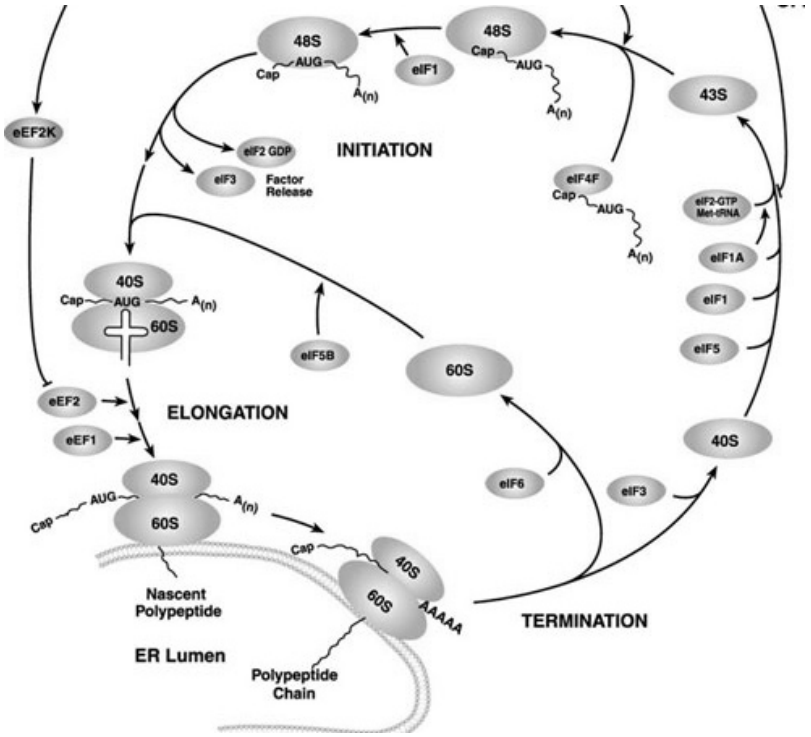

Figure 2: Schematic overview of translation.
An overview of factors used during initiation and elongation of translation is shown in this figure. Figure is adapted from www.cellsignal.com/reference/pathway/TC_Overview.asp.

The mechanism of inhibition of translation by a heat shock (as well as by other types of stress; Sheikh and Fornace, Jr. 1999), and thus also the mechanism of translational thermotolerance, is still not clear. Since there is a difference in heat sensitivity between cap- and IRES-dependent initiation of translation, both processes will be explained in more detail.

Cap-dependent translation

The cap-structure is the m^7GpppX at the 5'end of the mRNA. The 40S ribosomal subunit can bind here in association with initiation factors (see Figure 3) and then start scanning to find the initiation AUG. Cap-dependent initiation of translation under normal conditions involves the eIF4F complex. This protein complex mediates the recruitment of the small ribosomal subunit to the mRNA and consists of eIF4E, eIF4G and eIF4A. eIF4G is the scaffolding protein in this complex and can bind several other eIF's. eIF4E is the protein that recognizes and binds to the cap-structure and eIF4A is an ATP-dependent RNA helicase that unwinds secondary structures in the mRNA.

As a first step in cap-dependent initiation of translation, eIF4E binds to the 5' m^7GpppX cap-structure. It is still not clear whether this binding occurs in the eIF4F complex or eIF4E first binds to the cap and then the complex is formed (Gingras et al. 1999). eIF4G interacts with eIF3, which recruits the 40S ribosomal subunit complexed with eIF2/GTP/Met-tRNA, to the mRNA. eIF4A in the eIF4F complex has unwound the secondary structures in the mRNA so that the ribosomal subunit can interact with it. The eIF2/GTP/ $tRNA_i^{Met}$ complex positions the initiator tRNA on the 40S ribosomal subunit and is necessary for stable binding of the 40S ribosomal subunit to the mRNA. For this initiation complex to be able to start scanning towards the initiation codon, binding of eIF1 and eIF1A is needed. These two factors are necessary for the formation of a stable 48S complex on the initiation codon. When the 48S complex is located at the initiation codon, eIF5 and eIF5B couple the 60S ribosomal subunit to the initiation complex, several eIF's are released and the 80S ribosome can start translation (Pain 1996; Gingras et al. 1999; Pestova et al. 2001; Dever 2002).

Interaction of eIF4G with the poly A binding protein (PABP) is thought to facilitate reinitiation of the ribosomes on the cap-structure (Preiss and Hentze 1999). When PABP and eIF4G interact, the 5' and 3' ends of the mRNA are brought in close proximity of each other. The facilitation of reinitiation of ribosomes on the cap-structure by circularization is thought to be by simply transferring the 40S subunit directly to the 5' cap after it reaches the poly A tail. PABP is also known to interact with eIF4B (a factor that facilitates binding of the 40S ribosomal subunit to the mRNA). Circularization of the mRNA can thus be mediated by different

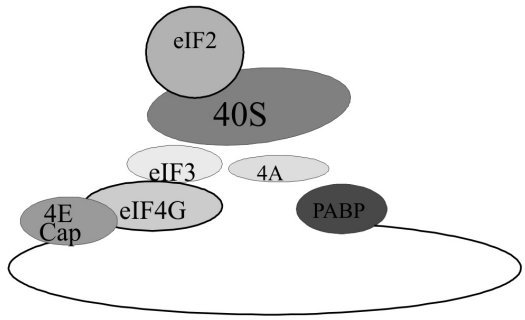

Figure 3: Simplified model of cap-dependent initiation of translation.
The initiation complex bound to the cap-structure is shown here, other factors are needed for scanning to and recognition of the initiation codon after this complex is bound to the cap (see also Figure 2). Picture is adapted from Sachs (2000).

factors, and this supports the idea that this circularization is an important process in regulation of the rate of initiation of translation (Le et al. 2000).

IRES-dependent translation

IRES-dependent initiation of translation is best known from viral RNA's but also some cellular mRNA's are thought to be translated via IRES's, for example the mRNA's of BiP, fibroblast growth factor-2 (FGF-2), c-Myc, ornithine decarboxylase (ODC) and vascular endothelial growth factor (VEGF). In addition, a cell cycle specific use of IRES mediated translation initiation has been described (Sachs 2000). IRES's are thought to form large tertiary structures that are able to bind the initiation complex (Figure 4), but no consensus in sequence or structure for IRES's has been found yet (Gingras et al. 1999). For IRES dependent translation there are several different mechanisms. As viral IRES's are the best studied, two different examples of viral IRES's will be given here.

Picorna virus RNA genomes are uncapped and contain highly structured barriers for the scanning ribosome in their 5'UTR. They contain IRES's of about 400 nt that form specific secondary structures.

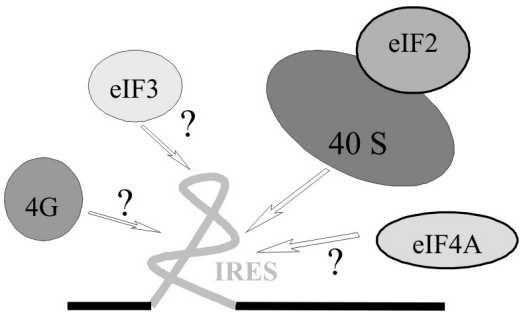

Figure 4: Simplified model of IRES-dependent initiation of translation.
What factors are needed for initiation is dependent on the type of IRES. Adapted from Sachs (2000)

In these IRES's the initiation codon is either located immediately 3' of the IRES and ribosomes do not need to scan to the initiation codon, or the initiation codon is situated about 160 nt from the IRES and the ribosome scans or shunts to this codon after attachment to the IRES. The mechanism of initiation used by Picorna virus IRES's is dependent on several initiation factors. The eIF4A and the eIF4A binding part of eIF4G are needed, together with eIF2 and 3 to form a 48S complex on the mRNA. eIF4B and the poly-pyrimidine-tract binding protein (PTB) enhance this formation. PTB is a factor for IRES-dependent translation that is encoded by a cellular gene and is thus not specific for viral IRES's. PTB stabilizes the IRES in its optimal conformation for binding of the essential factors and the 43S complex. eIF1 is necessary for selection of the proper initiation codon (Gingras et al. 1999; Pestova et al. 2001).

IRES's from for example the 5'NTR's of hepatitis C virus (HCV), classical swine fever (CSFV) and bovine viral diarrhea virus (BVDB) have very complex structures including a pseudoknot. The initiation AUG is located immediately 3' of the IRES. This IRES binds the 40S subunit stably and specifically in absence of all initiation factors. Addition of the eIF2/GTP/met-tRNA ternary complex is sufficient for bound 40S to lock onto the initiation codon in vitro. In vivo it is likely that also eIF3 is associated with the ribosomal subunit, since it has been reported to be associated with free 40S subunits in the cytoplasm. Also, eIF3 can bind specifically to the IRES. eIF4A, 4B, 4E or 4G are not necessary for initiation on these types of IRES's (Pestova et al. 2001).

Thus, viral IRES's use different mechanisms to initiate translation. These mechanisms have in common that eIF4E is not needed for initiation of translation.

As mentioned above, translation initiation on mammalian Hsp70 mRNA has been demonstrated to have a relaxed cap-dependence and a reduced dependence on eIF4F. Moreover, the 5'UTR of Hsp70 mRNA has been shown to have a high G/C content. Secondary structures in mRNA with high G/C content are more stable than those in mRNA with high A/T content. The 5' UTR of Hsp70 mRNA thus seems to be more compatible with IRES dependent translation than cap-dependent translation where secondary structures need to be removed from the mRNA by the scanning ribosome. These features of the Hsp70 mRNA all indicate the possibility of an IRES element in this 5'UTR. Rubtsova et al (2003) used a bicistronic messenger to show that Hsp70 mRNA indeed contains an IRES element in its 5'UTR. Unlike many other IRES's, the activity of the Hsp70 mRNA IRES requires the integrity of almost the entire sequence of the 5'UTR. The secondary structure formed by the Hsp70 IRES was suggested to have a relatively loose configuration. Both the 5' proximal and 3' terminal segments of the 5'UTR are essential elements and binding to the IRES is thought to occur in a three dimensional way ensuring a close proximity of the sequence surrounding the start

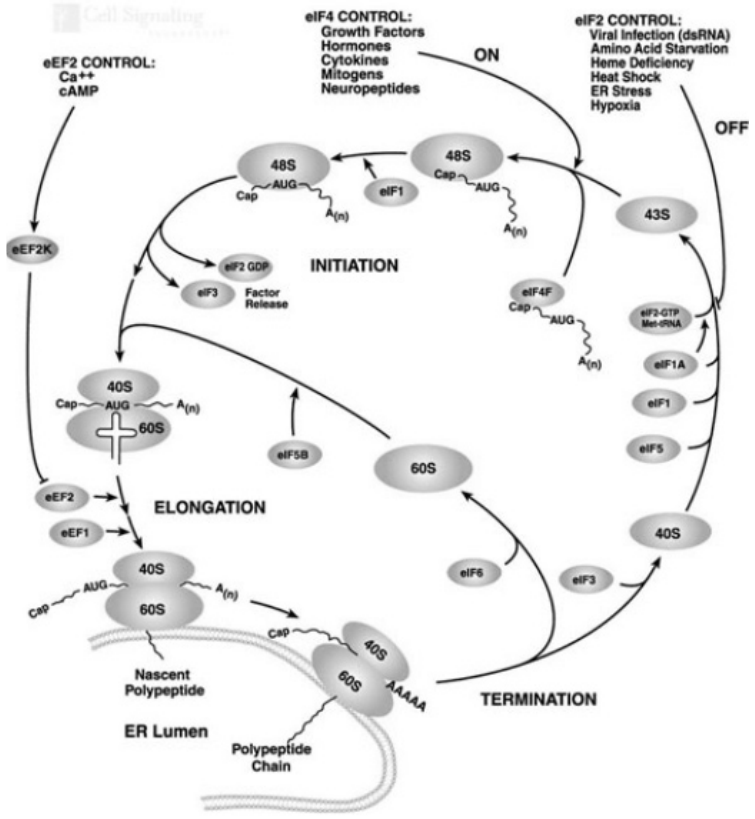

Figure 5: Schematic overview of translational control.
An overview of the translation factors which are targets of regulation is shown in this figure. More detailed schemes of regulation of activity of the eIF4 and eIF2 translation factors are given in Figures 6 and 7 resp. Adapted from www.cellsignal.com/reference/pathway/TC_Overview.asp.

codon to the scanning apparatus (Rubtsova et al. 2003). The exact mechanism and the requirement for the different initiation factors in IRES dependent translation of Hsp70 mRNA has not been elucidated yet.

Regulation of translation

The activity of many factors in the initiation complex can be regulated, thereby increasing or decreasing the translational activity of the cell. Increase in translational activity is for example induced by growth factors, hormones and cytokines, decreased translation is seen for example after viral infection, amino acid starvation and heat shock (see Figure 5).

Regulation of eIF4E activity

eIF4E activity can be regulated by two different processes: phosphorylation and interaction with eIF4E-binding proteins (4E-BP's). MNK1 (MAP-kinase-interacting protein kinase-1) phosphorylates eIF4E as a response to growth factors, hormones, mitogens, cytokines and stress. Thus phosphorylation of eIF4E is a response to a variety of stimuli, of which most (except stress) are correlated with an increase in translational activity (Figure 6; for ref: Gingras et al. 1999; Dever 2002; Scheper and Proud 2002). The physiological role of eIF4E phosphorylation is not clear. Scheper et al (2002) showed that phosphorylation of eIF4E reduces its affinity for the cap due to an increased rate of dissociation. For in vitro translation to take place, eIF4E does not need to be phosphorylated and the phosphorylation state of eIF4E does not affect its ability to rescue the lethal phenotype of eIF4E deletion in yeast (McKendrick et al. 2001). However, Lachance et al (2002) showed that *Drosophila melanogaster* homozygous for a lethal eIF4E mutation arrest growth during larval development and that transgenic *Drosophila* overexpressing non-phosphorylatable eIF4E^{Ser251Ala} in this eIF4E mutant background have reduced viability when compared to wild type *Drosophila*. Escapers develop more slowly than control siblings and are smaller. This shows that eIF4E phosphorylation is biologically significant and essential for normal growth and development (Lachance et al. 2002). These data taken together show that regulation of eIF4E activity by phosphorylation is still not completely clear.

In contrast to the regulation of activity by phosphorylation, the regulation of eIF4E activity by binding of 4E-binding proteins (4E-BPs) is well understood. The binding of 4E-BP to eIF4E results in an eIF4E complex that cannot bind eIF4G anymore and thus the cap-binding complex cannot be formed. Binding of 4E-BP to eIF4E thus results in decreased cap-dependent protein synthesis. The binding activity of 4E-BPs is dependent on their phosphorylation state, where phosphorylation reduces their affinity for eIF4E (Gingras et al. 1999). At least three different 4E-BPs are known in mammals: 4E-BP1, -2 and -3. This indicates that 4E-BPs are important factors in regulation of eIF4E activity. 4E-BP1 and -2 have been shown to be regulated by the same stimuli (including hormones, growth factors, mitogens and cytokines), the phosphorylation pattern, however, differs between the two. Little is known about regulation of 4E-BP3 (Gingras et al. 2001). Possibly these three 4E-BPs are regulated by different pathways resulting in different phosphorylation patterns. This phosphorylation pattern is important for the release of 4E-BPs from eIF4E (Gingras et al. 2001) and the different phosphorylation patterns may allow a fine-tuning of the activity of eIF4E.

Translation initiation factors are also regulated in their activity when cells are stressed. For example, as described before, heat shock results in inhibition of cap-dependent translation. Thus the activity of translation initiation factors must be regulated by stress. A most likely target for regulation, given its role in cap-dependent translation initiation, is eIF4E. Indeed, when eIF4E is deficient in HeLa cells, general translation in these cells is strongly inhibited but translation of Hsp mRNAs and cap-independent mRNAs can still take place (Joshi-Barve et al. 1992). This is the same expression pattern seen when cells are heat shocked. In CHO.K1 cells, heat shock, arsenite, sorbitol and hydrogen peroxide treatments result in an increase in the association of eIF4E-BP with eIF4E, resulting in a rapid inhibition of protein synthesis (Vries et al. 1997; Patel et al. 2002). Regulation of 4E-BP binding to eIF4E thus seems to be the most important in regulating eIF4E activity during stress.

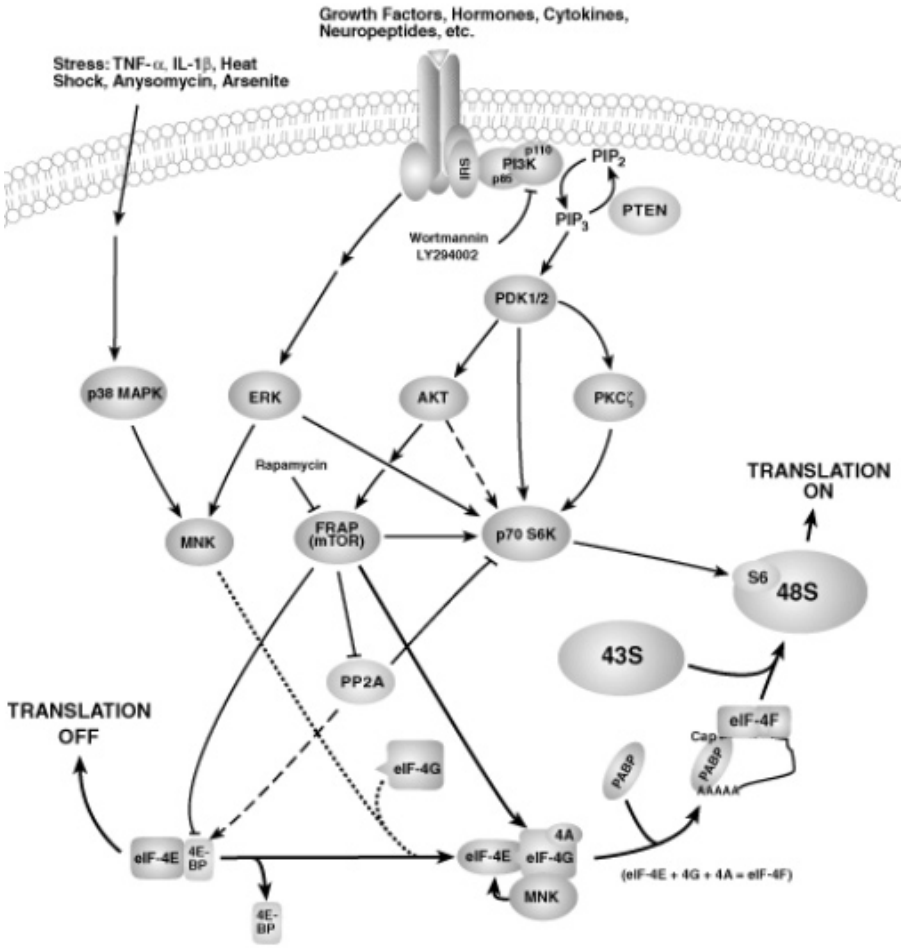

Figure 6: An overview of regulation of activity of eIF4 and p70 S6 kinase.
Environmental factors such as growth factors, hormones, cytokines and stresses influence the translational activity in the cell by activating or inactivating the translation factors as depicted in the scheme. Adapted from www.cellsignal.com/reference/pathway/TC_Reg_eIF4E.asp

Phosphorylation of eIF4E does not appear to play a role in the downregulation of eIF4E activity by stress, as eIF4E has been reported to be either phosphorylated or dephosphorylated after stress, dependent on the cell type and on the type of stress. For example, hypertonic stress or aminoacid starvation results in dephosphorylation of eIF4E (Morley and Naegele 2002), oxidative stress in vascular cells, however, results in phosphorylation of this factor (Duncan et al. 2003). When *Xenopus* cells are heat shocked, this results in phosphorylation of eIF4E (Fraser et al. 1999); but heat shock of CHO.K1 cells results in dephosphorylation of eIF4E. Thus, there is no clear correlation between phosphorylation of eIF4E, inhibition of cap-dependent protein synthesis and stress.

Since IRES dependent translation does not require eIF4E activity, regulation of eIF4E activity will have little effect on IRES dependent translation. However, inhibition of cap-dependent translation might result in a higher availability of translation factors for IRES-dependent translation and thus an increased translation of IRES containing mRNAs (Fernandez et al. 2001).

Regulation of eIF4G activity

eIF4G is the scaffolding protein in initiation of translation. Of this protein two isoforms are known: eIF4GI and eIF4GII. These two exhibit moderate sequence similarity (46% in human) but share similar overall biochemical activities. eIF4GI is expressed at higher levels than eIF4GII and is thought of as the prototype member of the family (Prevot et al. 2003). In vitro eIF4G can be phosphorylated by PKA, PKC and the protease-activated kinase II. In vivo increased phosphorylation has been observed when cells are treated with TPA, okadaic acid or EGF or when cells are infected with influenza viruses. Phosphorylation of eIF4G results in an increase in eIF4F complex formation (Gingras et al. 1999) and thus an increase in translation.

In cells infected by some viruses or cells undergoing apoptosis, eIF4GI can be cleaved, albeit at different sites and with different results. Cleavage of eIF4GI by viral proteins results in a loss of the eIF4E binding site and cap-dependent protein synthesis is shut down. The cleaved eIF4GI fragment can still associate with eIF3 and eIF4A and is able to promote internal initiation. However, viral cleavage of eIF4GI is not sufficient for complete shut-off of host cell translation, probably eIF4GII needs to be cleaved as well (Gingras et al. 1999; Dever 2002).

Cleavage of eIF4GI after apoptosis occurs at a different site than cleavage after viral infection. Caspase-3 has been shown to cleave eIF4GI in vitro and certain caspase inhibitors have been shown to inhibit apoptosis and cleavage of eIF4G. Thus proteins that are needed for apoptosis do cleave eIF4GI, however, it remains unclear whether or not cleavage of eIF4GI plays an important role in apoptosis. The eIF4GI fragments generated during apoptosis are thought to be inactive in translation initiation. This presumption is in agreement with the fact that protein synthesis is reduced in apoptotic cells (Gingras et al. 1999).

eIF4G activity can also be regulated by binding of other proteins. In vitro, purified Hsp27 specifically binds to eIF4G, prevents in vitro translation, eliminates interactions of eIF4G with other factors and promotes eIF4G insolubilization (Cuesta et al. 2000). In vivo, eIF4G can be bound by Hsp27 during heat shock, resulting in insoluble complexes of

eIF4G. This insolubilization of eIF4G results in inhibition of translation during heat shock since initiation complexes cannot be properly formed without eIF4G (Cuesta et al. 2000). During heat shock Hsp27 can thus inhibit translation by binding to eIF4G. In heat shocked cells, however, IRES-dependent translation does take place. Possibly the IRES-dependent initiation complex has a higher affinity for eIF4G, when compared to the cap-dependent complex. Thus the little eIF4G that is present in a stressed cell could be trapped by the IRES dependent initiation complex and IRES-dependent translation initiation can take place. Following a heat shock, however, Hsp27 was shown to stimulate recovery of RNA and protein synthesis (Carper et al. 1997 and chapter 5 of this thesis).

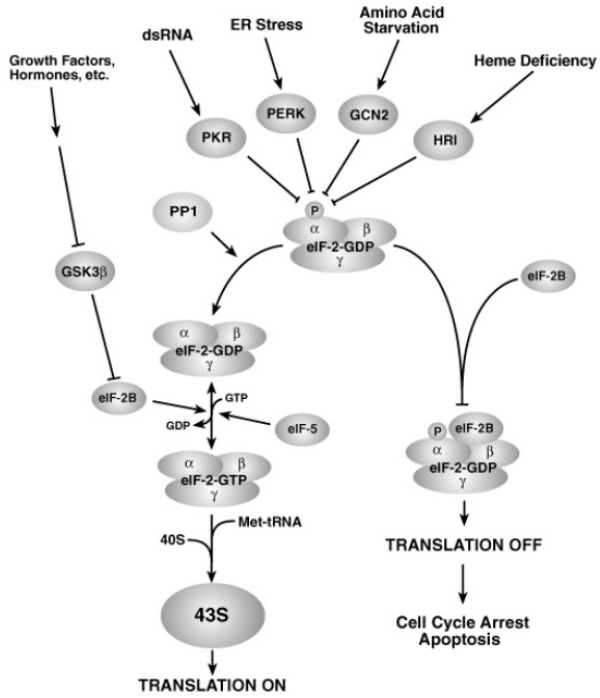

Figure 7: Schematic overview of the regulation of eIF2. eIF2 activity can be regulated by kinases that phosphorylate the eIF2α subunit. This results in higher affinity of eIF2B for eIF2 and thus inactivation of the eIF2 complex which shuts down translation initiation. Adapted from www.cellsignal.com/reference/pathway/TC_Reg_EIF2.asp.

Regulation of eIF2 activity

eIF2 is a complex consisting of three polypeptide chains, α, β and γ, which appear to be associated throughout the initiation cycle. This eIF2 complex recruits the initiator tRNA and conducts it as a Met-tRNA$_i$·eIF2·GTP to the 43S ribosomal subunit. It then participates in the recognition of the start codon on the mRNA. Upon joining of the 60S ribosomal subunit, GTP is hydrolysed and eIF2 is released as eIF2·GDP. The GDP in this complex must be replaced by GTP before eIF2 can join Met-tRNA$_i$ for another round of initiation. This exchange is catalysed by a complex that contains five polypeptide chains and is called eIF2B. The activity of eIF2B is limiting for peptide chain initiation since it is present at low stoichiometric ratios with respect to eIF2. Both eIF2 and eIF2B can be regulated in their activity through phosphorylation. Phosphorylation of eIF2α on Ser51 results in higher affinity of eIF2B for eIF2, which blocks the exchange of GDP for GTP and thus translation initiation (Figure 7; for review see: Pain 1996; Brostrom and Brostrom 1998).

During stress eIF2α kinases are activated. For example, PKR is activated during heat stress by RAX/PACT and can then phosphorylate eIF2α (Ito et al. 1999; Patel et al. 2000; Ruvolo et al. 2001). Activation of kinases such as general control nonrepressed 2 (GCN2), heme regulated inhibitor (HRI) and PKR like endoplasmatic reticulum kinase (PERK/PEK), by amino acid starvation, heme deficiency and ER stress, respectively, also results in phosphorylation of eIF2α. HRI can be activated by a heat shock as well (Sheikh and Fornace, Jr. 1999; Deng et al. 2002). In general eIF2α phosphorylation

blocks translation (Sudhakar et al. 2000). However, phosphorylation of eIF2α can differentially affect the use of IRES's. The effect depends on the IRES and on the time of eIF2α phosphorylation. Some IRES's, like the cat-1 and cricket paralysis virus IRES's are stimulated in their activity when eIF2α is phosphorylated. Activities of other IRES's like those of the BiP and Pim-1 mRNAs are not influenced by eIF2α phosphorylation (Fernandez et al. 2002). Although the Met-tRNA$_i$·eIF2·GTP complex is necessary for all translation initiation, irrespective of its mechanism, IRES-dependent translation seems to be less affected by inhibition of formation of this complex. Possibly this is caused by a higher affinity of the IRES-dependent initiation complex for the Met-tRNA$_i$·eIF2·GTP complex, when compared to the cap-dependent complex. Thus the little Met-tRNA$_i$·eIF2·GTP that is present in a stressed cell could be trapped by the IRES dependent initiation complex and thus not available to the cap-dependent initiation complex.

During stress eIF2B activity can also be down regulated by phosphorylation (Scheper et al. 1997; Williams et al. 2001). At mild heat shock temperatures this seems to be the major mechanism of inhibition of protein synthesis (Scheper et al. 1997). Both these modifications of eIF2α and eIF2B result in a down regulation of the eIF2 activity and inhibition of translation.

Stress granules

When cells are exposed to a heat shock, their morphology changes. In the cytoplasm the cytoskeleton is rearranged (attached cells start to round up and detach from their surface) and in the nucleus actin filaments condense to form rod-shaped bodies. In the cytoplasm phase dense granules can be found. These granules were first observed in 1983 in Peruvian tomato cells after heat shock and were called heat shock granules (Nover et al. 1983). As they are also found after other kinds of stress, they are now called more generally "stress granules" (SGs). Poly(A)$^+$ mRNA, translation factors and sHsps have been found to be located in these SGs (for review: Anderson and Kedersha 2002). These SGs thus could be important sites of regulation of translation after stress.

After environmental stress the two RNA-binding proteins TIA-1 and TIAR co-localise with poly (A)$^+$ mRNA in these cytoplasmic SGs. Formation of the SGs has been reported to be initiated by phosphorylation of eIF2α. Both TIA-1 and PABP-I shuttle in and out the SGs, indicating that the assembly of SGs is a highly dynamic process. Accumulation of proteins and mRNA in these SGs is irreversible when cells are lethally stressed, but SGs are reversible in cells recovering from sublethal stresses (Kedersha et al. 1999; Kedersha et al. 2000).

Arsenite treatment results in recruitment of most components of the 48S preinitiation complex (i.e. 40S ribosomal subunit, eIF3, eIF4E and eIF4G, but not of eIF2) and their associated mRNA transcripts in the SGs. Thus preinitiation complexes deficient of eIF2/GTP/tRNA$_i^{Met}$ locate to SGs after stress. When the SGs are disassembling, however, phosphorylated eIF2α can be found in these SGs as well (Kedersha et al. 2002). Proteins that stabilize (HuR) or destabilize (tristetraprolin) mRNA molecules are also present in SGs after stress. To disaggregate (the TIA proteins in) these SGs, Hsp70 and ATP are necessary (Anderson and Kedersha 2002). In plant

SGs sHsps are present as well (Nover et al. 1983; Nover and Scharf 1984). After arsenite treatment Hsp27 has been shown to be present in SGs in DU145 cells (human prostate cell line; Kedersha et al. 1999).

Mammalian SGs might be sites at which untranslated mRNAs are sorted and processed for reinitiation, degradation or packaging into stable, nonpolysomal RNP complexes. These SGs might thus serve as a translational checkpoint in stressed cells where sHsps can actively protect the translation by interactions with the translation machinery or by allowing specific mRNAs to be shuttled from the SGs to the cytoplasm.

The inhibition of protein synthesis by stress is not due to a single factor but the combined effect of many processes. Where Hsps interfere and protect in providing thermotolerance is not clear. Chapter 5 of this thesis shows that overexpression of αB-crystallin and Hsp27 results in protection of cap-dependent translation after heat shock. In chapter 6 the mechanism of this protection is investigated.

Outline of thesis

In the second chapter of this thesis transcriptional regulation of βB2-crystallin, one of the crystallins expressed in lens is being investigated. βB2-crystallin is a structural lens protein that does not belong to the sHsp family. The third chapter is about two sHsp genes: the αB-crystallin and HspB2 genes which are located only 1 kb apart in a head-to-head manner. Only the αB-crystallin gene is expressed in lens, both genes are active in muscle. The elements for lens expression must thus be restricted to the αB-crystallin promoter, but the two genes could share the muscle specific elements in the intergenic region. The fourth chapter focuses on the cellular distribution of αB-crystallin and Hsp25. Both these proteins traffic to the nucleus under normal and stress conditions but to visualize this trafficking a nuclear export inhibitor is needed. In the fifth chapter the contribution of αB-crystallin and Hsp27 to translational thermotolerance is assayed. Here we show that both αB-crystallin and Hsp27 protect cap-dependent, but not IRES-dependent translation. The sixth chapter describes the search for a mechanism of protection of cap-dependent translation mediated by αB-crystallin and Hsp27 and the effect of overexpression of αB-crystallin on the dynamics of SGs. In the appendix some of the properties of the T-REx cells stably transfected with αB-crystallin or Hsp27 are described.

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Regulatory Elements in the Rat βB2-crystallin Promoter

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Chapter 2

Regulatory Elements in the Rat βB2-crystallin Promoter

Abstract. The suggested common regulator of the eye lens crystallin genes is c-Maf. Maf responsive elements have been detected in a number of crystallin promoters including that of the rat βB2-crystallin gene. The βB2-crystallin gene is active in the post-natal lens and its mRNA reaches its maximal level in the rat lens 6 months after birth. Yet c-Maf has been reported to be present in the rat lens only up to 3 months of age. This discrepancy prompted an investigation into the role of the Maf responsive element (MARE) in the regulation of activity of the rat βB2-crystallin promoter in rat lens fiber cells. Although βB2 promoter activity is enhanced by c-Maf in both in vitro differentiating rat lens fiber cells and CHO cells, deletion of the βB2 MARE, which was mapped to -143/-123, does not decrease βB2 promoter activity in lens fiber cells. Furthermore, a dominant negative c-Maf construct did not inhibit activity of the βB2 promoter in lens fiber cells. The data suggest that the βB2 MARE does not play a major role in regulating activity of the βB2 promoter. Rather, a putative Sox binding site at -64/-159 and a positive element at -14/-7 seem to be the prime regulatory elements.

Introduction

During evolution the lens has recruited various proteins to serve as constituents of its refractive medium. These highly abundant water soluble lens proteins are collectively known as the crystallins. All vertebrate lenses examined contain the so-called ubiquitous crystallins, the α-, β- and γ-crystallins. These crystallins are encoded by gene families of which the expression is regulated both in space and in time. For example, the mammalian β-crystallins are encoded by a six membered gene family. These genes are active in the lens fiber cells, not in the lens epithelial cells, and are subject to developmental regulation: some β-crystallin genes, such as the βB1-crystallin gene, are expressed in the embryonic rodent lens, others, such as the βB2-crystallin gene, are expressed in the post-natal lens. It has previously been shown that the fiber cell specific expression of the βB2-crystallin gene is achieved through a complex interplay of upstream and intronic elements (Dirks et al., 1996b); how this gene is developmentally regulated is not known.

One element that has been suggested to play a major role in the activation of the β-crystallin promoters is the Maf Responsive Element (MARE), which is recognized by members of the Maf family of transcription factors but also by other transcription factors such as members of the AP1 family. The importance of Maf in regulating transcription in the lens was first realized with the isolation of L-Maf, a factor binding to a chicken αA-crystallin promoter element (Ogino and Yasuda, 1998). L-Maf is lens-specific and ectopic expression of L-Maf induces lens formation and expression

of crystallin genes. No homologue of L-Maf has been detected in the murine lens. These lenses do contain the related MafB and c-Maf (Sakai et al., 1997). Using transgenic mice, c-Maf was shown to be required for lens fiber formation and concomitant crystallin synthesis (Kawauchi et al., 1999; Kim et al., 1999; Ring et al., 2000). Maf response elements (MAREs) have been detected in α-, β- and γ-crystallin promoters. In the chicken βB1-crystallin promoter region a MARE sequence is found at -121/-102 (Ogino and Yasuda, 1998). Deletion or mutation of this region reduces promoter activity severely (Duncan et al., 1996). A putative MARE in the mouse βB2- crystallin promoter was identified by Ring et al. (2000) at -110/-98: a synthetic oligonucleotide containing this sequence interacted with recombinant c-Maf. The mouse and rat βB2-crystallin promoter sequences are virtually identical (Chambers et al., 1995; Dirks et al., 1996b) and the same putative MARE sequence is found in the rat βB2-crystallin promoter at -131/-119. These data suggest that c-Maf is a common activator of the β-crystallin gene promoters. However, c-Maf is expressed only in the embryonic and young post-natal rat lens, it is no longer detectable in the 3 month old lens (Yoshida et al., 1997). This expression pattern agrees with that of the βB1-crystallin gene, which is expressed in the embryonic lens, but not with that of the βB2-crystallin gene, as in the rat lens βB2-crystallin mRNA does not reach its maximum level until 6 months after birth (Aarts, Lubsen and Schoenmakers, 1989). Furthermore, no in vivo footprint over the putative -131/-119 rat βB2 MARE was seen (Dirks et al., 1996b), while in similar experiments the footprint over the rat γD-crystallin MARE was easily detected (Dirks et al., 1996a). The authors have therefore examined the role of the putative βB2-crystallin MARE: does it act as a MARE in the context of its promoter and is it required for activity of the βB2-crystallin promoter in the lens fiber cell? The authors have used in vitro differentiating explanted rat lens cells, the only available in vitro cell system in which the endogenous β-crystallin genes are active (Chamberlain and McAvoy, 1989; Peek et al., 1992; Civil et al., 2000), to show that, although c-Maf can activate the rat βB2-crystallin promoter in the context of the lens fiber cell, the Maf responsive region of the rat βB2-crystallin gene is not an essential promoter element. During these experiments an upstream regulatory element was detected that may well be a recognition site for the Sox transcription factor family. The mapping of a positive element around -20 was also refined.

Materials and Methods

Cell culture and transfection

Lens epithelial cell explants were prepared from newborn rat lenses as previously described. Explants were cultured in M199 (Life Technologies, Breda, The Netherlands) with 0.1% BSA and 25 ng ml-1 FGF-2 (a kind gift from Scios, Mountain View, CA, U.S.A.) for 5-10 days to allow fibre cell differentiation to occur. Two to four explants per plate were transfected using the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA, U.S.A.) as described (Klok et al., 1998) using 0.75 mg of DNA (0.6 mg of the reporter and expression constructs, as indicated, and 0.15 mg of CMV-β-gal). The transfection efficiency reached is about 10%. Reporter gene activities are about 10% of those obtained with CHO cells, in part due to the lower transfection efficiency of explants, in part due to the fact that fewer cells are

used. Explants were harvested 3 days after transfection, lysed and assayed as described (Dirks et al., 1996a,b; Klok et al., 1998). Luciferase activity was determined using the Luciferase Assay System from Promega according to the manufacturer's instruction. CHO-IR800 cells (CHO cells stably expressing the insulin receptor) were cultured in DMEM (Life Technologies) with 10% fetal calf serum. Cells were passaged 24 hr before transfection and plated in 35 mm plates. Cells were transfected with 1.5 mg of DNA (0.6 mg of the reporter construct, 0.6 mg of the appropriate expression construct or empty vector and 0.3 mg of CMV-β-gal) using 3 ml of Fugene 6 under the conditions recommended by the manufacturer (Roche Boehringer, Mannheim, Germany). Cells were harvested 48 hr after transfection and assayed for β-galactosidase activity and chloramphenicol acetyl transferas or luciferase activity. All transfections were done in duplicate or triplicate. All data reported are the average of at least three independent experiments in the case of explants and of at least two independent experiments in the case of CHO cells. Reporter gene activity was corrected for differences in transfection activity on the basis of the activity of the co- transfected CMV-β-gal. Reporter gene activity was then calculated relative to a standard promoter construct, of which the activity was set to 1 as indicated in the legend to the figures, and the averages (with standard deviation) of the duplicate or triplicate measurements were then calculated. The results reported are the averages, and their standard deviation, of the average results of repeat experiments.

Mutagenesis and reporter gene constructs

Some of the βB2 promoter-CAT fusion genes used in this study have been previously described (Dirks et al., 1996b). The -240/+1016 construct was made by digesting the -750/+1016 construct with SacI (located 5' of the insert) and SphI (located at -240). Sites were blunted with T4 DNA polymerase and religated. For the internal deletions, the -240/+1016 construct was linearized at the SmaI site at -123, digested with exonuclease III, blunted, reclosed and sequenced. For the -167Δ(-143/-123)/+1016 construct, a double stranded synthetic oligonucleotide with the -167 to -144 sequence was inserted between the 50 SacI site and the SmaI site at -123. Site directed mutagenesis was performed using the QuikChange kit (Stratagene, LaJolla, CA, U.S.A.) according to the manufacturer's instruction. The sense sequences of the primers used were: -22/-19 CACC->AGGA: GGAAGGTATAAATACAGGATCCCAC CGGCCTGGC; -18/-15 TCCC->CGGT: GGTATAAATACCACCCGGTACCGGCCT GGCTTCA; -14/-11 ACCG->TGGC: TAAA TACCACCTCCCTGGCGCCTGGCTTCA CGGC; -10/-7 GCC->CGG: ATACCACCT CCCACCGCGGTGGCTTCACGGCACT; -162/ -161 TT->AA: GCTAATGACATTAAA GTGTGGAGACAC; and -148/-147 AA->TT: GTGTGGAGACACTTTGCTTGTGGGC. All constructs were sequenced to confirm the presence of the desired mutations. For the βB2 promoter luciferase reporter gene constructs, the promoter regions were recloned from the CAT fusion genes to the pGL2 vector (Promega, Madison, WI, U.S.A.).

Trans-acting factor expression clones

The expression clones for rat Maf-1 (MafB) and Maf-2 (c-Maf) were obtained from Dr M. Sakai; in these clones MafB or c-Maf expression is driven by the β-actin promoter (Sakai et al., 1997). For the c-Maf binding and dimerization domain expression

clone, the β-actin/c-Maf clone was digested with StuI (which cuts the sequence seven nucleotides upstream of the beginning of the extended DNA binding domain) and BamHI (which cuts 30 of the insert). The StuI/BamHI fragment was inserted in the SmaI/BamHI digested pAS2-1 vector (Clontech, Palo Alto, CA, U.S.A.) to obtain an in-frame 5' NcoI site; the NcoI/BamHI fragment was then inserted in a NcoI/BamHI digested β-actin vector.

The chicken Sox-2 expression clone was obtained from Dr H. Kondoh and has been described in Kamachi et al. (1995).

Results

Explanted rat lens epithelial cells differentiate to fiber cells in vitro upon the addition of FGF-2. This system faithfully reproduces in vivo fiber cell differentiation with respect to morphogenesis and the activation of the crystallin genes (McAvoy and Chamberlain, 1989; Chamberlain, McAvoy and Richardson, 1991; Peek et al., 1992). The rate of differentiation of the explanted epithelial cells is dependent upon the developmental age of the cells: explants from neonatal rats differentiate faster and accumulate more crystallin mRNA and protein than explants taken from older rats (Richardson and McAvoy, 1990; Peek, Lubsen and Schoenmakers, 1991). The in vitro differentiated fiber cells mimic secondary fiber cells, i.e. the fiber cells that in vivo differentiate from the epithelial cells at the equator of the lens, rather than primary cells, i.e. the fiber cells that result from differentiation of the cells from the lens vesicle.

The advantage of this system is that promoter elements and transcription factors can be studied in an environment that closely resembles the in vivo situation but can still be easily manipulated. The disadvantage is that the transfection efficiency of these primary cultures is low leading to scatter in the data.

The βB2 MARE

To confirm that the rat βB2-crystallin promoter contains a functional MARE, the -240/+1016 βB2 promoter CAT fusion gene, previously shown to contain all elements required for proper expression in lens fiber cells (Dirks et al., 1996b), was co-transfected in explanted lens cells with either a MafB or a c-Maf expression construct. As shown in Fig. 1, MafB activated the βB2 promoter about two-fold, while c-Maf yielded a five-fold activation suggesting not only that a MARE is indeed present in the βB2 promoter but also that its preferred partner in the context of lens fiber cells is c-Maf rather than MafB.

To map the site required for activation by co-transfected c-Maf, the authors turned to cells more easily handled than in vitro differentiating explanted lens cells, namely CHO cells. In these cells, the βB2 promoter is also activated by co-transfection of c-Maf (Fig. 2). Promoter truncation mutants (with or without the first intron) were co-transfected with the c-Maf expression construct into CHO cells. For maximal activation by c-Maf the region between -240 and -123 is required [Fig. 2(A) and (B)]. Further mapping, using internal deletions, pinpointed the MARE to the region between -143 and -125. Surprisingly, deleting -123/-115 did not affect the activation of the βB2 promoter by c-Maf, even though this deletion removes the last 4 bp of the MARE identified by Ring et al. (2000). It must be noted, though, that of the sequence identified by Ring et al. (2000) only the first 7 bp (TGCTGAC

at -131/-126 in the rat βB2 promoter) match the consensus TRE-type MARE (Kataoka, Noda and Nishizawa, 1994). The downstream sequence (CCGGGC) shows little resemblance to the remainder of the TRE-type MARE (TCAGCA).

The previous analysis of the rat βB2-crystallin promoter in differentiating explanted lens cells had shown that deletion to -123 lowered the activity by about 75 %, consistent with deleting an activating MARE (Dirks et al., 1996b). To confirm that the loss of activity is indeed due to deletion of the MARE, the activity of the -167(Δ143-123)/+1016 construct in explanted lens cells was tested. Surprisingly, this construct was as active as the -240/+1016 parental construct (Fig. 3). Hence, the -131/-119 MARE is not essential for the activity of the βB2-crystallin promoter in differentiating explanted lens cells. It must be emphasized that in the cells used for transfection the endogenous βB2-crystallin gene is active and that copious amounts of the cognate mRNA and protein are being synthesized (Chamberlain, and McAvoy, 1989; Peek et al., 1992; Civil et al., 2000).

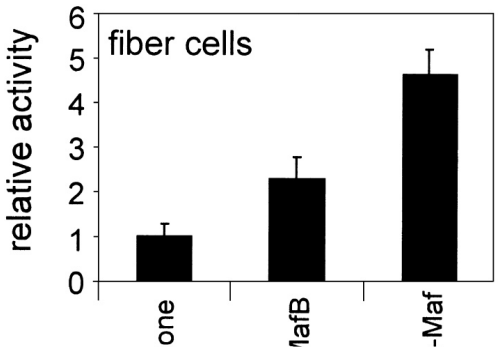

Fig 1. Activation of the βB2-crystallin promoter by MafB or c-Maf in explanted lens cells.
The -240/+1016 βB2-crystallin promoter construct was co-transfected with either empty β-actin vector (none), the MafB expression vector (MafB) or the c-Maf expression vector (c-Maf), in explants cultured for 7-10 days with FGF-2 as described in the Materials and Methods section. Activity is expressed relative to that of the -240/+1016 promoter construct co-transfected with empty vector, which was set at 1. Bars indicate the standard deviation.

The experiments reported above do not rigorously exclude the possibility that, in explanted lens cells, c-Maf targets a sequence other than the -131/-119 MARE. Hence a strategy was devised to inhibit the endogenous c-Maf: it was argued that expression of the DNA binding/dimerization domain of c-Maf should inhibit c-Maf activity by competing for the MARE. This strategy was tested in CHO cells. As shown in Fig. 4(A), expression of the c-Maf C-terminal domain (c-MafBD) inhibits the activation of the βB2 promoter by added c-Maf in CHO cells, showing that the c-Maf DNA binding/dimerization domain does act as a dominant negative mutant. The c-MafBD construct did not inhibit the βB2 promoter activity in the absence of added c-Maf, in agreement with the finding that measurable activity of this promoter in CHO cells is dependent upon adding c-Maf. In differentiating lens explants, where endogenous c-Maf is present, the c-MafBD mutant inhibited the activity of the -240/+1016 βB2 CAT fusion gene only slightly, by about 20% [Fig. 4(B)], confirming that c-Maf is not essential for the activity of the βB2 promoter under these conditions.

The -167/-143 region of the βB2 promoter contains a Sox binding site

The data presented in Fig. 3 show that the -167/-143 region must contain activating element(s). Inspection of the sequence showed that this region contained two putative targets of the Sox family of transcription factors. To test the importance of these sites, the TT at -162/-161 was mutated in AA, thus inactivating the putative Sox binding site (-164/-159) and the AA at -148/-147 was mutated in TT, thereby inactivating the element -145/-150. The activity of these mutants, and of

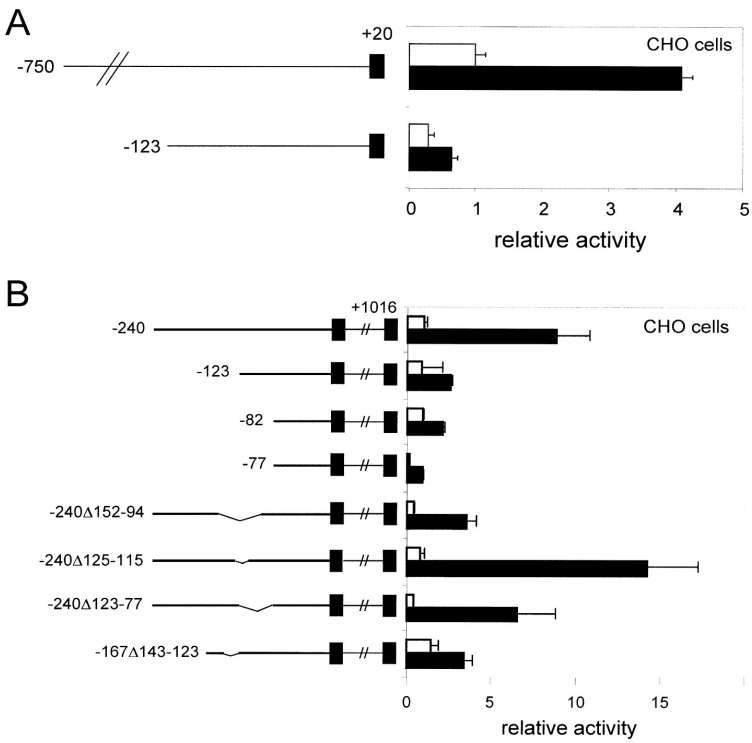

Fig 2. Mapping of the βB2-crystallin MARE in CHO cells. Deletion and truncation mutants of the βB2 promoter, as indicated in the figure, were co-transfected with (j) or without the c-Maf expression vector (h). Activity is expressed relative to that of the -750/+20 construct (A) or the -240/+1016 construct (B), which were set at 1. Bars indicate the standard deviation.

the double mutant, is shown in Fig. 5(A). The TT->AA mutation at -162/-161 (mut1) caused a reduction in promoter activity by about 60% while the activity of the -148/-147 AA->TT mutant (mut2) did not differ significantly from that of the wild type promoter. As expected from the decreased activity of the -162/-161 TT->AA mutation, the double mutant (mut1/2) was also less active than the wild type construct. These results show that the putative Sox binding site at -164/-159 is essential for full promoter activity. If the -164/-159 element is indeed a Sox binding site, one would expect that co-transfection with Sox would increase promoter activity. Thus the -240/+1016 βB2 promoter construct together with a chicken Sox-2 expression construct was co-transfected. A 50% increase in the activity of the wild type promoter and the -148/-147 AA->TT mutant promoter was seen, while no effect on the activity of the -162/-161 TT->AA or the double mutant promoter was found [Fig. 5(B)]. In these experiments, the amount of the Sox expression construct co-transfected was only 4 ng. As also observed by others (see also Kamachi et al., 1995; Kamachi, Cheah and Kondoh, 1999), higher amounts inhibited expression. The lens cells may already contain almost saturating amounts of endogenous Sox.

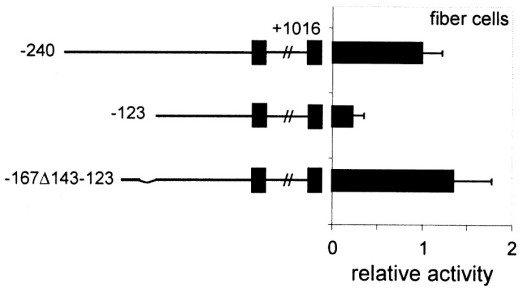

Fig 3. Deleting the -143/- 123 region does not affect βB2 promoter activity in differentiating lens fiber cells. The -240/+1016 βB2-crystallin promoter construct or deletions thereof as indicated in the figure was transfected in explants cultured for 7-10 days with FGF-2 as described in the Materials and Methods section. Activity is expressed relative to that of the -240/+1016 promoter construct vector, which was set at 1. Bars indicate the standard deviation.

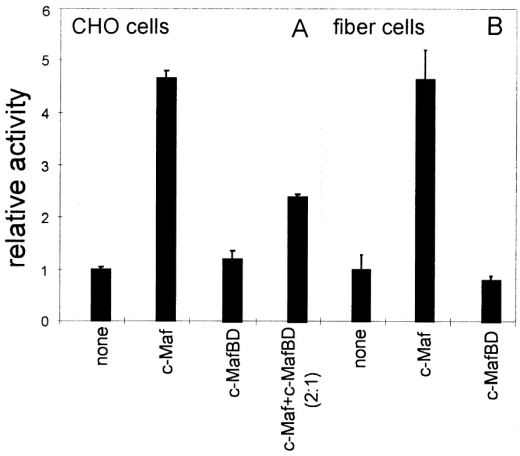

Fig 4. The effect of expression of the c-Maf C-terminal DNA binding and dimerization domain.
CHO cells (A) or explanted lens cells cultured with FGF-2 (B) were transfected with the -240/+1016 βB2-crystallin promoter construct and either empty vector (none), the c-Maf expression vector (c-Maf), or a vector expressing only the c-Maf DNA binding and dimerization domain (c-MafBD), or the c-Maf expression and the c-MafBD mutant in a 2 : 1 ratio as described in Materials and Methods. Activity is expressed relative to that of the -240/+1016 construct, which was set at 1. Bars indicate the standard deviation.

The -38/+10 βB2-crystallin promoter region

Previous studies had shown that the -38/+10 βB2 promoter construct retains residual activity and also contains a protein interaction site, indicating that this region of the promoter contains a regulatory element (Dirks et al., 1996b). Bandshift experiments mapped the protein interaction site to the region -25/-5 (data not shown). To pinpoint this element further, the element was mapped by introducing mutations in the region -20/-5. For ease of manipulation, in these experiments a promoter construct was used in which the first intron sequence was inserted upstream at -750. It has previously been shown that this construct is as active as the promoter clone containing the first intron in its proper position (Dirks et al., 1996b). All sequence changes

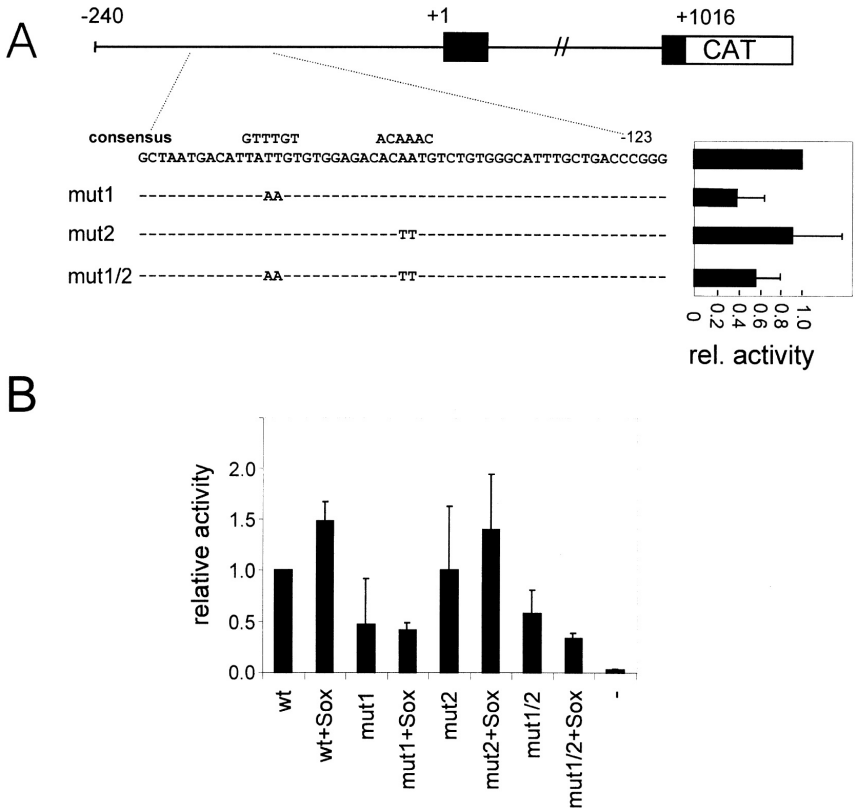

Fig 5. Sox binding sites in the rat βB2-crystallin promoter.
(A) Activity of βB2 promoter mutants. Mutations were introduced as indicated in the figure and the mutant βB2 promoter CAT fusion genes were transfected in explants cultured for 3-6 days with FGF as described in the Materials and Methods section. Activity is expressed relative to that of the -240/+1016 construct, which was set at 1. Bars indicate the standard deviation. (B) Effect of co-transfection of an expression construct of chicken Sox-2. The (mutated) promoter constructs described in (A) were transfected in explants with or without the Sox-2 expression construct (Kamachi et al., 1995), the ratio between amount of the Sox-2 expression construct and that of the promoter construct transfected was 5 x 10-3. Activity is expressed relative to that of the -240/+1016 construct, which was set at 1. Bars indicate the standard deviation. The lane denoted indicates the activity of a promoter-less construct.

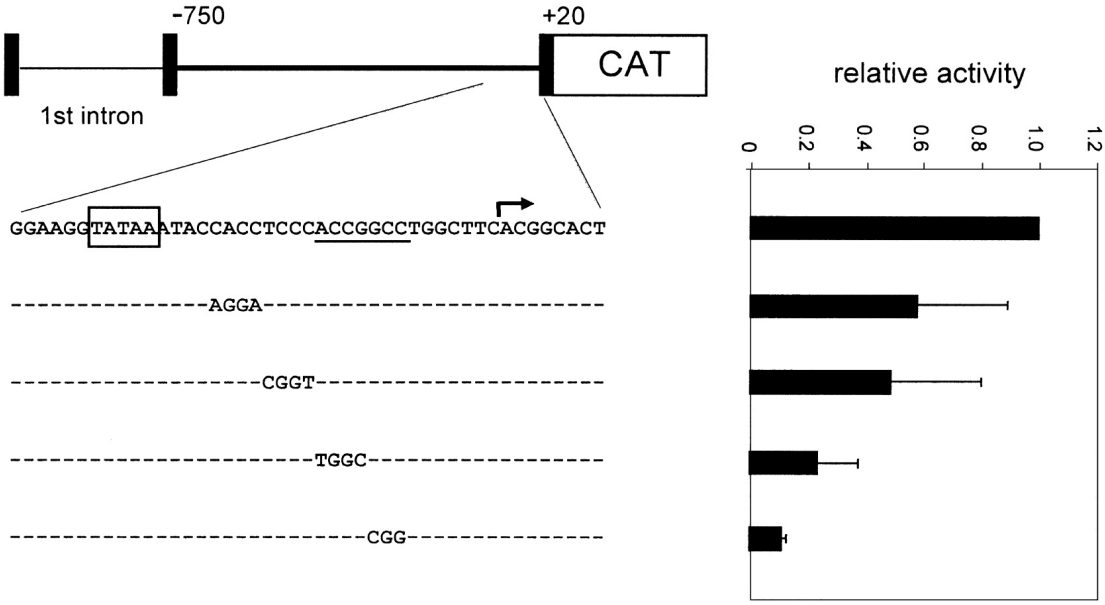

Fig 6. Mapping of the -20 activating element in the βB2-crystallin promoter.
Mutations were introduced in the (ints) - 750/+20 βB2 promoter construct (in this construct the first intron is inserted in the sense orientation at -750) as indicated in the figure. Activity of the mutated promoter regions was measured in explanted lens cells cultured for 7 days with FGF-2 as described in the Materials and Methods section. The TATA box is boxed, the transcription start site is indicated by the arrow, the region identified by the mutations as important for activity is underlined. Activity is expressed relative to that of the (ints) - 750/+20 βB2 promoter construct, which was set at 1. Bars indicate the standard deviation.

downstream from the TATA box decreased promoter activity, but the most debilitating changes were those around -10 (see Fig. 6): changing GCC to CGG caused a 90% drop in activity. We conclude that the -10 region of the βB2 promoter contains an activating element.

Discussion

At first glance the findings with respect to the importance of the MARE in the activity of the βB2-crystallin promoter seem contradictory: on the one hand it was found that added c-Maf can activate the promoter, on the other hand that neither deletion of the MARE nor transfection of the c-MafBD mutant affects promoter activity. One possible explanation for this discrepancy is that the transfection host, the in vitro differentiating lens fiber cells, does not contain endogenous c-Maf. The authors have been unable to isolate nuclear extracts from these cells containing factors able to bind to a MARE. However, this is more likely due to an experimental problem (the paucity of cells) than to a real absence of c-Maf. The most compelling argument for the presence of c-Maf in these cells is that the MARE of the γ-crystallin promoter is occupied in these cells as determined by in vivo foot printing (Dirks et al., 1996b). Furthermore, all the endogenous crystallin genes are active in the in vitro differentiating cells (Chamberlain and McAvoy, 1989; Peek et al., 1992; Civil et al., 2000). Hence, either these cells contain c-Maf, or c-Maf is not required for activity of the endogenous crystallin promoters. A more likely explanation for the results is that the βB2 MARE has a low affinity for c-Maf: the endogenous levels of c-Maf could then be insufficient to occupy the βB2 MARE, as evidenced by the lack of an in vivo footprint

over the putative -131/-119 βB2 MARE (Dirks et al., 1996b) but transfection of the expression plasmid would increase levels such that the βB2 MARE is occupied and becomes active. The results thus suggest that although c-Maf can activate the βB2-crystallin promoter it is not an essential factor for the activity of the βB2-crystallin promoter in the lens. The lack of βB2-crystallin mRNA in the c-Maf knock-out mice (Ring et al., 2000) is probably due to the fact that the lenses in these mice do not reach the stage of development at which βB2-crystallin is expressed. The extent of reliance on c-Maf as activating factor may well be one of the determinants that specifies the developmental profile of expression of the β-crystallin genes. For example, the activity of the -152/+30 chicken βB1 promoter construct (with a MARE at -121/-102; Ogino and Yasuda, 1998) in transgenic mice is restricted to the primary fiber cells (Duncan et al., 1996). A similar situation is found in the γ-crystallin gene family: a gene that is shut down early in development, the γD gene, contains a high affinity MARE, while a gene that continues to be expressed late in development, the γB gene, has a low affinity MARE flanked by a AP1 site (Klok et al., 1998). Note that the βB2-crystallin gene is unusual in that it is also expressed outside the lens and the MARE may well be involved in the activity of this promoter in other tissues, for example the brain (Head, Sedowofia and Clayton, 1995; Dirks et al., 1998; Magabo et al., 2000).

The results do point to another factor as being important for the expression of the βB2-crystallin gene, namely Sox. The importance of Sox in the activation of the crystallin promoters was first noted for the chicken δ-crystallin promoter and the mouse γF-crystallin promoter (Kamachi et al., 1995; see also Kondoh, 1999). In the mouse, both Sox-1 and Sox-2 are found in the lens, with Sox-1 replacing Sox-2 during fetal development (Kamachi et al., 1998). Sox-1 and Sox-2 are closely related and likely to be functionally redundant (Nishiguchi et al., 1998; Wegner, 1999). Mice lacking Sox-1 also lack γ-crystallins (Nishiguchi et al., 1998). They do contain a normal level of βA3/A1-crystallin mRNA, which led to the suggestion that Sox-1 is not required for β-crystallin synthesis. However, the data show that a Sox recognition site is required for maximal activity of the βB2 promoter. As the Sox-1 knock out mice are microphthalmic and do not complete differentiation of the primary fibre cells (Nishiguchi et al., 1998), they are unlikely to contain secondary fibre cells, the site of expression of the βB2-crystallin gene.

The ubiquitous crystallin genes are thought to have been recruited from stress responsive genes. The general occurrence of Maf and Sox target sites in the promoters of these genes suggests that Maf and Sox were once, and may well still be, regulators of the stress response. No such connection for Sox has yet been reported (for review, see Wegner, 1999; Pevny and Lovell-Badge, 1997). For Maf there is a role in the stress response, as subject - expression of at least some Maf mRNA increases during stress (Suzuki et al., 2001) - and as regulator - MafK represses the anti-oxidant response element (ARE) (Nguyen, Huang and Pickett, 2000). MafK is a small Maf protein that lacks an activation domain, if the ARE can also be bound by a large Maf, i.e. a Maf protein with an activation domain, then Maf could also activate the oxidative stress response. The Maf family of transcription factors could thus not only play a role in development (for review see Blank and Andrews, 1997; Motohashi et al., 1997), but also in the regulation of the stress response.

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Sequence and functional conservation of the intergenic region between the head-to-head genes encoding the small heat shock proteins αB-crystallin and HspB2 in the mammalian lineage.

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Chapter 3

Sequence and functional conservation of the intergenic region between the head-to-head genes encoding the small heat shock proteins αB-crystallin and HspB2 in the mammalian lineage.

Abstract. An unexpected feature of the large mammalian genome is the frequent occurrence of closely linked head-to-head gene pairs. Close apposition of such gene pairs has been suggested to be due to sharing of regulatory elements. We show here that the head-to-head gene pair encoding two small heat shock proteins, αB-crystallin and HspB2, is closely linked in all major mammalian clades, suggesting that this close linkage is of selective advantage. Yet αB-crystallin is abundantly expressed in lens and muscle and in response to a heat shock, while HspB2 is abundant only in muscle and not upregulated by a heat shock. The intergenic distance between the genes for these two proteins in mammals ranges from 645 bp (platypus) to 1069 bp (opossum), with an average of about 900 bp; in chicken the distance was the same as in duck (1.6 kb). Phylogenetic footprinting and sequence alignment identified a number of conserved sequence elements close to the HspB2 promoter and two further upstream. All known regulatory elements of the mouse αB-crystallin promoter are conserved, except in platypus and birds. The lens-specific-region 1 (LSR1) and the heat shock elements (HSE's) lack in birds; in platypus the LSR1 is reduced to a Pax-6 site, while the Pax-6 site in LSR2 and a HSE are absent. Most likely the primordial mammalian αB-crystallin promoter had two LSR's and two HSE's.

In transfection experiments the platypus αB-crystallin promoter retained heat shock responsiveness and lens expression. It also directed lens expression in *Xenopus laevis* transgenes as did the HspB2 promoter of rat or blind mole rat. Deletion of the middle of the intergenic region including the upstream enhancer affected the activity of both the rat αB-crystallin and HspB2 promoters, suggesting sharing of the enhancer region by the two promoters.

Introduction

Bidirectional gene pairs, located so close that promoter regions overlap, are surprisingly common in eukaryotes. For example, Adachi and Lieber (2002) recently found that almost 30% of the housekeeping genes in man were located in a bidirectional fashion less than 1 kb apart. As eukaryotic genomes are large relative to the number of genes, one would expect genes to drift apart unless there is a selective advantage to maintaining the gene pair. A recent analysis of divergent genes in the human chromosomes 20, 21 and 22 showed a biphasic distribution of the intergenic distance between such genes, with most genes being separated by an average of 25 kb and a minority by an average of 0.3 kb (Takai and Jones 2003), strongly suggesting a selective pressure in maintaining a close apposition between at least some head-to-head gene pairs. It is commonly thought that such selective pressure is imposed

by the sharing of regulatory elements by the gene pair (see for example Labrador and Corces 2002; Takai and Jones 2003). Indeed, overlapping and shared promoter elements have been identified in a number of cases (see for examples Shinya and Shimada 1994; Yoshida et al. 2002; Hansen et al. 2003; Meyer et al. 2003; Otte et al. 2003; Shin et al. 2003; Zhang et al. 2003). However, close apposition of a gene pair as seen in one genome could also be due to chance. Hence, before a functional significance can be attached to a gene pair, conservation of the close linkage needs to be shown first. Tracing these evolutionary conserved gene pairs is of considerable interest as it will help delineate the regulatory modules used in the eukaryotic genomes. In addition, comparison of the intergenic region between a conserved gene pair will provide insight into the evolution of eukaryotic promoter regions.

Here we have focused on the intergenic region between the αB-crystallin and HspB2 genes. The proteins encoded by these genes both have the α-crystallin domain characteristic of small heat shock proteins (sHsps) and form together with eight others the sHsp family in man (Kappé et al. 2003). The αB-crystallin and HspB2 genes most likely arose from an inverted duplication of an ancestral sHsp gene. This event must have occurred early in the vertebrate lineage as orthologs of these genes are found in the zebra fish genome. The αB-crystallin and HspB2 genes are located only about 0.9 kb apart in a head-to-head manner in the human, mouse and rat genomes and could thus share (at least part of) their promoter regions (Iwaki et al. 1997). The expression patterns of these two genes are, however, quite different. Products from both genes are found in heart and muscle, but only αB-crystallin and not HspB2 is expressed in lens (Iwaki et al. 1997). Also, αB-crystallin, unlike HspB2, is stress inducible (Suzuki et al. 1998). Putative elements involved in the regulation of expression of the HspB2 gene have been identified only at the sequence level (Fig. 1). In contrast, elements important for the regulation of expression of the mouse αB-crystallin gene have been well documented experimentally (Fig. 1). The two promoter proximal lens-specific regions (LSR's) both contain Pax-6 and RAR/RXR binding sites (Gopal-Srivastava et al. 1996, 1998).The upstream enhancer encompasses four αBE elements and a muscle response factor (MRF) binding site. The αBE elements are important for expression in both lens and muscle, while the MRF is only involved in muscle expression (Dubin et al. 1991; Gopal-Srivastava and Piatigorsky 1993, 1994; Srinivasan and Bhat 1994; Gopal-Srivastava et al. 1995, 1996, 1998). A second, minor transcription initiation site is located just upstream from the enhancer, at -474 in the mouse (Dubin et al. 1991; Gopal-Srivastava et al. 1995). Two heat shock elements (HSE's) have been found in the αB-crystallin promoter (Srinivasan and Bhat 1994): one within the LSR2 and one in the upstream enhancer region (Fig.1).

As only the αB-crystallin gene is expressed in the lens or upregulated after heat shock, the elements for lens expression or heat shock responsiveness must be restricted to the αB-crystallin promoter and be isolated from the HspB2 promoter. Both αB-crystallin and HspB2 are expressed in muscle and could share the muscle specific elements in the intergenic region. However, Swamynathan and Piatigorsky (2002) showed that deletion or inversion of the intergenic enhancer region inhibited the αB-crystallin promoter activity between 20 to 50 fold more than the HspB2 promoter activity

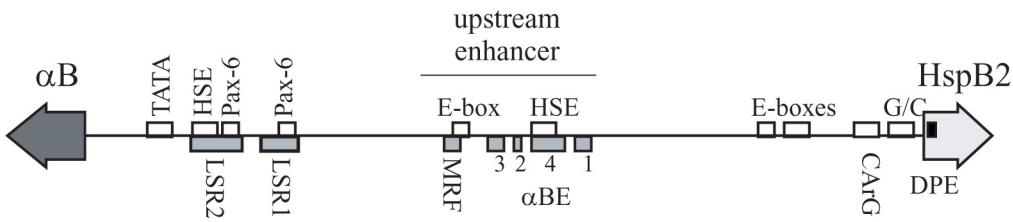

Fig. 1. Schematic representation of the intergenic region between the αB-crystallin and the HspB2 genes.
The known regulatory sites of the mouse αB-crystallin promoter are indicated in the figure as follows: TATA: TATA-box; HSE's: heat shock elements (Srinivasan and Bhat 1994; consensus sequence: inverted repeat of nGAAm); LSR: Lens specific regions (contain Pax-6 and RAR/RXR binding sites, Gopal-Srivastava and Piatigorsky 1994; Gopal-Srivastava et al. 1996, 1998); MRF: binding site for the muscle response factor, binding to this element increases transcription in muscle (Gopal-Srivastava, and Piatigorsky 1993); αBE: αB-crystallin elements 1-4, enhance promoter activity in lens, muscle and heart (Gopal-Srivastava and Piatigorsky 1993; Gopal-Srivastava et al. 1995). The putative regulatory elements of the HspB2 promoter have been identified only on the basis of sequence analysis (Iwaki et al. 1997; Swamynathan and Piatigorsky 2002): E-box: binding site for MyoD family members; CArG-box: binding site for SRF; G/C: G/C-rich promoter region; DPE: Downstream promoter element.

and therefore concluded that the muscle enhancer acts unidirectionally towards the αB-crystallin promoter only. If the αB-crystallin and the HspB2 promoters do not share regulatory elements, there would be no obvious need to maintain the close distance between or the head-to-head orientation of these genes. We therefore checked the GenBank database whether the close head-to-head orientation was also present in species other than man and rodents. As this search showed that these genes had drifted further apart in duck, we sampled the major mammalian clades to determine whether the intergenic distance between this gene pair is also variable in mammals. We show here that the intergenic distance between the αB-crystallin/HspB2 gene pair shows little variation in mammals but is significantly larger in chicken and duck. As sequence alignment of the intergenic regions showed the absence of one of the HSE's and divergence of the LSR elements in platypus, we have measured the heat shock response and determined the lenticular activity of the platypus αB-crystallin promoter. Finally, we have tested whether shortening the intergenic region, and thus decreasing the distance between a HSE and the HspB2 promoter, could confer heat shock responsiveness to the rat HspB2 promoter.

Materials and Methods

Intergenic region sequences.

Sequences of the intergenic regions of human (*Homo sapiens*, AP000907), rat (*Rattus norvegicus*, U04320), mouse (*Mus musculus*, NT_039473.1) and duck (*Anas platyrhynchos*, U16124) were retrieved from the GenBank database. For other species we performed PCRs on genomic DNA to obtain sequences containing the intergenic region between αB-crystallin and HspB2. One set of degenerated primers αB-1rev (TCTGA GAGYCCMGTSTCNADCCA) and Hsp2B-1rev (GGGTTGGCAAAYTCRTAYTC) was used for blind mole rat (*Nannospalax ehrenbergi*, AJ617819), rabbit (*Oryctolagus cuniculus*, AJ617821), pika (*Ochotona princeps*, AJ617822), cat (*Felis catus*, AJ617823), leaf-nosed bat (*Macrotus californicus*, AJ617817), shrew (Crocidura russula, AJ617818), anteater (*Cyclopes didactylus*, AJ617820), manatee (*Trichechus manatus*, AJ617824), opossum (*Didelphis marsupialis*, AJ617826), platypus (*Ornithorhynchus anatinus*, AJ617827) and chicken (*Gallus gallus*, AJ617828). Another set of degenerated primers was used for mole (*Talpa europaea*, AJ617825): αB-2rev (ATTCARCAGGTGYTCYCCRAAGA) and HspB2-2rev (GTGGCTGGGTGGGCATGY GYYA). PCRs were performed using the

Expand HF kit (Roche). The DNA was first denatured at 94ºC for 3 min, and then cycles of denaturation (1 min at 94ºC), annealing of the primers (90 s at 55ºC) and elongation (2 min at 68ºC) were performed. PCR samples were taken after 40 cycles. PCR-fragments were cloned into the pGEM-T vector and sequenced.

Phylogenetic analysis and footprinting.

A global pair-wise alignment of the intergenic regions was made using the Bayes Aligner (a Bayesian block aligner; available at http://www.Bayesweb.wadsworth.org). The histogram indicates the probability that any given base j in one sequence aligns to any base k in the other sequence. Probabilities are determined from a set of alignments representative of all possible alignments of the two sequences (Zhu et al. 1998; Wasserman et al. 2000). The Match program, available at http://www.gene-regulation.com, was used to find additional possible transcription factor binding sites in the conserved regions. Direct sequence alignments were produced using ClustalW and adjusted manually using Genedoc. Phylogenetic analyses were performed on an alignment in which all gaps present in more than half of the sequences have been deleted. This resulted in an alignment of the most conserved parts of the intergenic sequences, with a final length of 1048 base pairs. All remaining gaps were treated as missing data. PAUP* 4.0b10 (Swofford 2002) was used to reconstruct phylogenetic trees under the maximum likelihood (ML) criterion. The Hierarchical Likelihood Ratio Test as implemented in MODELTEST (Posada and Crandall 1998) was used to determine the model of sequence evolution fitting the data the best under ML. The best fit was a HKY85 + Γ4 model with a transition/transversion ratio of 1.44 and a gamma shape parameter of 1.35. This model was used to search for the best ML tree and in ML nonparametric bootstrap analyses. The bootstrap analyses included 100 replicates. The tree bisection reconnection branch swapping option was used to swap branches.

Constructs.

The starting vector for the bidirectional reporter constructs was made by inserting the coding sequence of β-galactosidase from pCH110 (Pharmacia Biotech) HindIII blunt and BamHI blunt into the SmaI site of the pGL3 basic vector (Promega).

Rat luc-αB-HspB2-β-gal: The rat intergenic region was amplified from rat genomic DNA by PCR using the Rat αB BglII primer (GAAGATCTGAGTGTAGAGTCGGTTAGC) at position +35 relative to the αB-crystallin transcription start site and the Rat HspB2 XhoI primer (CCGCTCGAGTGTAGCCCCAACAAGATC) at position +62 relative to the HspB2 transcription start site. When cloning this fragment XhoI/BglII into the bidirectional reporter vector this results in the luciferase reporter gene being driven by the αB-crystallin promoter and the β-galactosidase reporter gene being driven by the HspB2 promoter. For the *Rat β-gal-αB-HspB2-luc* (the reversed construct) the primers were: Rat αB XhoI (CCGCTCGAGTGTAGAGTCGGTTAG) and Rat HspB2 BglII (GAAGATCTAGTGTAGCCCCAACAAGA) and the PCR product was also inserted XhoI/BglII in the bidirectional reporter construct.

The rat deletion constructs were made by PCR using the Rat αB BglII primer and primers located at position -393 relative to +1 of the αB-crystallin gene (Δ630: AACTGCAGCCCAGGAAGATTCCAGC) for the *Rat Δ630 luc-αB-HspB2-β-gal* clone and at position -177 relative to +1 of the αB-crystallin

gene (Δ850: AACTGCAGCCCTGCCCCGT GTTTC) for the *Rat Δ850 luc-αB-HspB2-β-gal* clone. These PCR fragments were recloned into the rat luc-αB-HspB2-β-gal construct using the PstI at position -682 relative to +1 of the αB-crystallin gene and BglII at αB-crystallin side of the construct. The reversed constructs were made by PCR on these deletion constructs using the primers for the rat β-gal-αB-HspB2-luc construct and inserting XhoI/BglII into the bidirectional reporter construct.

The sequences of the platypus and blind mole rat intergenic regions obtained as described above were used to design primers to clone the respective intergenic regions into the bidirectional reporter vector. For *Platypus luc-αB-HspB2-β-gal* the primers used were: Platypus αB BglII (GAAGATCTGCTCTGGCTGGCTGGGCG) and Platypus B2 XhoI (CCGCTCGAGGGA CACTGGCCGGACGC), and for *Platypus β-gal-αB-HspB2-luc*, the primers used were: Platypus αB XhoI (CCGCTCGAGGCTCTG GCTGGCTGGGCG) and Platypus B2 BglII (GAAGATCTGCGCTGCGGACACTGGCC) For the *Blind mole rat luc-αB-HspB2-β-gal* construct: Blind mole rat αB BglII (GAAG ATCTAATGTAGGGGGTCAGCTGG) and Blind mole rat B2 XhoI (CCGCTCGAGGCA GCCCCAACAAGCTCAGTA) primers were used and for the *Blind mole rat β-gal-αB-HspB2-luc* construct: Blind mole rat αB XhoI (CCGCTCGAGAATGTAGGGGGTCAGCT G) and Blind mole rat B2 BglII (GAAGATC TGCAGCCCCAACAAGCTCAGTA) primers were used. PCR fragments were inserted XhoI/BglII into the bidirectional reporter vector.

pCSGFP2-intergenic region constructs: For *Xenopus laevis* transgenesis the intergenic regions of rat, blind mole rat and platypus were cloned blunt into pBluescript (Stratagene) and then either orientation was cloned into the pCSGFP2 construct (kindly provided by Dr. E. Amaya, Wellcome, Cambridge, UK) using SalI/BamHI for the rat and blind mole rat and SalI/EcoRI for the platypus constructs. This resulted in constructs in which either the αB-crystallin promoter or the HspB2 promoter of the intergenic region drives EGFP expression.

Cell culture.

C2 cells (mouse myoblast cells) were cultured in Dulbecco's modified Eagle's medium (Gibco) with penicillin and streptomycin (Roche) and supplemented with 20% fetal calf serum (PAA laboratories) to prevent differentiation of these cells. To obtain lens fiber cells, four lens epithelial cell explants from newborn rats were cultured in M199 medium (Sigma) supplemented with 0.1% BSA (Roche), penicillin and streptomycin, glutamax-1 (Gibco) and 50 ng/ml FGF-2 for 2 days prior to transfection (Chamberlain and McAvoy 1987; Klok et al. 1998).

Transfection.

C2 cells and lens explants were transfected using lipofectAMINE plus (Invitrogen). Approximately $6.5x10^4$ C2 cells were plated in Dulbecco's modified Eagle's medium with penicillin and streptomycin and 10% fetal calf serum in 6 well plates and cultured for 24 h. Lens fiber cells (4 explants per 35 mm dish) were transfected after 48 h FGF-2 treatment. Both C2 cells and lens explants were transfected with a total of 1 µg DNA per well using 6 µl plus reagent and 4 µl of lipofectAMINE in Dulbecco's modified Eagle's medium. The total of 1 µg DNA per well was divided into 0.9 µg of the various bidirectional reporter constructs and 0.1 µg of pEGFP (Clontech) as a transfection control. After 4 hours, the medium was replaced by Dulbecco's modified Eagle's

medium with 10% FCS for C2 cells or M199 with 0.1% BSA and 50 ng/ml FGF-2 for lens explants. C2 cells were harvested 48 h after transfection or heat shocked 48 h after transfection and harvested after 6 h of recovery at 37ºC and assayed for reporter gene activity. Lens explants were harvested 72 h after transfection and assayed for reporter gene activity.

Heat shock.

C2 cells were heat shocked 48 hours after transfection by submerging the 6 well plates into a 45ºC water bath for 30 min, harvested after 6 h of recovery at 37ºC and assayed for reporter activities (β-galactosidase and luciferase) as described in the next section.

Reporter assays.

Cells were harvested by vigorously shacking in 200 µl reporter lysis mix (25 mM Bicine pH 7.5, 0.05% Tween-20 and 0.05% Tween-80) per well. Lens explants were harvested and lysed by vigorously shaking in an eppendorf tube with 100 µl of reporter lysis mix. 20 µl of these lysed cells or explants was used for the reporter assays.

For the β-galactosidase assay, galacton (Tropix) was diluted 1:100 in 100 mM phosphate buffer pH 8.1, 5 mM MgCl2; of this dilution 200 µl was added to 20 µl of the cell lysate. After 30 minutes incubation at room temperature, 300 µl of light emission accelerator (Tropix) was added. For the luciferase assay 100 µl of luciferase reagent (Promega) was added to 20 µl of the lysate immediately before measurement. Measurements were performed on a Lumat LB 9507 luminometer for 10 seconds. All experiments were performed at least in duplicate, all data shown are the averages of at least two independent experiments.

Xenopus transgenesis.

Xenopus laevis unfertilized eggs and sperm nuclei were obtained as described previously (Jansen et al. 2002). The intergenic region-EGFP DNA fragments obtained by digesting the pCSGFP2-intergenic region constructs with SalI and NotI, were purified using a Qiaex II gel extraction kit (Qiagen). The fragment (250 ng/5 µl) was mixed with $2.5x10^5$/2.5 µl sperm nuclei, diluted to 500 µl and ~10 nl was injected per egg as described previously (Jansen et al. 2002). After 3 hours at 18ºC, embryos at the 4 cell stage were separated and put in 0.1x MMR, 6% Ficoll and 50 µg/ml gentamycin; after 24 hr at 18ºC gastrulas were again transferred to a new dish containing 0.1x MMR and gentamycin and kept at 22ºC. Pictures of several differentiation stages were taken using Leica MZFLIII fluorescence microscope.

Results

Phylogenetic footprinting and alignment of mammalian and avian sequences of the intergenic region between the αB-crystallin and HspB2 genes shows conservation and divergence of regulatory elements.

An initial GenBank database search for genomic sequences of the mammalian αB-crystallin and HspB2 genes showed that sufficient sequence information to map these two genes head-to-head with an intergenic distance of about 0.9 kb was only available from man, mouse, and rat. A head-to-head orientation was also found in the sequence from duck. Here, the 5' region of the HspB2 gene was present about 1.6 kb upstream of the αB-crystallin gene (N.B. at the time the duck αB-crystallin sequence

was published [Wistow and Graham 1995], the link with the HspB2 gene could not be noted as the HspB2 gene was only identified later [Iwaki et al. 1997]). The larger distance between the αB-crystallin and the HspB2 genes in duck raised the question whether the close linkage found in man and rodents was the exception or the rule in mammals. Sampling of a broad variety of mammalian taxa, including a marsupial (opossum) and a monotreme (platypus), showed that the average length of the intergenic region in mammalian species is about 0.9 kb, with platypus having the shortest one (645 bp) and opossum the longest (1069 bp; Table 1). The length of the intergenic region in chicken was found to be almost the same as in duck (1687 bp versus 1640 bp; Table 1). Thus close linkage of the αB-crystallin/HspB2 gene pair is a conserved feature of the mammalian genome, while a larger distance is found in two avian genomes. A phylogenetic tree based on the conserved sequences in the intergenic regions shows the expected topology of mammalian relationships (Springer et al. 2004), although the branch lengths indicate an accelerated rate of change in the branches to platypus and opossum (Fig. 2A).

To identify conserved sequence elements, the intergenic regions and the 5' non-coding regions of the HspB2 gene were aligned using the Bayes Aligner (Zhu et al. 1998; Wasserman et al. 2000; for examples, see Fig. 2B). A remarkable conservation of sequence, particularly of the 5' flanking sequence of the αB-crystallin gene, was found (see for example the comparison between the human and opossum sequence, Fig. 2B-2). Only when more distant species were compared, are phylogenetic footprints corresponding to most of the known regulatory sites of the mouse αB-crystallin promoter (see Fig. 1) clearly visible (Fig. 2B). Sequence similarity decays faster on the HspB2 side of the intergenic region. The phylogenetic footprints mark putative E- and CArG boxes, a G/C rich promoter region and a conserved region in the 5' non-coding region of the HspB2 gene (see also Fig. 1). Two additional possible regulatory sites emerged from this analysis, one around 600 and one around 700 (see asterisks Fig. 2B-2, numbering for the human sequence). The first contained the consensus binding site for c-Rel, a member of the REL/NF-κB/IκB superfamily of transcription factors which are involved in (anti-)apoptosis and cellular transformation (Foo and Nolan 1999). The second region matched the consensus sequence for the octamer sequence recognized by Oct transcription factors, members of the POU protein family (for review, see Phillips and Luisi 2000).

Table 1: Length of the intergenic regions between the αB-crystallin and HspB2 genes.

Species	Length of intergenic region (bp)*
Mouse	866
Rat	908
Blind mole rat	906
Rabbit	928
Pika	928
Human	964
Cat	938
Bat	884
Mole	973
Shrew	912
Anteater	958
Manatee	923
Opossum	1069
Platypus	645
Chicken	1687
Duck	1640

* Distances between the transcription start sites of the αB-crystallin and HspB2 genes.

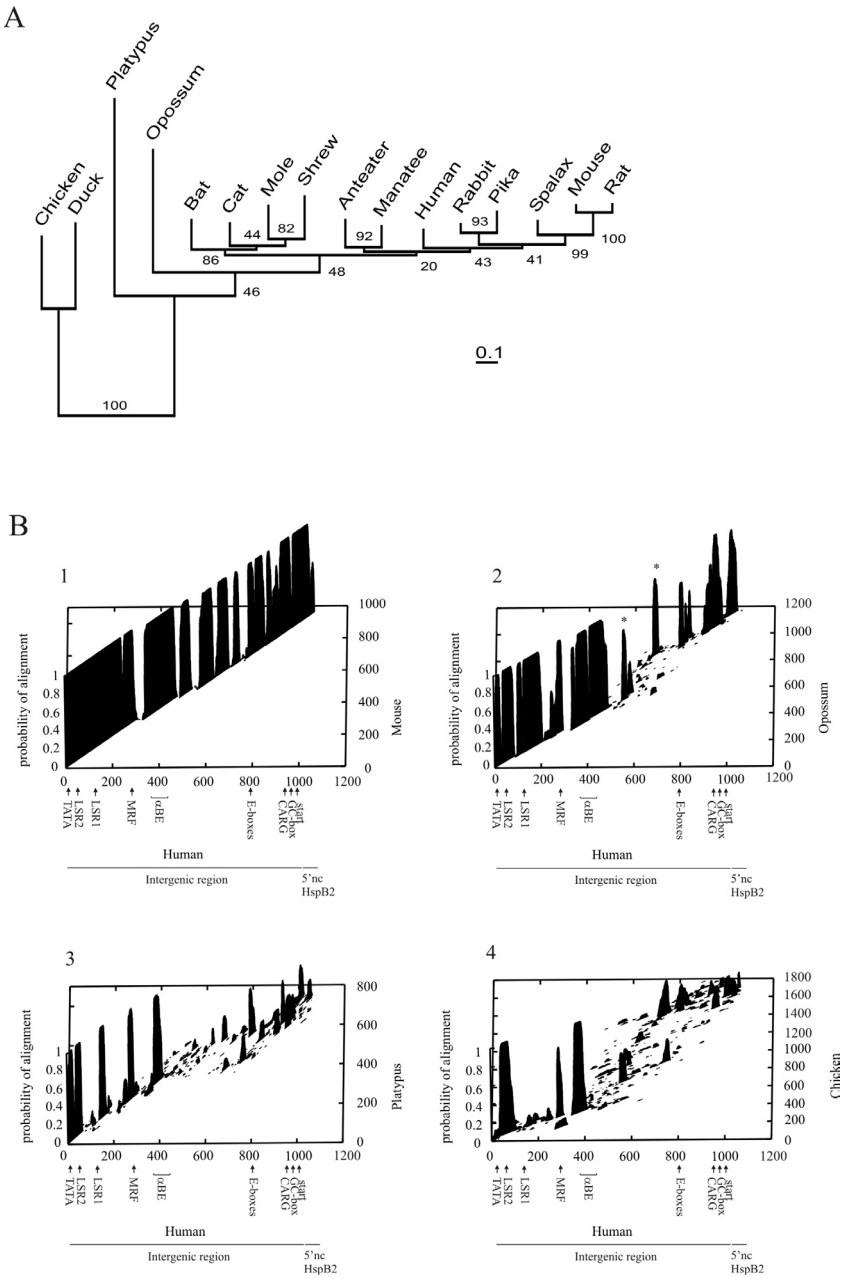

Fig. 2. Phylogenetic analysis of the intergenic region between the αB-crystallin and HspB2 genes.

A: Maximum likelihood tree (-lnL = 10965.74) of the intergenic regions between the αB-crystallin and the HspB2 genes. The tree is based on conserved intergenic regions using a HKY85 + Γ4 model of sequence evolution (see Materials and Methods for details). Branch lengths are proportional to the numbers of base substitutions, and the bar corresponds to 0.1 nucleotide change per site. Numbers indicate nodal nonparametric bootstrap support under the maximum likelihood criterion. B: Phylogenetic footprinting of the intergenic region between the αB-crystallin and HspB2 genes. Two-dimensional histogram output of the Bayesian block aligner (Bayes Aligner; Zhu et al. 1998; Wasserman et al. 2000). The histogram indicates the probability that any given base j in the human sequence aligns to any base k in the mouse (1), opossum (2), platypus (3) or chicken (4) sequence. Probabilities are determined from a set of alignments representative of all possible alignments of the two sequences. The known regulatory sites of the mouse αB-crystallin promoter are indicated in the figure, the sites indicated close to the HspB2 promoter are based on sequence analysis only (Iwaki et al. 1997). The two conserved elements identified by the human-opossum sequence comparison are indicated with an asterisks (*).

This region also contained the sequence recognized by Elk-1, one of the proteins which forms part of the ternary complex with the Serum Response Factor (SRF), but lacks the SRF recognition site, which is usually adjacent (for review, see Shaw and Saxton 2003). A search for the recognition site of the CTCF transcription factor (consensus sequence CCGCNNGGNGGCAG; Ishihara and Sasaki 2002), which mediates insulator activity (for review, see Ohlsson et al. 2001; Burgess-Beusse et al. 2002; Kuhn and Geyer 2003), yielded a number of matches but none in a conserved region.

The phylogenetic footprinting together with direct inspection of the complete sequence alignment (supplementary material) revealed some notable changes in

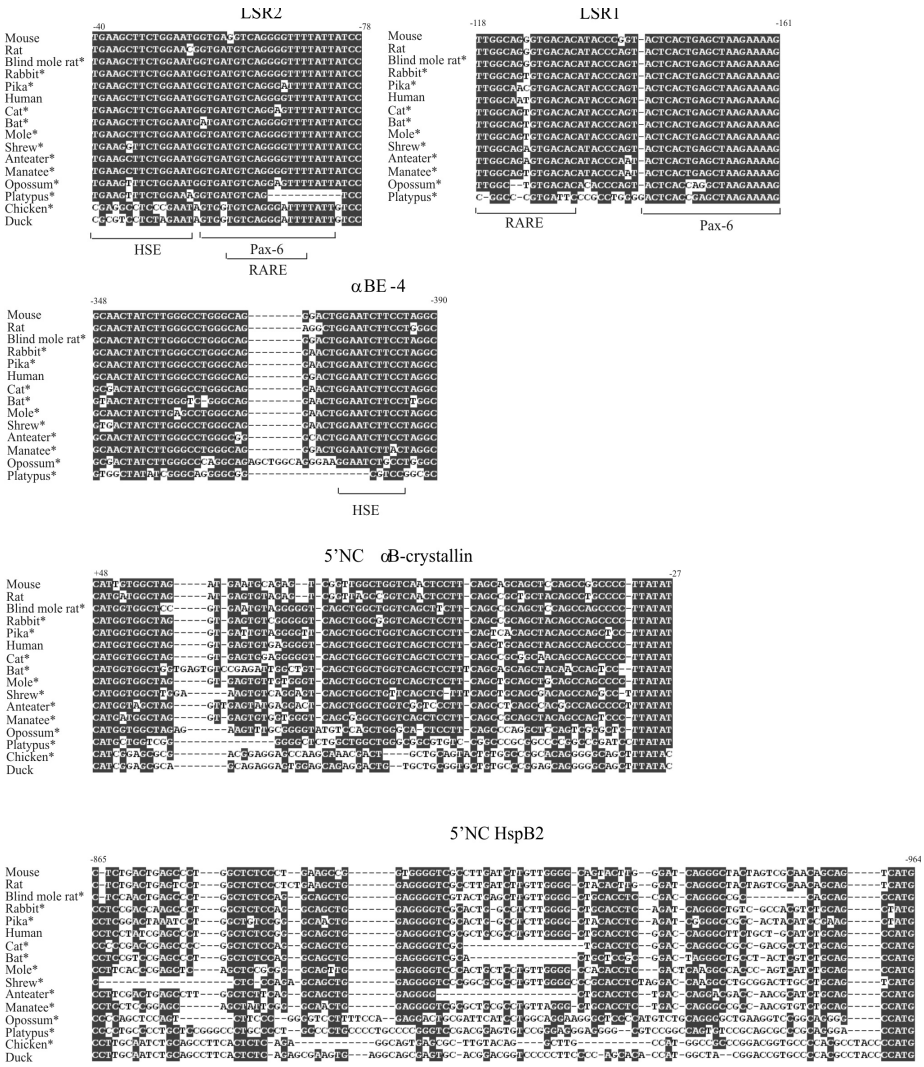

Fig. 3. Alignment of some conserved elements in the intergenic regions between the αB-crystallin and the HspB2 genes. Sequences of the intergenic regions between mammalian and avian αB-crystallin and the HspB2 genes were aligned using ClustalW and adjusted manually using Genedoc (see supplementary material for the complete alignment). Here only the alignments of the LSR1 and 2, the αBE-4 element, and the 5' UTR's of the αB-crystallin and HspB2 genes are shown; the full alignment is shown in the supplementary material. Chicken and duck sequences were omitted from the LSR1 and αBE-4 alignments, since the LSR1 is not present (see Fig. 2B) and the αBE-elements contain insertions (see supplementary material). Position numbers are given for the mouse sequences and are relative to αB-crystallin transcription start site (+1). Asterisks (*) indicate newly determined sequences.

(putative) regulatory elements: the complete loss of LSR1 in chicken (and duck; Fig. 2B-4 and supplementary material), the sequence divergence of the 5' part of LSR1 in platypus (Fig. 3), the deletion of the Pax-6 site in the platypus LSR2 (Fig. 3) and the deletion of the MRF binding sequence in the blind mole rat (Hough et al. 2002; supplementary material). The avian sequences have insertions in the elements in the αBE region, separating conserved sequence blocks (supplementary material). In addition, the avian sequences lack HSE's, in agreement with the lack of heat shock response of the duck αB-crystallin gene (Wistow and Graham 1995). In platypus the HSE in the so-called

αB-4 element in the αBE region is absent (Fig. 3), while mutations in the manatee and opossum sequences possibly inactivate the HSE in this element. All mammals have a HSE consensus sequence in LSR2 (Fig. 3). The sequence alignment also shows a conservation of the +20 to +40 region of the HspB2 gene (Fig. 3). This could be indicative of a DPE element, as suggested by Swamynathan and Piatigorsky (2002), although the DPE consensus sequence (GNNN[A/G][A/T][C/T][G/A/C]; Kadonaga 2002) is not easily discerned. There is a striking conservation of the 5' non-coding region of the αB-crystallin gene relative to that of the HspB2 gene in mammals (Fig. 3).

The platypus αB-crystallin promoter is less active relative to the HspB2 promoter than the αB-crystallin promoters of rat and blind mole rat.

The data presented above (Table 1, Fig. 2B and 3) show that the length of the intergenic region between the αB-crystallin and the HspB2 genes is conserved in mammals and that the known regulatory elements of the mouse αB-crystallin promoter are conserved as well, with the exception of platypus. To determine the functional significance of the sequence divergence of the platypus intergenic region, the activity of the platypus αB-crystallin and HspB2 promoters in lens and muscle cells was measured. To that end, we cloned the intergenic region of platypus in a bidirectional reporter construct containing the luciferase coding region on one side of the intergenic region and β-galactosidase coding region on the other side. For comparison, we used the rat intergenic region cloned into the bidirectional reporter construct, while the blind mole rat intergenic region in the bidirectional reporter construct was used to test whether our assay systems could detect the switch from lens to muscle expression of the blind mole rat αB-crystallin promoter described by Hough et al. (2002). The intergenic regions were inserted in both directions between the luciferase and β-galactosidase reporter genes to make sure that differences in activity of αB-crystallin and HspB2 promoters are not due to differences in reporter gene activity and sensitivity of the assays. Constructs are called luc-αB-HspB2-β-gal when the αB-crystallin promoter drives luciferase expression and the HspB2 promoter drives β-galactosidase expression; the reverse construct is called β-gal-αB-HspB2-luc. The relative activity of the αB-crystallin promoter with respect to that of the HspB2 promoter, or vice versa, can then be determined from the ratio of luciferase to β-galactosidase activity.

The activity of the promoter constructs in C2 myoblasts, indicative of expression in muscle, is shown in Fig. 4A. For unknown reasons there was a difference between the relative promoter activities when either luciferase or β-galactosidase was used as reporter gene, making comparisons more difficult. However, a clear trend was seen. The platypus αB-crystallin promoter is between 50 and 75% (depending on the reporter gene) weaker with respect to the platypus HspB2 promoter than the corresponding rat promoters. For the blind mole rat, the ratio of the activity of the αB-crystallin promoter to that of the HspB2 promoter was two-fold higher than that of the corresponding rat promoters when β-galactosidase was driven by the αB-crystallin promoter. The difference was marginal when the αB-crystallin promoter drives the luciferase gene. These data suggest, in agreement

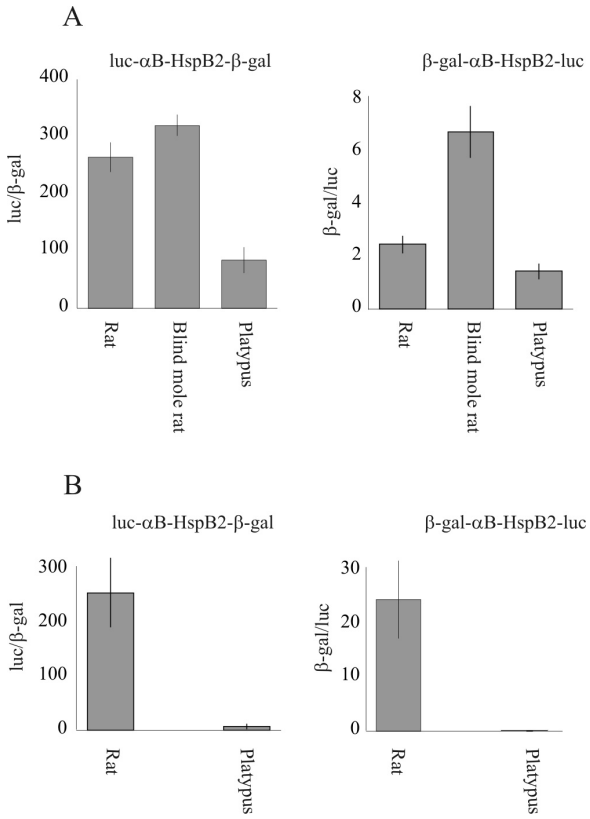

Fig. 4. The activity of the αB-crystallin promoter relative to that of the HspB2 promoter of the rat, blind mole rat and platypus in C2 or lens cells.
A: C2 cells were transfected with constructs containing the intergenic region of rat, blind mole rat or platypus. In these experiments transfection efficiencies were monitored by co-transfection of an EGFP expression construct. Visual inspection showed no major differences in the efficiency of transfection of the various constructs. The ratio of the αB-crystallin promoter activity relative to that of the HspB2 promoter (luc/β-gal or β-gal/luc) was calculated by dividing the activity of the reporter gene driven by the αB-crystallin promoter (luciferase in the case of the luc-αB-HspB2-β-gal construct and β-galactosidase in the case of β-gal-αB-HspB2-luc construct) by the activity of the reporter gene driven by the HspB2 promoter (β-galactosidase in the case of the luc-αB-HspB2-β-gal construct and luciferase in the case of β-gal-αB-HspB2-luc construct). Error bars indicate the standard deviation. B: Differentiated lens explants (lens fiber cells) were transfected with constructs containing the intergenic region of rat or platypus. αB-crystallin promoter activities were calculated relative to HspB2 promoter activities for both luc-αB-HspB2-β-gal and β-gal-αB-HspB2-luc constructs as in A. Error bars indicate the standard deviation.

with the work of Hough et al. (2002), that the blind mole rat αB-crystallin promoter is more active in muscle cells than the rat αB-crystallin promoter.

To test the activity of the αB-crystallin and HspB2 promoters in lens cells, the bidirectional constructs were transfected into rat lens fiber cells, obtained by in vitro differentiation of explanted newborn rat lens epithelial cells. The rat and blind mole rat αB-crystallin promoters were very active in this system, as expected. Less activity was obtained from the platypus αB-crystallin promoter. When the relative activity of the αB-crystallin promoter with respect to the HspB2 promoter is calculated, then the expression directed by the rat αB-crystallin promoter relative to HspB2 promoter is about 30 times higher than that directed by the platypus αB-crystallin promoter relative to the platypus HspB2 promoter (Fig. 4B). For the blind mole rat, the activity of the reporter genes driven by the HspB2 promoter in lens is not significantly above background. Thus, no relative activities for the blind mole rat promoters could be calculated.

The platypus αB-crystallin promoter is activated by a heat shock

Since the platypus intergenic region lacks the HSE in the αB-4 element (Fig. 3), we tested whether there are differences in heat shock response between rat, blind mole rat and platypus αB-crystallin and HspB2 promoters. The bidirectional reporter constructs were transfected into C2 cells, these cells were heat shocked 48 h after transfection and assayed for luciferase and β-galactosidase activity after 6 h of

recovery at 37°C. An experimental problem in these experiments is that the promoters are already active before the heat shock is applied and a distinction must thus be made between the amount of reporter gene product (protein or mRNA) present before the heat shock and the additional amount made in response to the heat shock. To circumvent this problem we argued as follows. Luciferase is heat labile, hence after a heat shock only newly synthesized luciferase (and not luciferase present before heat shock) will be measured. However, the pre-existing luciferase mRNA is still present. To determine how much luciferase is made after heat shock from pre-existing mRNA, we used the rat HspB2 promoter as reference as this promoter has already been shown to be the non-heat shock inducible (Iwaki et al. 1997). When this promoter drives luciferase expression (the rat β-gal-αB-HspB2-luc construct), the luciferase activity after 6 hours of recovery from a heat shock was about 5% of that found in non-heat shocked cells (Fig. 5). Hence recovery of 5% of the initial luciferase activity was taken as indicative of a lack of heat shock response. In contrast to luciferase, β-galactosidase is not heat labile. Any additional increase in β-galactosidase activity due to the heat shock will be marginal compared to the activity already present in the cell and the level of β-galactosidase activity was thus taken as a control for the transfection efficiency.

Figure 5 shows the relative amount of luciferase after a heat shock compared to that without a heat shock for the bidirectional promoter constructs from the three species. For the rat, the amount of luciferase activity from the rat luc-αB-HspB2-β-gal construct after heat shock was 33% of the control, indicating a heat shock response. For platypus the relative amount of luciferase activity obtained from the αB-crystallin promoter in the construct luc-αB-HspB2-β-gal was twice as high (64%), indicative of a strong heat shock response. The relative amount of luciferase activity recovered after heat shock from the platypus HspB2 promoter (the β-gal-αB-HspB2-luc) was even less than that from the rat HspB2 promoter and the platypus HspB2 promoter is thus unlikely to be heat shock inducible. Finally, the blind mole rat αB-crystallin promoter was relatively less active after a heat shock than the rat or platypus αB-crystallin promoters, while the blind mole rat HspB2 promoter seems to be more active. A possible explanation for the latter finding is that in the blind mole rat the HSE's act towards both the αB-crystallin and the HspB2 promoter thereby decreasing the effect on the αB-crystallin promoter.

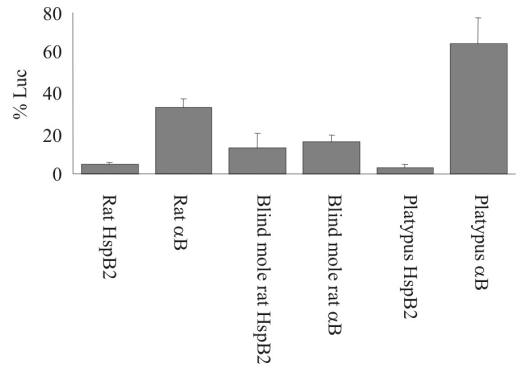

Fig. 5. Heat shock response of the rat, blind mole rat and platypus αB-crystallin and HspB2 promoters in C2 cells.
C2 cells transfected with the constructs containing the intergenic region of rat, blind mole rat or platypus were heat shocked 48 h after transfection for 30 min at 45ºC and harvested after 6 h of recovery at 37ºC; control cells were not heat shocked. The luciferase values were corrected for differences in transfection efficiency using the β-galactosidase values. The luciferase activity (Luc) measured after heat shock and recovery is expressed relative to that of non-heat shocked cells, which was set at 100%. Error bars indicate the standard deviation.

Deletion of the rat intergenic region decreases the relative activity of the αB-crystallin promoter and makes the HspB2 promoter slightly heat shock responsive

The data presented in Fig. 5 show that in vitro, as in vivo, the HSE's present in the rat or platypus intergenic region act unidirectionally towards the αB-crystallin promoter. To determine whether the rat HspB2 promoter becomes heat shock sensitive if the HSE's are moved closer to this promoter, two deletion constructs were made (Fig. 6). In the Δ630 construct, 290 bp spanning the region between the E-boxes at the HspB2 side of the promoter and the upstream HSE were deleted, moving this element close to the HspB2 promoter. In the Δ850 construct the region between the E-boxes and the LSR1 was deleted, thus placing the HSE in LSR2 close to the HspB2 promoter. In non heat shocked C2 cells (Fig. 6A), the Δ850 deletion adversely affected the activity of both promoters (data not shown) with the αB-crystallin promoter relatively more inhibited than the HspB2 promoter (Fig. 6A). The Δ630 deletion had little effect on the relative activity of the αB-crystallin promoter when that promoter was driving the β-gal reporter gene, while it appeared to have a strong negative effect when the αB-crystallin promoter was driving the luciferase reporter gene, making it difficult to interpret the effect of this deletion on promoter activity (Fig. 6A). After heat shock (Fig. 6B), the HspB2 promoter in either one of the two deletion constructs was more active than in the wild type construct. The activity of the αB-crystallin promoter after heat shock was not significantly affected by either the Δ630 or the Δ850 deletion (Fig. 6B).

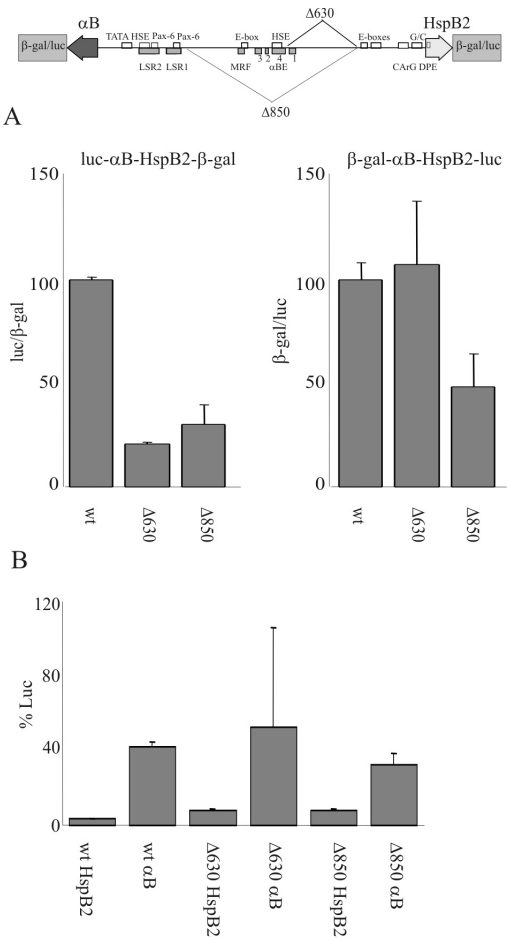

Fig. 6. Effect of deleting part of the intergenic region on the activity and heat shock response of the rat αB-crystallin and HspB2 promoters in C2 cells.
A: Deletion constructs of the rat intergenic region (schematic representation) in the bidirectional reporter vector were transfected into C2 cells. The figure shows the ratio of activity of the αB-crystallin and HspB2 promoters as calculated from the levels of luciferase and β-galactosidase relative to that obtained from the full length constructs. B: C2 cells transfected with the full length or deletion constructs of the rat intergenic region were heat shocked 48 h after transfection for 30 min at 45°C and harvested after 6 h of recovery at 37°C, control cells were not heat shocked. The luciferase values were corrected for differences in transfection efficiency using the β-galactosidase values. The luciferase activity (Luc) measured after heat shock and recovery is expressed relative to that of non-heat shocked cells, which was set at 100%. Error bars indicate the standard deviation.

The αB-crystallin and HspB2 promoters are active in the lens of *Xenopus laevis* transgenic animals.

In *in vitro* differentiated lens fiber cells the platypus αB-crystallin promoter was only poorly active (Fig. 4B). To rule out that the activity of this promoter was only due to leakiness of the transfection system and not to lens-specific recognition, we turned to an in vivo system based upon an animal which is evolutionarily equidistant from rat, blind mol rat and platypus, namely *Xenopus laevis.* We have previously shown that a rat γ-crystallin promoter is recognized as a lens-specific promoter in *Xenopus laevis* (Brakenhoff et al. 1991). Constructs containing the whole intergenic region and in which either the αB-crystallin or the HspB2 promoter drives an EGFP reporter gene were injected into *Xenopus* oocytes. Expression was monitored during development. When EGFP expression was regulated by the rat γD-crystallin promoter, expression is mainly seen in lens, but a slight background staining was also found during the first 8 days of development. In contrast, EGFP expression driven by either the HspB2 or the αB-crystallin promoter shows specific staining of the somites as well as lens after 2 days (Fig. 7). After 4-8 days not only muscle and lens but also heart and some brain and neuronal tissue can be seen to be fluorescent (note that the expression level -but not the expression pattern - varied between embryos and that the pictures shown in Fig. 7 are qualitative not quantitative). The most striking observation was that in these *Xenopus* larvae there was little difference in the pattern of EGFP expression from the αB-crystallin or from the HspB2-crystallin promoter, in particular, clear lens staining was seen as well when EGFP expression was driven by the HspB2 promoter. These data suggest that in *Xenopus laevis* the HspB2 promoter is also recognized as a lens-specific promoter since we only observed staining of the lens in *Xenopus* transgenes when lens-specific (e.g. crystallin) promoters are used, not when muscle- or brain-specific promoters are driving EGFP expression (data not shown).

Discussion

We have shown here that the head-to-head orientation of the αB-crystallin/HspB2 gene pair is present in all mammalian lineages as well as in birds. Assuming that this gene pair originated from an inverted duplication of an ancestral sHsp gene, then our results show that the close linkage has been retained through mammalian evolution. It is commonly suggested that maintenance of a close apposition of head-to-head promoter regions is due to the selective pressure imposed by the sharing of promoter elements (see for example Shinya and Shimada 1994; Labrador and Corces 2002; Yoshida et al. 2002; Meyer et al. 2003; Otte et al. 2003; Shin et al. 2003; Takai and Jones 2003; Zhang et al. 2003). Given the difference in expression pattern between the αB-crystallin and the HspB2 genes, one would then have to postulate that the expression in muscle requires shared elements, while the lens specific regions and the HSE's are directed at the αB-crystallin promoter only. The elements that control the activity of the HspB2 promoter have not been mapped but the available evidence suggests that at least some are located within the upstream enhancer region (the αBE region and the MRF), which is involved in the expression of the αB-crystallin gene in muscle, as

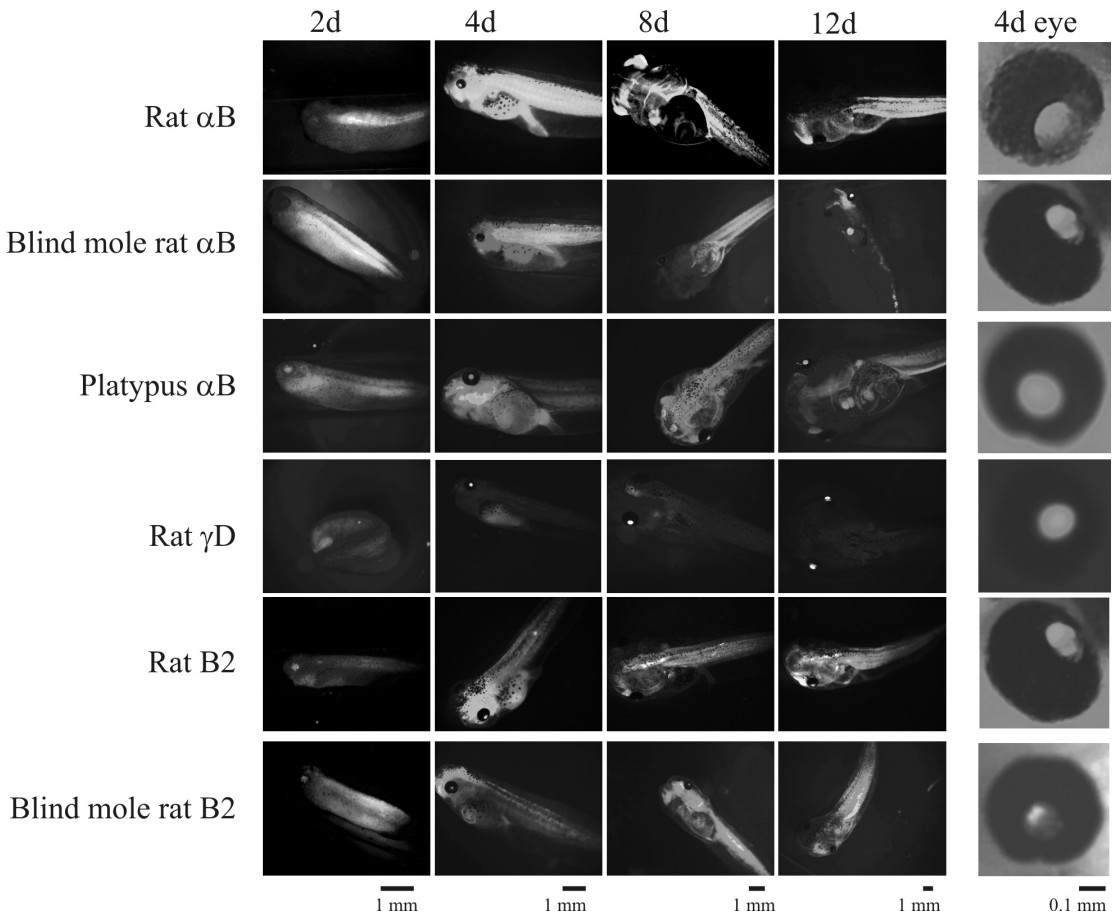

Fig. 7. Expression of EGFP directed by the αB-crystallin and HspB2 promoters in transgenic Xenopus laevis.
The rat, blind mole rat and platypus intergenic regions cloned in either direction (in the case of rat and blind mole rat) or a single direction (in the case of platypus) in front of the EGFP reporter gene were used to generate transgenic X. laevis tadpoles. Transgenic animals with the rat γD-crystallin promoter driving EGFP expression are shown as a control. Transgenic animals were photographed after 2, 4, 8 and 12-15 days of development. An enlargement of the eye is shown of animals at the 12-15 days stage of development. Representative photographs are shown.

deletion of this region does lower the activity of the HspB2 promoter, although to a much lesser extent than it lowers the activity of the αB-crystallin promoter (Swamynathan and Piatigorsky 2002). Similarly, we found that deletion of the enhancer region (the Δ850 deletion) inhibited the activity of both the αB-crystallin and the HspB2 promoters in mouse myoblasts cells, with the αB-crystallin promoter being the most affected. As deletion of the enhancer region has a more severe effect on the activity of the αB-crystallin promoter, most of the elements within this complex are required only for expression of the αB-crystallin promoter. A few elements acting on the HspB2 promoter could be interspersed with those acting on the αB-crystallin promoter. If occupancy of one element working towards one promoter promotes occupancy of a second element working towards the other promoter, then intermingling of elements and synergistic binding of transcription factors to the different elements would explain the selective pressure to maintain the complex enhancer region and the head-to-head orientation. Alternatively, the selective pressure could be due to as yet unknown

regulatory phenomena. An obvious possibility is that one or both of the two conserved regions between the upstream enhancer region and the HspB2 promoter detected by phylogenetic footprinting is required for optimal function of both the αB-crystallin and the HspB2 promoter. Neither of these two regions is absolutely required for promoter activity as deletion of these regions in the Δ630 or Δ850 deletion constructs did not silence either the αB-crystallin or the HspB2 promoter. However, the first region could play a crucial role in the transcriptional response to stress or apoptotic insults, as it contains a binding site for members of the REL/NF-κB/IκB family of transcription factors (Foo and Nolan 1999) which play a role in this response; the second region could be involved in setting the proper level of expression during development, one of the functions of the Oct transcription factors (Phillips and Luisi 2000) for which a target site is present in this region. Clearly, the role, if any, of these two conserved elements needs to be elucidated and the functional elements of the HspB2 promoter need to be mapped before a model of the regulatory functions embedded in the intergenic region between the αB-crystallin and HspB2 genes can be formulated.

The LSR's and the HSE's present in the intergenic region between the αB-crystallin and the HspB2 gene work towards the αB-crystallin gene only, as only this gene is expressed in the lens and only this gene is heat shock inducible. Yet the textbook definition of an enhancer is that its stimulatory effect on the rate of transcription initiation is independent of the location or orientation of the enhancer. There are a number of ways in which the HspB2 promoter could be shielded from the LSR's and HSE's. One possibility is that the HspB2 promoter is silenced in the lens by an epigenetic mechanism. DNA methylation has been suggested to be responsible for the inactivity of γ-crystallin promoters in the lens epithelial cells (Peek et al. 1991), while differential histone acetylation controls the alternative activation of the murine bidirectional TK and KF promoters (Schuettengruber et al. 2003). A second possibility is that the transcription factors which mediate lens-specific or heat shock induced transcription may require a TATA box promoter and be incompatible with the GC rich promoter region of the HspB2 promoter. In the case of the heat shock induced transcription this is unlikely as the bidirectional promoter region of the Hsp60 and Hsp10 genes lacks TATA boxes and contains only a single HSE that acts on both promoters (Hansen et al. 2003). The third possibility is that, as suggested by Swamynathan and Piatigorsky (2002), the enhancing effect is blocked by an insulator. A common blocker of enhancer activity in mammals is CTCF (Bell et al. 1999; Ohlsson et al. 2001; Burgess-Beusse et al. 2002; Kuhn and Geyer 2003). If CTCF is involved, then one would expect the cognate binding site to be located within a phylogenetic footprint. However, none of our phylogenetic footprints contained the CTCF recognition sequence. Our transfection experiments do not address the question whether CTCF is involved in shielding the HspB2 promoter in the mammalian lens since CTCF insulator activity is mediated by chromatin structure (Burgess-Beusse et al. 2002; Labrador and Corces 2002; Kuhn and Geyer 2003) and is unlikely to be detected in transient transfection assays. In the *Xenopus* transgenes, the constructs are integrated in the genome, but, at least during embryogenesis, CTCF expression in *Xenopus laevis* is restricted and absent

in lens fiber or muscle cells (Burke et al. 2002).

In a comparison of 51 rodent and human promoter regions, Dermitzakis and Clark (2002) found that about 30-40% of the transcription factor binding sites in the human promoter regions were not functional in the orthologous rodent promoter regions. Such a divergence between species is unlikely in the case of the αB-crystallin promoter. All elements important for expression of this promoter in either the lens or muscle as determined for the murine promoter (Dubin et al. 1991; Gopal-Srivastava and Piatigorsky 1993, 1994; Srinivasan and Bhat 1994; Gopal-Srivastava et al. 1995, 1996, 1998), are conserved in the human sequence as well as in other mammals, with the exception of platypus. The platypus promoter has an intriguing pattern of divergence: the LSR2 is present, except for the Pax-6 site, while of the LSR1 only the Pax-6 site is found. If Pax-6 interacts with the other factors binding to the LSR2, one would expect that loss of the Pax-6 site would relieve the selective pressure to maintain the binding site of other factors and that LSR2 would be lost, yet in the platypus promoter lacks most of the LSR1. As both the LSRs bind RAR/RXR and Pax-6 (Gopal-Srivastava and Piatigorsky 1994; Gopal-Srivastava et al. 1996, 1998), these two regions could have originated from a duplication of an ancestral LSR. Initially then, two redundant complexes would have been present: a complex of Pax-6 with the factors interacting with the LSR1 and a complex of Pax-6 with the same factors binding to the LSR2. Loss of the Pax-6 site from LSR2 could have resulted in an interaction of the Pax-6 bound to LSR1 with the factors binding to LSR2. The remainder of LSR1 would then no longer be functional and that sequence could diverge freely. In the avian promoter region only the LSR2 is found; the LSR1 is missing. The platypus, chicken and duck promoter sequences do illustrate that a single LSR complex suffices for lens expression of the αB-crystallin promoter. It has been shown experimentally that the LSR2 can direct expression of the mouse αB-promoter to the lens, although the level of expression is low (Gopal-Srivastava et al. 1996). The platypus promoter also lacks the upstream HSE but does have the proximal HSE which explains why the promoter is heat shock responsive. The avian αB-crystallin promoters lack both HSE's and at least the duck αB-crystallin promoter has been reported to be insensitive to a heat shock (Wistow and Graham 1995). Although it is difficult to trace the evolution of promoter regions (for discussion and review, see Wray et al. 2003), our results argue that the primordial mammalian αB-crystallin promoter had two LSR's and two HSE's and that the loss of one of the two LSR's and one of the two HSE's is secondary.

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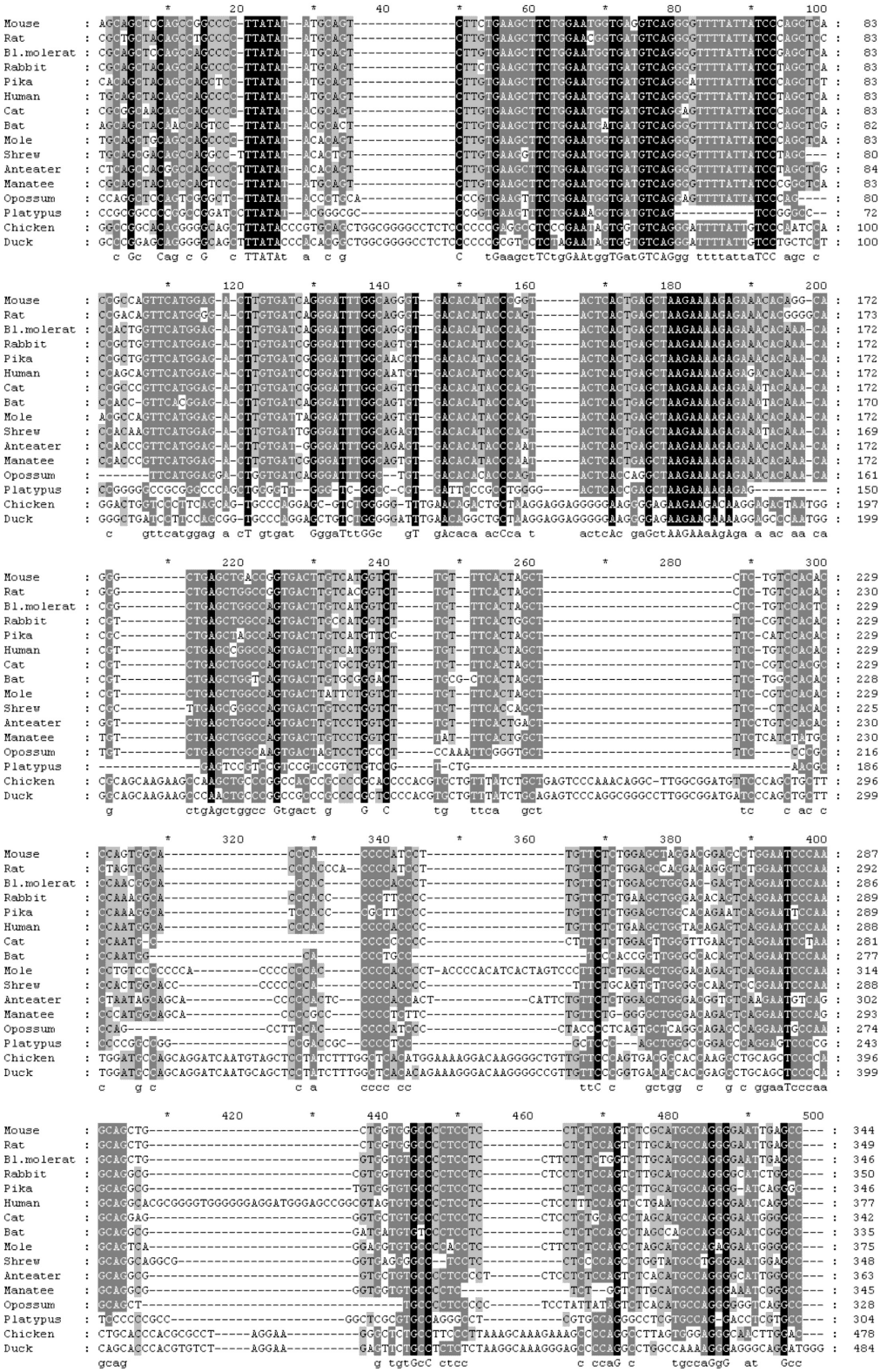
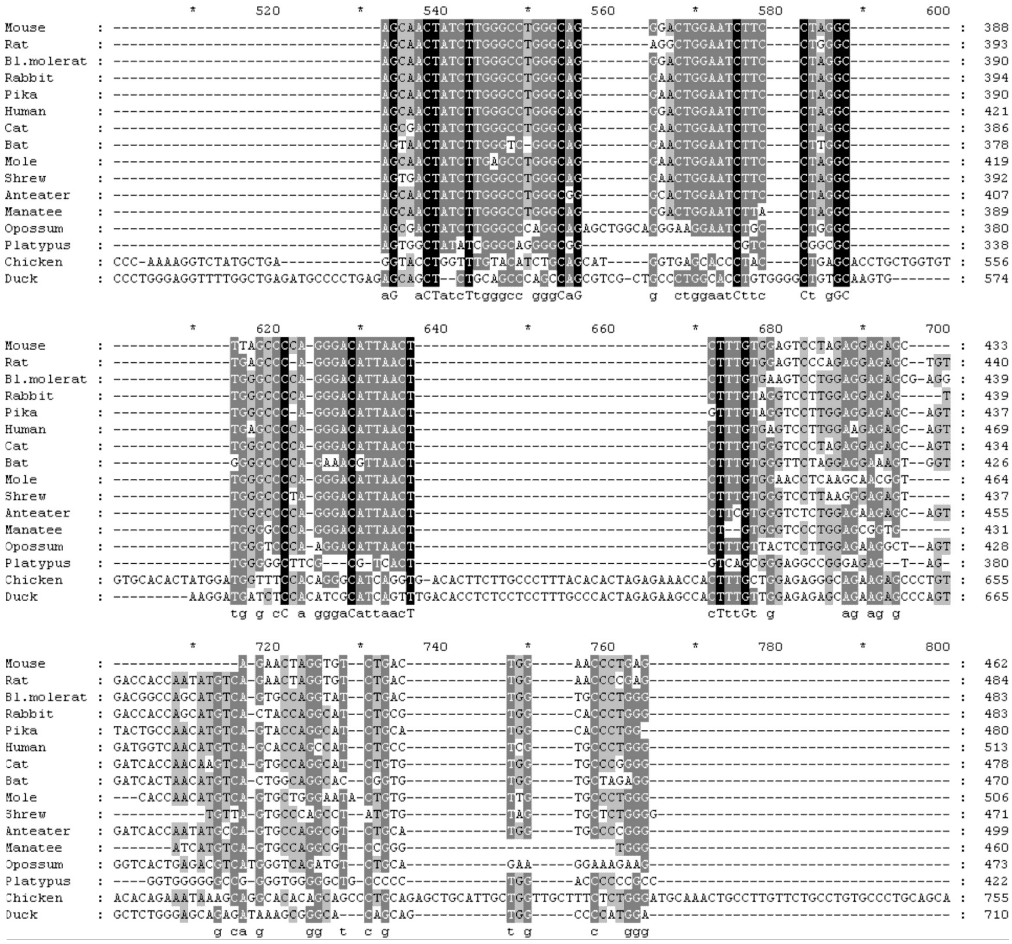
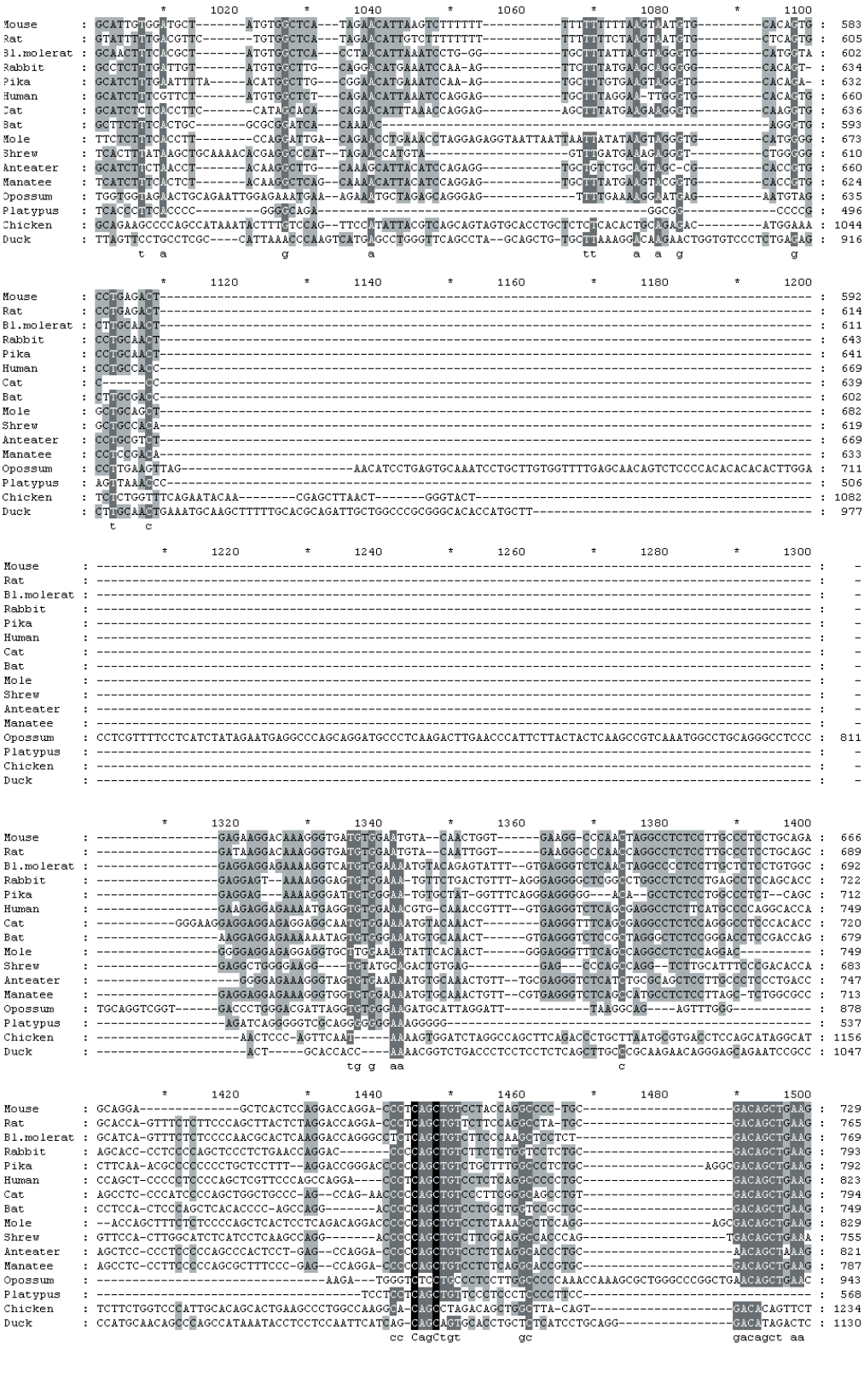
Supplementary material

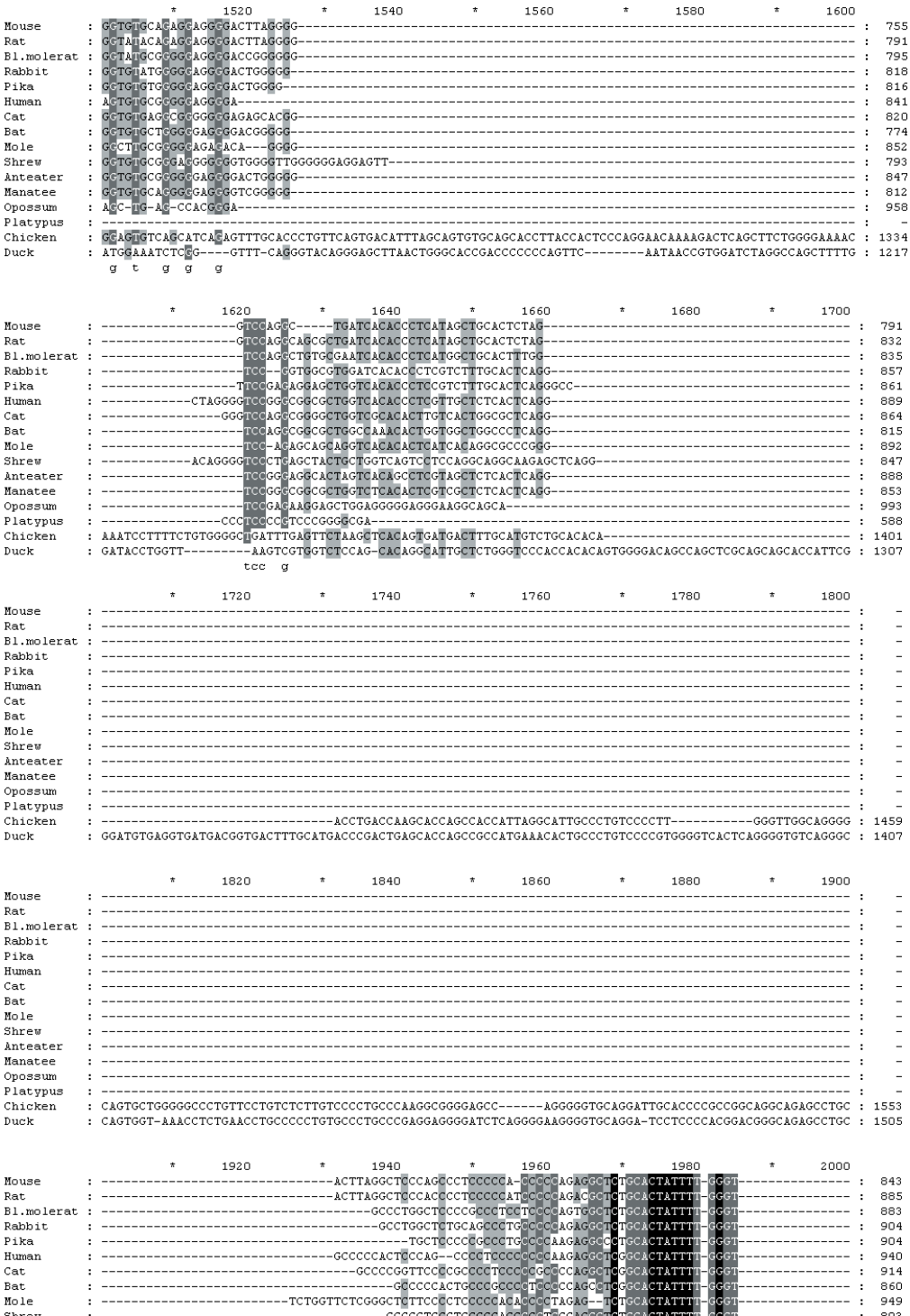
Intergenic region between αB-crystallin and HspB2 genes
Transcription start sites are indicated with an arrow.
The known regulatory sitesof the mouse αB-crystallin promoter are indicated in the figure as follows:
TATA: TATA-box;
HSE's: heat shock elements (consensus sequence: inverted repeat of nGAAm);
LSR: Lens specific regions (contain Pax-6 and RAR/RXR binding sites);
MRF: binding site for the muscle response factor, binding to this element increases transcription in muscle;
aBE: αB-crystallin elements 1-4, enhance promoter activity in lens, muscle and heart.

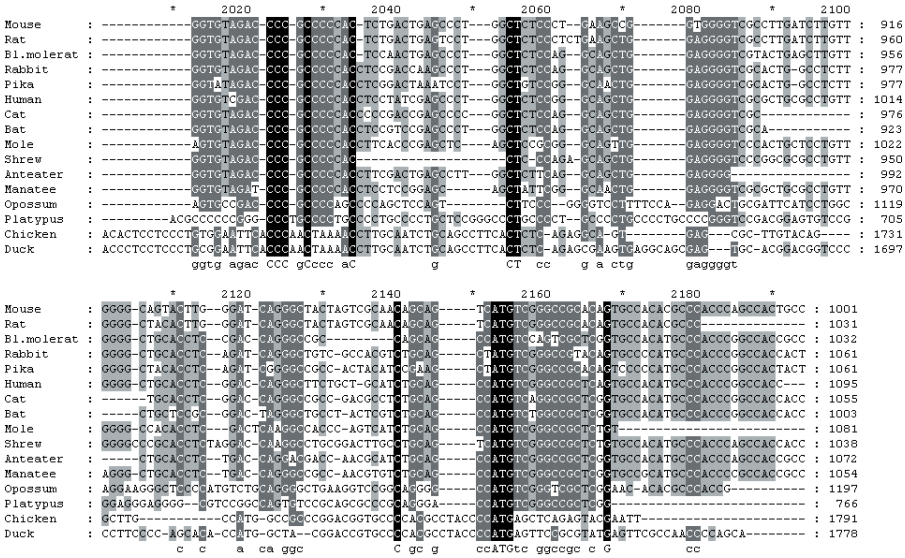
The putative regulatory elements of the HspB2 promoter have been identified only on the basis of sequence analysis:
E-box: binding site for MyoD family members;
CArG-box: binding site for SRF;
G/C: G/C-rich promoter region.











αB-crystallin and Hsp25 traffic to the nucleus in stressed and non-stressed cells.

Linda Doerwald, John den Engelsman, Wilfried W. de Jong and Nicolette H. Lubsen

Chapter 4

αB-crystallin and Hsp25 traffic to the nucleus in stressed and non-stressed cells.

Abstract. The small heat shock proteins αB-crystallin and Hsp25 have been reported previously to be located in the cytoplasm in non-stressed cells and to be located in (Hsp25) or around (αB-crystallin) the nucleus in stressed cells. Here we show that during treatment with the nuclear export inhibitor leptomycin B, αB-crystallin as well as Hsp25 accumulated in the nucleus of murine myoblasts (C2 cells) under normal conditions as well as during stress imposed either by a heat shock or by treating the cells with arsenite. The nuclear staining pattern of these proteins was homogeneous and no evidence for an association with a nuclear structure was found. Our results indicate that αB-crystallin and Hsp25 shuttle to the nucleus under normal conditions as well as during stress.

Introduction

αB-crystallin and Hsp25 are members of the small heat shock protein family (sHsps). These proteins are known to be upregulated during stress and are generally thought to be active as chaperones (van Montfort et al 2001; Narberhaus 2002; Arrigo and Müller 2002). Both proteins are also constitutively expressed in a number of tissues (van Montfort et al 2001; Arrigo and Müller 2002). In non-stressed cells, both αB-crystallin and Hsp25 appear to be located mainly in the cytoplasm. For αB-crystallin nuclear staining seems to be dependent on the antibody; only when an antibody raised against the C-terminal tail of αB-crystallin is used, can nuclear spots be seen (Kato et al 1996, van Rijk et al 2003).

Upon stress Hsp25 translocates to the nucleus, a process that is phosphorylation dependent (Geum et al 2002). αB-crystallin has not been found in the nucleus in heat shocked cells, rather it has a perinuclear location (Djabali et al 1997, Wiesmann et al 1998). In addition Hsp25 and αB-crystallin interact with several cytoskeletal structures after stress and are thought to be involved in maintaining the assembly and stability of the cytoskeletal structures (van Montfort et al 2001, Quinlan 2002).

When αB-crystallin is over-expressed, some is found in the nucleus as well (Bhat et al 1999), showing that this protein is not excluded from the nuclear compartment. Hence it is possible that endogenous αB-crystallin (and/or Hsp25) does shuttle from cytoplasm to the nucleus and back but that the levels in the nucleus are too low to detect. Shuttling proteins can be trapped in the nucleus by adding the nuclear export inhibitor leptomycin B (LMB; Kudo et al 1999). Nuclear import can still take place in LMB treated cells and if αB-crystallin and/or Hsp25 do shuttle between cytoplasm and nucleus, this should result in an increase of these proteins in the nucleus. Here we show that in LMB treated control, heat shocked or arsenite stressed cells αB-crystallin as well as Hsp25 is found in the nucleus.

Results and discussion

Cellular location of αB-crystallin and Hsp25 in non-stressed cells.

Mouse myoblast (C2) cells contain high endogenous levels of αB-crystallin and Hsp25 and staining of these cells for αB-crystallin (using a monoclonal antibody raised against the full length chicken αB-crystallin; Sawada et al 1993) or Hsp25 yields a strong cytoplasmic signal while the nucleus appears weakly stained if at all (Fig 1A). However, after blocking nuclear export with LMB for 1.5 h, both αB-crystallin and Hsp25 are clearly detectable in the nucleus (Fig 1B). The relative staining intensity of αB-crystallin and Hsp25 in the nucleus increased in time from 1.5 hours of LMB treatment to 6 hours treatment (latest time point tested, data not shown, also compare Fig1B with 2A and 2B for αB-crystallin and Hsp25, respectively).

These data show that in non-stressed cells αB-crystallin as well as Hsp25 traffic to the nucleus. The finding that long incubation periods with LMB are required to trap significant amounts of the proteins in the nucleus suggests that the nuclear import of these proteins during normal conditions is slow.

αB-crystallin, Hsp25 and nuclear domains?

The nuclear staining of αB-crystallin or Hsp25 in LMB treated cells is homogeneous, no association with nuclear structures can be seen. To enhance detection of the possible association with nuclear structures, cells were treated with Triton before fixing them. This results in a decrease of Triton-soluble proteins and only Triton-insoluble proteins will be seen. Thus if these proteins are located in specific domains in the nucleus of these LMB treated cells, Triton treatment should show a clear nuclear

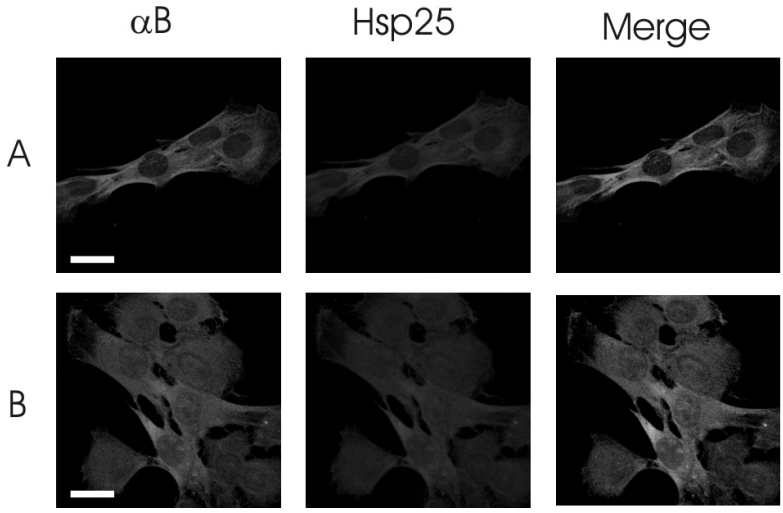


Fig 1. Localization of αB-crystallin and Hsp25 in control (A) and LMB treated (B) cells.
Approximately 2x10^4 C2 cells (mouse myoblasts) were plated on coverslips (18 x18 mm, Knittel) in DMEM supplemented with 10% FCS and penicillin/streptomycin. Twenty-four hours after plating, the cells were treated with 2 ng/ml LMB for 1.5 hr. Cells were fixed in 3% paraformaldehyde in PBS and then permeabilized with 0.2% Triton X-100 in PBS. For detection of αB-crystallin a monoclonal antibody (raised against the complete chicken αB-crystallin (Riken Cell Bank nr. 1304); when polyclonal antibody raised against the C-terminal tail of αB-crystallin is used, nuclear spots can be seen; Kato et al 1996, van Rijk et al 2003) and FITC conjugated secondary antibodies (DAKO A/S, Copenhagen, Denmark) were used. For detection of Hsp25, a polyclonal antibody and TRITC conjugated secondary antibody (DAKO A/S, Copenhagen, Denmark) was used. All images were obtained by confocal laser scanning microscopy on a BIO-RAD MRC1024 using a Kr/Ar laser and sequential scanning. Confocal data are stacked to present whole cells in these pictures. Bars indicate 20 μm. (A) control cells; (B) LMB treated cells.

pattern. However, cells treated with Triton again showed a homogeneous staining of αB-crystallin and Hsp25 in the nucleus (Fig 2A and B).

To test whether a nuclear pattern can be seen if the level of αB-crystallin in the nucleus is enhanced, αB-crystallin with a nuclear localization signal (αBnls) was transfected in C2 cells. αB-crystallin could be clearly seen in the nucleus of over-expressing cells, the staining however also does not show a clear pattern (Fig 2C). When these αBnls over-expressing cells were treated with Triton, αB-crystallin staining shows a more punctuated pattern, but staining can still be seen throughout the whole nucleus (Fig 2C).

These results show that in non-stressed cells neither αB-crystallin nor Hsp25 is located in distinct nuclear domains or associated with particular nuclear structures.

Cellular localization of αB-crystallin after heat shock or arsenite stress.

We showed above that in non-stressed cells treated with LMB, both αB-crystallin and Hsp25 could be found in the nucleus. What happens when cells are stressed?

When C2 cells were heat shocked, Hsp25 but not αB-crystallin can be found in the nucleus (Fig 3A). However, when cells were heat shocked in the presence of LMB, intense staining of αB-crystallin as well as Hsp25, is seen in the nucleus (Fig 3B). In the merged picture, αB-crystallin and Hsp25 can be seen to co-localise. Similarly, when arsenite was used to stress the cells, Hsp25 accumulates in the nucleus, while αB-crystallin does not (Fig 3C, merged picture). When arsenite stressed cells were treated with LMB, a clear nuclear staining of both αB-crystallin and Hsp25 can be seen (Fig 3D).

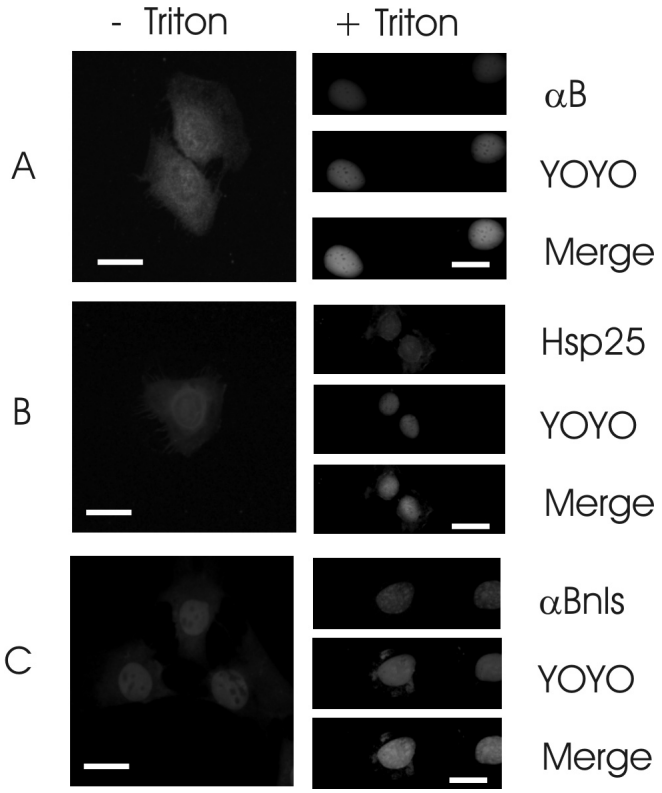


Fig 2. Nuclear-staining pattern of αB-crystallin and Hsp25 in LMB treated cells and in cells overexpressing αBnls.
Cells were treated 24 hours after plating (see legend to Fig 1.) with 10 ng/ml LMB for 6 hr. Cells were treated with 0.2% Triton X-100 in PBS and then fixed in paraformaldehyde (+Triton). As a control LMB treated cells first fixed with 3% paraformaldehyde in PBS and then permeabilised with 0.2% Triton X-100 are shown (-Triton). Cells were stained for αB-crystallin (A) or Hsp25 (B). An expression vector of αB-crystallin with the nuclear localization signal of the 23 kDa FGF-2 isoform (αBnls) was constructed by cloning αB-crystallin cDNA NcoI/XhoI into pLRbFGFk (Leenders et al 1997) and then recloning NdeI blunt/XhoI into NcoI blunt/SalI β-actin expression vector (Gunning et al 1987). C2 cells were transfected with 1 μg αBnls construct per well (C), using lipofectamin plus (Invitrogen) and 48 hours after transfection cells were harvested. Cells were stained using a monoclonal antibody against αB-crystallin (A and C) or polyclonal antibody for Hsp25 (B) using TRITC conjugated secondary antibodies (DAKO A/S, Copenhagen, Denmark). Cells treated with Triton X-100 prior to fixation were stained with 1 μM YOYO to stain the nucleus. Images were obtained as described in the legend to Fig 1.

It is noteworthy that the relative intensity of the nuclear αB-crystallin and Hsp25 staining in stressed cells is higher than in non-stressed cells after 1.5 hours of LMB treatment (compare Fig 3B and D with Fig 1B). This suggests that translocation of αB-crystallin and Hsp25 to the nucleus during stress was faster than in non-stressed cells. Furthermore, as Hsp25 can be detected in the nucleus of stressed cells without LMB treatment whereas αB-crystallin cannot (Fig 3A and C merged picture), Hsp25 is retained in the nucleus in stressed cells, while αB-crystallin is not. For Hsp25 it has been shown that the nuclear location during stress depends on the phosphorylation state (Geum et al 2002). Our data then suggest that phosphorylated Hsp25 interacts with a nuclear structure, thus causing retention, while non-phosphorylated Hsp25 shuttles to and from the nucleus. An association of Hsp25 with nuclear structures in stressed cells is also suggested by the more punctuated staining in the nuclei of stressed cells. αB-crystallin is phosphorylated as well after stress (Ito et al 1997; Arrigo and Müller 2002), but phosphorylation of Hsp25 is more pronounced than that of αB-crystallin. It is possible that phosphorylated αB-crystallin is also retained in the nucleus. However, due to the low level of phosphorylation we probably would not detect such an interaction against the background of non-phosphorylated αB-crystallin.

Our data show that αB-crystallin and Hsp25 traffic to the nucleus under normal conditions as well as during stress. The functional significance of the nuclear location of αB-crystallin and Hsp25 in normal and stressed cells remains to be elucidated.

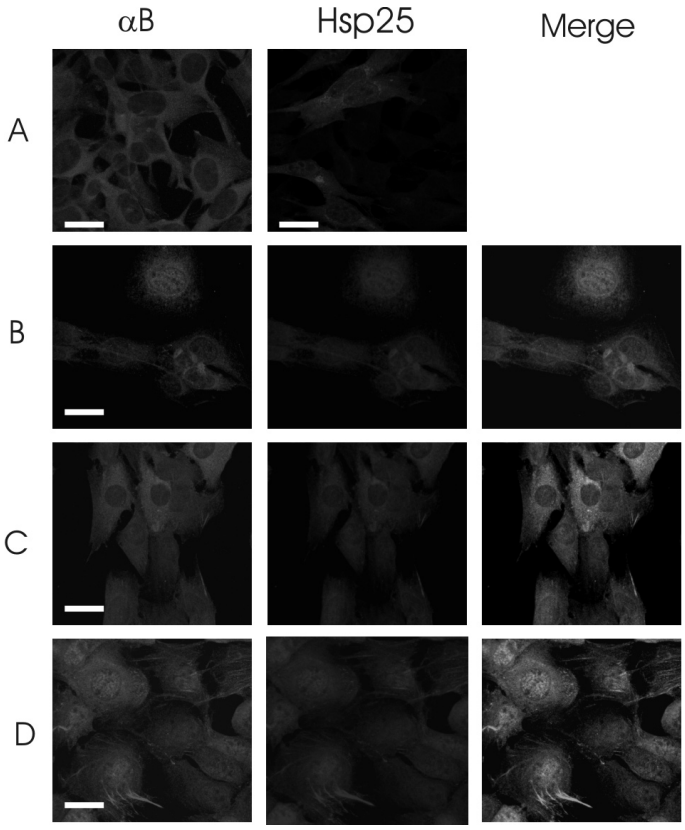


Fig 3. Nuclear localization of αB-crystallin and Hsp25 after stress.
Cells were stressed 24 hours after plating (see legend to Fig 1). For the heat shock, control cells were heat shocked for 60 minutes at 45°C (A), 30 minutes before heat shock 2 ng/ml LMB was added to the cells (B). Cells were harvested immediately after heat shock. For the arsenite treatment, 100 mM arsenite was added to the cells for 1 hour (C), for LMB treated cells 100 mM of arsenite was added 30 minutes after addition of 2 ng/ml LMB (D). After 1 hour of arsenite treatment cells were harvested.
Cells were fixed and stained and images were obtained as described in the legend to Fig 1.

Acknowledgement These experiments were supported by the BioMed program of the European Community (BMH4-CT98-3895).

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Translational thermotolerance provided by small heat shock proteins is limited to cap-dependent initiation and inhibited by 2-aminopurine.

Linda Doerwald, Carla Onnekink, Siebe T. van Genesen, Wilfried W. de Jong and Nicolette H. Lubsen.

JBC. 2003. 278, p.49743-49750.

Chapter 5

Translational thermotolerance provided by small heat shock proteins is limited to cap-dependent initiation and inhibited by 2-aminopurine.

Abstract. Heat shock results in inhibition of general protein synthesis. In thermotolerant cells protein synthesis is still rapidly inhibited by heat stress but protein synthesis recovers faster than in naive heat shocked cells, a phenomenon known as translational thermotolerance. Here we investigate the effect of overexpressing a single heat shock protein on cap-dependent and cap-independent initiation of translation during recovery from a heat shock. When overexpressing αB-crystallin or Hsp27, cap-dependent initiation of translation was protected but no effect was seen on cap-independent initiation of translation. When Hsp70 was overexpressed however, both cap-dependent and -independent translation were protected. This indicates a difference in the mechanism of protection mediated by small or large heat shock proteins. Phosphorylation of αB-crystallin and Hsp27 is known to significantly decrease their chaperone activity; therefore we tested phosphorylation mutants of these proteins in this system. αB-crystallin needs to be in its non-phosphorylated state to give protection whereas phosphorylated Hsp27 is more potent in protection than the unphosphorylatable form. This indicates that chaperone activity is not a prerequisite for protection of translation by small heat shock proteins after heat shock. Furthermore we show that in the presence of 2-aminopurine, an inhibitor of kinases, amongst which PKR, the protective effect of overexpressing αB-crystallin is abolished. The synthesis of the endogeneous Hsps induced by the heat shock to test thermotolerance is also blocked by 2-aminopurine. Most likely the protective effect of αB-crystallin requires synthesis of the endogenous heat shock proteins. Translational thermotolerance would then be a co-operative effect of different heat shock proteins.

Introduction

Cells facing stress divert their resources to combating and surviving that stress. For example during a heat shock general macromolecular synthesis and processing is inhibited and the set of transcription units that encode the heat shock proteins (Hsps) is activated (1,2). Synthesis of the Hsps is required for optimal survival of heat (or other) stress. The Hsps are a complex group of proteins, ranging in size between 90 and 20 kD. The small heat shock proteins (sHsps) belong to a family of proteins distinguished by sharing a common protein domain, the so-called α-crystallin domain (for review, see ref. (3). In man, there are ten different sHsps (4), of these Hsp27 and αB-crystallin are traditional heat shock proteins in the sense that their synthesis is induced by a heat shock (5). All sHsps, including the stress inducible sHsps, are constitutively expressed in different tissues; αB-crystallin for example is abundant in lens, heart, skeletal muscle and brain (5-7). The best known property of Hsp27 and of αB-crystallin is their in vitro chaperone activity: they keep their substrates in solution but cannot refold them (for review, see ref. 6,8). In vivo, they might act as a reservoir of unfolded proteins for the large Hsps, which are ATP dependent refoldases.

The sHsps are also thought to stabilize the cytoskeleton during stress (9), in addition they interact with some components of the apoptotic pathway, thereby protecting the cell from apoptosis (10).

sHsps can be regulated in their activity by phosphorylation. αB-crystallin has three phosphorylation sites. Serine 59 is phosphorylated when cells are stressed, serines 19 and 45 are found phosphorylated when cells are going into mitosis (11,12). The overall level of phosphorylation of αB-crystallin remains low (13). Hsp27 has two (rodents) or three (man) phosphorylation sites. At normal temperature Hsp27 is mainly unphosphorylated, after different kinds of stress, including heat shock, Hsp27 is extensively phosphorylated (5,14). For Hsp27 and αB-crystallin phosphorylation has been shown to prevent complex formation in vitro (15-17), resulting in significant decrease in chaperone activity (16-18). Phosphorylation mimicked Hsp27 protects cells from heat stress but not from oxidative stress (16,19,20).

Cells having a full complement of Hsps due to an earlier stress are more resistant to subsequent heat stress, a phenomenon known as thermotolerance. Thermotolerance can be induced by expression of a single Hsp, such as Hsp70, Hsp27 or αB-crystallin (21-25). In thermotolerant cells protein synthesis is still rapidly inhibited by heat stress but protein synthesis recovers faster than in naive heat shocked cells, a phenomenon known as translational thermotolerance (26-29). The mechanism of inhibition of translation by a heat shock (as well as by other types of stress; ref. 30), and thus also the mechanism of translational thermotolerance, is still a matter of debate. The main block appears to be at the level of translation initiation. Phosphorylation and thus inhibition of eIF2α is commonly found after stress (31,32) but other factors must be affected as well since the inactivation of eIF2B in rat Hepatoma cells did not directly correlate with the level of phosphorylation of eIF2α (33). The cap-binding complex must also be affected, since cap-independent initiation of translation is more stress resistant than cap-dependent initiation of translation (34,35). Deficiency in eIF4E strongly inhibits general translation in HeLa cells but translation of Hsp mRNAs and cap-independent mRNAs still takes place (36), a situation that resembles the pattern of translation in cells recovering from heat stress and suggests that eIF4E is down regulated during a heat shock. Stalled translation initiation complexes containing almost all components of the 48S preinitiation complex, but not the 60S ribosomal subunit, accumulate in the cytoplasm as stress granulas (SG's). Hsp27 has been detected in SG's as well (37), possibly in complex with eIF4G (38). Assembly in SG's is a highly dynamic process and untranslated mRNA's are thought to be sorted and processed there for either reinitiation, degradation or packaging into nonpolysomal mRNP complexes (39). This indicates that during and after stress, SG's are important checkpoints for initiation of translation.

Thus far, translational tolerance has only been assayed at the level of overall rate of protein synthesis, no distinction has been made between cap-dependent and cap-independent translation initiation. We show here that overexpression of either αB-crystallin or Hsp27 protects cap-dependent but not cap-independent translation initiation, while overexpression of Hsp70 affects both. We further show that the phosphorylation state of αB-crystallin or Hsp27 affects its ability to confer translational tolerance. Finally we show

that 2-aminopurine, a kinase inhibitor which inhibits eIF2α kinases such as dsRNA activated kinase (PKR) (40,41), blocks the establishment of translational tolerance by overexpression of a sHsp.

Experimental procedures

Cell culture

C2 cells (mouse myoblast cells) were cultured in Dulbecco's modified Eagle's medium (Gibco) with penicillin and streptomycin (Roche) and supplemented with 20% fetal calf serum (PAA laboratories) to prevent differentiation of these cells. T-REx cells (HeLa cells stably transfected with tetracycline repressor protein (TetR), Invitrogen) were cultured in Minimum essential medium Eagle (Biowhittaker) with glutamax (Gibco), 10% fetal calf serum, penicillin, streptomycin and blasticydin (Invitrogen).

SDS PAGE and western blot analysis of heat shocked cells

For heat shock assays, 6 well plates were seeded with approximately $2.5x10^5$ cells per well for C2 cells and approximately $6.5x10^5$ cells per well for T-REx cells. The next day cells were submitted to a heat shock by submerging plates in a 45°C water bath. C2 cells were heat shocked for 30 min; T-REx cells were heat shocked for 60 min at 45°C. Cells were harvested at various times during recovery at 37°C by scraping in 100 µl lysis buffer (20% glycerol, 6% SDS and 120 mM Tris·HCl pH 6.8). Protein concentrations were measured using the BCA protein assay kit (Pierce) according to the manufacturer's protocol for 96 well plates. After addition of SDS sample buffer (20% glycerol, 4% SDS, 200 mM DTT, 200 mM Tris·HCl pH 6.8 and bromphenol blue), 40 µg of the samples was loaded on gel, separated and western blotted. Blots were blocked for 1 h in blocking buffer (10 mM Tris·HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 5% dried low fat milk) and incubated with primary antibodies in appropriate dilutions in blocking buffer (anti-αB-crystallin mouse monoclonal 1:500, anti-Hsp25 rabbit polyclonal 1:5000, anti-Hsp70 1:1000; Stressgen) for 1 h at room temperature. After three 10 min washes in blocking buffer, the blot was incubated with the secondary antibody with a conjugated alkaline phosphatase (Promega) for 1 h. The blots were washed again in blocking buffer, rinsed once with AP buffer (100 mM Tris·HCl pH 9.5, 100 mM NaCl and 5 mM MgCl2) and stained with NBT/BCIP in AP buffer, dried and analyzed with Molecular analyst software (BioRad) after scanning using a BioRad GS700 imaging densitometer.

Pulse labelling

C2 cells were cultured in Dulbecco's modified Eagle's medium with penicillin and streptomycin and 20% foetal calf serum to 80% confluence in 25 cm^2 flasks. Cells were starved for 2 h in Dulbecco's modified Eagle's medium without methionine with 20% foetal calf serum and penicillin and streptomycin. Cells were heat shocked for 30 min at 45°C and assayed for level of protein synthesis at different time points during recovery. At these time points medium was replaced by 2 ml Dulbecco's modified Eagle's medium without methionine with 20% foetal calf serum and penicillin and streptomycin and 20 µCi of [35S]-methionine (Amersham). After 1 h incubation at 37°C, cells were harvested by scraping in 1 ml PBS and centrifuging for 2 min at 5000 rpm. The cell pellet was resuspended in 50 µl of SDS sample

buffer. After 10 min at 95°C and 15 min in a sonicator bath, 25 µl of the sample was loaded on a SDS PAGE gel. To enhance the 35S signal, the gel was incubated in Amplify (Amersham) for 30 min, after fixing with 0.1% Coomassie brilliant blue in 40% methanol and 10% acetic acid, and destaining in 7.5% methanol and 7.5% acetic acid. After drying the gel was autoradiographed overnight and results were analysed with Molecular analyst software (BioRad) after scanning using a BioRad GS700 imaging densitometer.

Transfections

C2 cells were transfected using lipofectamine plus (Invitrogen). Approximately $6.5x10^4$ cells were plated in Dulbecco's modified Eagle's medium with penicillin and streptomycin and 10% fetal calf serum in 6 well plates. After 24 h cells were transfected with a total of 1 µg DNA per well using 4 µl lipofectamine and 6 µl plus reagent. As a transfection control 0.1 µg of CMV-β-galactosidase was co-transfected, the other 0.9 µg of DNA was divided over the various constructs, pHsp-Cap-Luc or pHsp-IRES-Luc and expression vectors for αB-crystallin, Hsp27, Hsp70, βB2-crystallin or the empty vectors as control in a 1:1 ratio (except when indicated otherwise). Forty-eight h after transfection cells were heat shocked for 30 min at 45°C (unless mentioned otherwise) and assayed for reporter gene activity during recovery at 37°C.

Approximately $2.5x10^5$ T-REx cells were plated in Minimum essential medium Eagle with glutamax, 10% foetal calf serum, penicillin and streptomycin in 6 well plates and transfected after 24 h with 1 µg DNA using 3 µl of Fugene reagent (Roche) per well. As a control for the transfection efficiency, 0.2 µg of the pGL3 control vector (Promega) was co-transfected. The pcDNA4TO LacZ (Invitrogen) and αB-crystallin, Hsp27 or empty expression vectors were used in a 1:1 ratio. Forty-eight h after transfection cells were heat shocked for 1 h at 45°C. Where indicated cells were pre-heat shocked for 30 min at 45°C 6 h earlier. Cells were treated with 1 µg/ml doxycyclin for 3 h, starting 3.5 h before the heat shock to induce the pcDNA4TO LacZ construct. After 3 h of induction, cells were washed with PBS and fresh Dulbecco's modified Eagle's medium with penicillin and streptomycin and 10% foetal calf serum was added. Cells were harvested during recovery at 37°C and assayed for β-galactosidase and luciferase activity.

Transfections were done in triplicate and repeated at least twice with different batches of DNA. Unless otherwise indicated results of representative experiments are shown.

RT-PCR

Transfected cells were scraped in 0.5 ml TRIzol (Invitrogen) per well. After transfer into a tube, 100 µl chloroform was added and the mixture was vortexed for 15 s. After 15 min on ice and centrifuging for 15 min at 13000 rpm at 4°C, 200 µl of the upper phase was precipitated with an equal amount of isopropanol. At this step material from 3 wells was pooled. Samples were left at -20°C for at least 3 hours. After centrifuging for 30 min at 13000 rpm at 4°C, the pellet was washed twice with cold 70% ethanol and then air-dried for 5 min. The pellet was dissolved in 45 µl H_2O and stored at -20°C. The RNA was treated with 7.5 U of RNAse free DNAse per µg of RNA for 15 min at 37°C and DNAse was then inactivated for 10 min at 70°C. The reverse transcription reaction was performed using the 1st strand cDNA synthesis kit for RT-PCR (Roche) according to the manufacturer's instructions

with 1 µg of RNA and random primers in a total volume of 20 µl.

Primers used for the PCR reaction were: luciferase mRNA primers (at position +1330: TGGATGGCTACATTCTGGAGAC and at position +1720: CCTTCTTGGCCT TTATGAGGATC) and as a transfection control β-galactosidase mRNA primers (at position +155: TGGCGTTACCCAACTTAATC and at position +630 TCAGACGGCAAACGAC TGT). The PCR was performed in a total of 25 µl, using 5 µl of cDNA, a mixture of luciferase and β-galactosidase mRNA primers, cDNA PCR reaction buffer, dNTPs and Advantage HF polymerase (Advantage-HF PCR kit, DB Biosciences). A parallel reaction was performed on 0.25 µg of the DNAse treated RNA to test for DNA contamination. The DNA was denatured at 94°C for 60 s, primers were annealed at 66°C for 30 s and then elongation was performed at 68°C for 90 s. PCR samples were taken after 20, 25 and 30 cycles and separated on 5% acrylamide gels in 1x TBE buffer. Results were quantitated using Molecular analyst software (BioRad) after scanning on a Bio Rad Geldoc 1000 system. Samples shown did not contain DNA contamination.

Treatment with 2-aminopurine

For the pre-heat shock, cells were transfected with pcDNA4TO LacZ and pGL3 control vector in a 2:1 ratio with a total of 1 µg DNA per well. Forty-eight h after transfection cells were pre-heat shocked for 30 min at 45°C. The LacZ construct was induced as described in the transfection section of the Experimental Procedures. 2-aminopurine (Sigma) was added to a final concentration of 10 mM after washing away the doxycyclin. 2-aminopurine was dissolved in PBS/HAc (17.5 M; 200:1) to a 100 mM stock and adjusted to pH 7.5 with NaOH. As a negative control, PBS/HAc (200:1 adjusted to pH 7.5 with NaOH) was added to non-treated cells. Cells were harvested after 6 h incubation at 37°C. For the αB-crystallin transfected cells 2-aminopurine was added 30 min before heat shock. Cells were harvested after 6 h of recovery with 2-aminopurine at 37°C. Cells were assayed for luciferase and β-galactosidase activity and western blotted for Hsp expression.

Reporter assays

After transfection in 6 well plates, heat shock and recovery, cells were harvested by scraping or vigorously shacking in 200 µl reporter lysis mix (25 mM Bicine pH 7.5, 0.05% Tween-20 and 0.05% Tween-80) per well.

For the β-galactosidase assay, galacton (Tropix) was added 1:100 to a 100 mM phosphate buffer pH 8.1 with 5 mM MgCl2, 200 µl of this mixture was added to 20 µl of the cell lysate. After 30 min incubation at room temperature, 300 µl of light emission accelerator (Tropix) was added. For the luciferase assay, 100 µl of luciferase reagent (Promega) was added to 20 µl of the cell lysate immediately before measurement. Measurements were performed on a Lumat LB 9507 luminometer for 10 sec. In C2 cells luciferase values were corrected for transfection efficiencies using the β-galactosidase values. In T-REx cells, β-galactosidase values were corrected for transfection efficiencies using the luciferase values of non-heat shocked cells transfected in parallel.

Constructs

The two reporter constructs (Fig. 1) were made in the pGL3 basic vector (Promega). For the pHsp-Cap-Luc, the D. melanogaster Hsp70 promoter was excised from the

pBN247 construct (42) using HindIII blunt/ SalI and cloned in front of the luciferase gene using SmaI and XhoI sites from the vector. For the pHsp-IRES-Luc construct the rat FGF-2 IRES (532 bp) was excised from pRObFGF503 (43) using HindIII/NcoI and inserted between the Hsp70 promoter and the luciferase reporter gene. Rat αB-crystallin cDNA was cloned NcoI-XhoI/SalI into β-actin vector (44). The human αB-crystallin coding sequence (kindly provided by P. Muchowski, University of Washington, Seattle) and phosphorylation mutants, made by site directed mutagenesis (Stratagene), were cloned into the pIRESneo vector (Clontech). The constructs pSV HaHsp27wt and phosphorylation mutants SA, AS, AA and EE were a kind gift from J. Landry (15). The Hsp70 clone was constructed by PCR on mRNA isolated from heat shocked HeLa cells. The primers used were at position +1 (CAAGCTAACGGCTAGCCTGAGGAGC) and at position +2390 (AAGGCCCCTAAT CTACCTCCTCAATGGTGGG) of the Hsp70 mRNA. PCR fragments were cloned into pGEM-T-Easy vector (Promega), and after sequencing, the Hsp70 coding sequence was cloned into the β-actin vector using SphI blunt/SalI for the insert and NcoI blunt/ SalI for the vector. The rat βB2-crystallin expression construct was described previously (45).

Results

αB-crystallin protects cap-dependent but not cap-independent translation

Overall translation in C2 cells (mouse myoblasts) is severely inhibited after a 30 min heat shock at 45°C and does not recover within seven h at 37°C (Fig. 2). Accumulation of the endogenous small heat shock proteins Hsp27 and αB-crystallin is first detectable after 3 h, the level of Hsp27 reaches a steady state after about 4.5 h while the level of αB-crystallin continues to increase up to 7.5 h (Fig. 2). Hence, even though general protein synthesis is inhibited, special mRNAs, such as those encoding heat shock proteins, are still translated under these conditions.

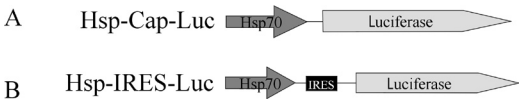

Fig 1. Reporter constructs to test translation efficiency after heat shock.
A: To test for cap-dependent translation after heat shock, the pHsp-Cap-Luc construct was made. This construct contains the luciferase gene of the pGL3 basic vector with the *D. melanogaster* Hsp70 promoter driving expression of the reporter gene. B: In the pHsp-IRES-Luc the 5' non-coding region of the pHsp-Cap-Luc is replaced by the 532 bp long 5' non-coding region of the FGF-2 mRNA. This region contains an IRES known to be used during stress (46). This construct thus reflects IRES dependent translation after stress.

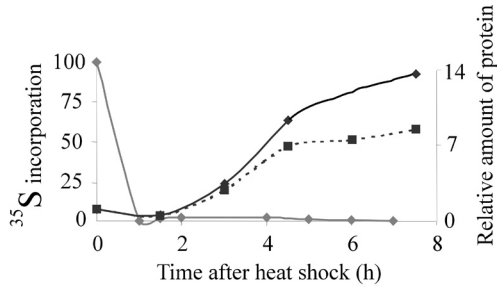

Fig 2. Effects of heat shock on translation in C2 cells.
Protein synthesis was measured after a 30 min 45°C heat shock as described in Experimental Procedures. The level of protein synthesis is expressed relative to synthesis before heat shock (*solid line* with *gray diamonds*). In a separate experiment, the endogenous levels of αB-crystallin (*solid line* with *black diamonds*) and Hsp27 (*dotted line* with *black squares*) were determined by analyzing heat shocked cells during recovery at 37°C, as described in Experimental Procedures. Levels of αB-crystallin and Hsp27 are expressed relative to their level before heat shock.

To determine whether prior expression of a small heat shock protein provides a measure of translational thermotolerance under these conditions, C2 cells were transfected with an expression construct for αB-crystallin together with constructs designed to report translation efficiency after heat shock (Fig. 1). Two different constructs were used. In one, pHsp-Cap-Luc, the luciferase coding region is placed under control of the Hsp70 promoter without altering the 5' non-coding region of the pGL3 basic vector; in the second, pHsp-IRES-Luc, the 5' non-coding region of the FGF-2 mRNA was inserted between the Hsp70 promoter and the luciferase coding region. Both reporter gene constructs are driven by the Hsp70 promoter and the reporter mRNA should thus accumulate only after a heat shock; to decrease background resulting from possible promoter leakage prior to the heat shock, luciferase was used as the reporter. This enzyme is heat labile and inactivated by the heat shock. The only difference between the mRNAs encoded by these two reporter constructs is in the 5' non-coding region. The pHsp-Cap-Luc mRNA has a short, non-structured 5' non-coding region and is predicted to be initiated via the default mechanism for translation initiation, namely cap-dependent translation initiation. The pHsp-IRES-Luc mRNA contains the FGF-2 internal ribosome entry site (IRES) known to be used during stress (46) and should be initiated via a cap-independent mechanism. In support of this proposed difference in the mechanism of translation initiation, we found that the luciferase expression from the pHsp-Cap-Luc construct is inhibited by rapamycin, while that from the pHsp-IRES-Luc is not (Fig. 3 and data not shown). Rapamycin has been reported to inhibit cap-dependent translation initiation but not cap-independent translation initiation (47).

During recovery from a heat shock the luciferase yield from the IRES-Luc mRNA was higher than that from the Cap-Luc mRNA. Overexpression of αB-crystallin significantly stimulated the luciferase expression from the Cap-Luc mRNA but it did not affect that from the IRES-Luc mRNA (Fig. 3). As the luciferase mRNA levels did not differ between control cells and cells overexpressing αB-crystallin (Fig. 3B and

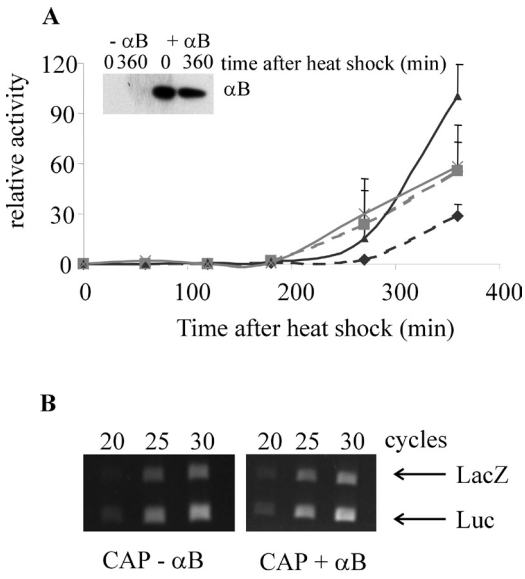

Fig 3. Expression of the cap- and IRES- dependent reporter constructs after heat shock and protection by αB-crystallin during recovery.
C2 cells were transfected with pHsp-Cap-Luc (*dotted line* with *black diamonds*), or with pHsp-IRES-Luc (*dotted line* with *black squares*) and empty expression vector or co-transfected with the αB-crystallin expression construct and the pHsp-Cap-Luc (*solid line* with *black triangles*) or pHsp-IRES-Luc (*solid line* with *Xs*) constructs. A: Reporter gene activity was measured at several time points during recovery at 37°C. Values shown are relative to those obtained in cells transfected with pHsp-Cap-Luc and the αB-crystallin expression construct after 360 min of recovery, error bars indicate the standard deviation. The insert shows the western blot for αB-crystallin after co-transfection with pHsp-Cap-Luc and empty vector (- αB) or the αB-crystallin expression vector (+ αB). B: RT-PCR (after 20, 25 and 30 cycles) of luciferase mRNA (Luc) and β-galactosidase mRNA (LacZ) after transfection of pHsp-Cap-Luc with (CAP + αB) or without (CAP - αB) the αB-crystallin expression construct. Cells were harvested 6 h after heat shock. Note that the β-galactosidase mRNA level represents the control for transfection efficiency as well as for the efficiency of the RT-PCR reaction.

data not shown), the increase in luciferase yield from the Cap-Luc mRNA must be due to an increase in the rate of translation.

The protective effect of αB-crystallin on the rate of translation of the Cap-Luc mRNA was dependent upon the severity of the heat shock: the level of protection was similar during recovery from a 30 or 45 min heat shock at 45°C but decreased during recovery from a longer or more severe heat shock (Fig. 4). In subsequent experiments with C2 cells a 30 min heat shock at 45°C was used (note that cell lines differ in their resistance to heat stress, to obtain comparable data with HeLa cells, the HeLa cells need to be exposed to 45°C for one h, data not shown).

The level of protection by αB-crystallin is dependent upon the amount of αB-crystallin. When more of the αB-crystallin expression construct was co-transfected, the amount of activity obtained from the pHsp-Cap-Luc reporter increased (Fig. 5). Due to the fact that there is a maximum to the amount of DNA that can be transfected,

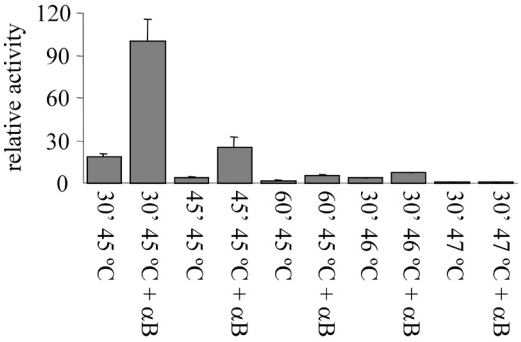

Figure 4. Translational thermotolerance provided by αB-crystallin depends on the severity of the heat shock.
C2 cells were transfected with the pHsp-Cap-Luc and the αB-crystallin expression construct or the empty vector. Forty-eight h after transfection, cells were subjected to different heat shocks: 30, 45 and 60 min at 45°C or 30 min at 46°C or 47°C. Six h after recovery at 37°C cells were harvested and assayed for reporter gene activity. Values shown are relative to those obtained in cells transfected with pHsp-Cap-Luc and the αB-crystallin expression construct after a 30 min heat shock at 45°C and 6 h of recovery at 37°C, error bars indicate the standard deviation.

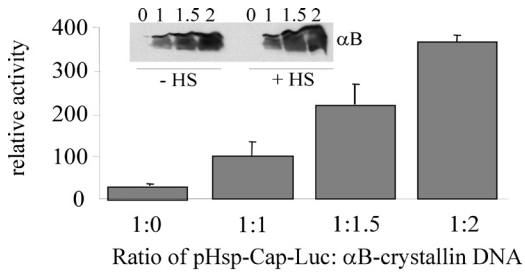

Fig 5. Dependency of protection on the level of αB-crystallin.
Different ratios of the pHsp-Cap-Luc and the αB-crystallin expression constructs were transfected. The total amount of DNA transfected was kept constant by adjusting for the amount of αB-crystallin expression construct with the empty vector. Cells were harvested after 6 h of recovery and assayed for reporter gene activity. Activities are expressed relative to the activity obtained after transfecting a 1:1 ratio of pHsp-Cap-Luc and αB-crystallin expression construct, error bars indicate the standard deviation. The insert shows the western blot for αB-crystallin in non-heat shocked (-HS) and in heat shocked cells (+ HS) transfected with corresponding ratios of the pHsp-Cap-Luc and the αB-expression constructs.

it was not possible to increase the amount of the co-transfected αB-crystallin expression construct above that shown in Fig. 5, so it is not known what the maximal level of protection provided by αB-crystallin under these conditions is.

Translation thermotolerance provided by Hsp70 and Hsp27

Hsp70 and Hsp27 have also been reported to provide cells with translational thermotolerance (21,27,48). We therefore tested whether expression of these two heat shock proteins also increased the rate of translation of the Cap-Luc mRNA during recovery from a heat shock. As shown in Fig. 6, the results obtained after overexpression of Hsp27 are very similar to those obtained after overexpression of αB-crystallin as shown above: Hsp27 did not affect the level of mRNA from either the pHsp-Cap-Luc or pHsp-IRES-Luc contruct (Fig. 6B and data not shown) but in the presence of Hsp27 the yield from the Cap-Luc mRNA is increased

while that from the IRES-Luc mRNA stayed the same. In contrast, in the presence of Hsp70 the yield from both the Cap-Luc and the IRES-Luc mRNAs is increased. As control, an expression construct for βB2-crystallin, a lens structural protein, was co-transfected. In the presence of βB2-crystallin neither Cap-Luc nor IRES-Luc mRNA yielded more luciferase. Protection is thus specific for Hsps. Although the two sHsps as well as the large Hsp provide translational thermotolerance, the protection provided by the sHsps is more limited and apparently directed at a step required for cap-dependent initiation only.

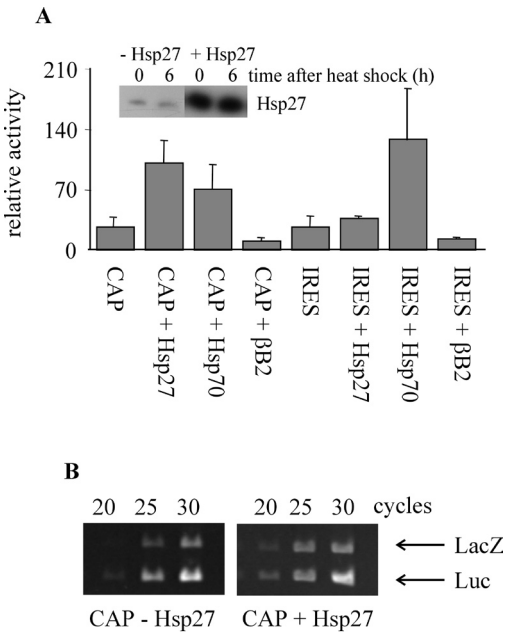

Fig 6. The effect of overexpression of Hsp27, Hsp70 and βB2-crystallin on protection of translation after heat shock.

A: C2 cells were transfected with the pHsp-Cap-Luc or pHsp-IRES-Luc together with Hsp27, Hsp70 or βB2 expression constructs or their empty vector. Forty-eight h after transfection, cells were heat shocked and harvested after 6 h of recovery at 37°C. Bars show the values relative to those obtained in cells transfected with pHsp-Cap-Luc and the Hsp27 expression construct, error bars indicate the standard deviation. The insert shows the western blot for Hsp27 in cells co-transfected with pHsp-Cap-Luc and empty vector (- Hsp27) or the Hsp27 expression construct (+ Hsp27). Data shown are from the same western blot, intermediate lanes were removed for clarity's sake.B: RT-PCR (after 20, 25 and 30 cycles) of luciferase mRNA (Luc) and β-galactosidase mRNA (LacZ) after transfection of pHsp-Cap-Luc with (CAP + Hsp27) or without (CAP - Hsp27) the Hsp27 expression construct. Cells were harvested 6 h after heat shock. Note that the β-galactosidase mRNA level represents the control for transfection efficiency as well as for the efficiency of the RT-PCR reaction.

The role of phosphorylation

Hsp27 and αB-crystallin are phosphorylated during stress. For Hsp27 the extent of phosphorylation is high (5,14) and phosphorylation is required for thermotolerance (19,49,50). For αB-crystallin the extent of phosphorylation is low (6,13) and the role of phosphorylation in thermotolerance is not known. To test whether the phosphorylation state of αB-crystallin or Hsp27 influences their ability to protect the translation of the Cap-Luc mRNA, constructs expressing mutants mimicking non-phosphorylated (αB S45A and S19,45,59A and Hsp27 AS, SA and AA) or constitutive phosphorylated (αB S19D, S45D, S59D and S19,45,59D and Hsp27 EE) αB-crystallin or Hsp27 were co-transfected with pHsp-Cap-Luc. As shown in Fig. 7A, mimicking constitutive phosphorylation of αB-crystallin abolishes the protection of translation, irrespective of the site at which phosphorylation is mimicked. The non-phosphorylatable mutants of αB-crystallin, however, are more active in our translation protection assay. In contrast, the non-phosphorylatable mutant of Hsp27 (Hsp27 AA) was inactive, while the mutant mimicking full phosphorylation (Hsp27 EE) was as active as wild type. The mutant in which the most 5' phosphorylation site was mutated (Hsp27 AS) was as active as wild type, while the mutant in which the second phosphorylation site was mutated (Hsp27 SA) was slightly less active than wild type (Fig. 7B). These data show that for full activity in translation thermotolerance, Hsp27 needs to be phosphorylated while αB-crystallin must be dephosphorylated.

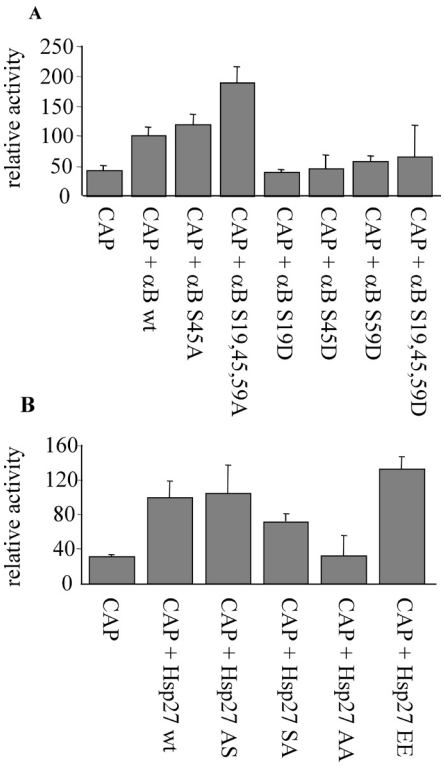

Fig 7. Effect of phosphorylation of αB-crystallin or Hsp27 on protection of Cap-dependent translation after heat shock.
A: Mutants mimicking phosphorylation of αB-crystallin are indicated as αB S19D, S45D, S59D and S19,45,59D, non-phosphorylatable αB-crystallin mutants as S45A and S19,45,59A, where the number indicates the amino acid residue mutated. Expression constructs of the mutants were co-transfected with pHsp-Cap-Luc. B: Three mutants mimicking (partially) unphosphorylated Hsp27 (Hsp27AS, Hsp27 SA and Hsp27 AA) or one mimicking phosphorylation (Hsp27EE) were co-transfected with the pHsp-Cap-Luc construct. Cells were heat shocked and harvested after 6 h of recovery. Bars show the activities in the presence of the mutant proteins relative to activity of pHsp-Cap-Luc in the presence of overexpressed wild type αB-crystallin or Hsp27 respectively. Error bars indicate the standard deviation.

Thermotolerance of translation of mRNA synthesized from a non-heat shock promoter

In the experiments described above we cannot give a quantitative measure of the extent of translational thermotolerance provided by αB-crystallin or Hsp27 as we have no way of determining what the yield of luciferase would be if translation were not inhibited since we used a heat shock promoter. Nor can we determine whether the extent of translational thermotolerance obtained when αB-crystallin or Hsp27 is overexpressed is similar to that seen in thermotolerant cells, as the use of the Hsp promoter and thus the expression of pHsp-Cap-Luc or pHsp-IRES-Luc may differ between naive and thermotolerant cells. To have at least some measure of the relative translational thermotolerance, we turned to T-REx HeLa cells, HeLa cells stably expressing the tetracycline repressor. In these cells we induced expression of a LacZ construct, placed under control of the tetracycline repressor, for three h to deliver a burst of β-galactosidase mRNA, we then washed away the inducer and 30 min later, challenged the cells with a heat shock of one h at 45°C. The rate of increase in β-galactosidase activity during recovery at 37°C was then measured. As shown in Fig. 8A in the presence of co-transfected αB-crystallin or Hsp27 the rate of accumulation of β-galactosidase was significantly higher than in cells co-transfected with an empty vector. As mRNA levels did not differ significantly (data not shown), this effect must be due to an increase in translation, i.e. translational thermotolerance provided by the small heat shock proteins. [Note that in this system the protective effect of αB-crystallin and Hsp27 persists for at least 20 h (Fig. 8A). In C2 cells, using the pHsp-Cap-Luc reporter described above, luciferase values in control cells had caught up with those in αB-crystallin expressing cells after 20 h of recovery. This difference is most likely due to differences in the stability of the reporter enzymes luciferase versus β-galactosidase and of their cognate mRNAs.]

The extent of translation of the β-galactosidase mRNA during recovery from a heat shock was also measured in thermotolerant cells, i.e. cells recovering from a mild prior heat shock. The rate of increase in β-galactosidase activity in these cells (preHS + HS) was not significantly different from that in cells overexpressing αB-crystallin alone (αB + HS). In the presence of αB-crystallin or after a pre-heat shock the translational activity was about 30% of that found in control, non-heat shocked, cells (-HS, Fig. 8B).

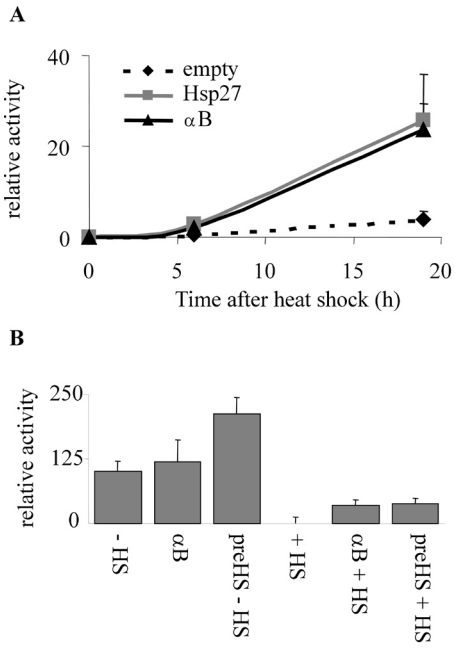

Fig 8. Thermotolerance of the translation of mRNA synthesized from a non-heat shock promoter.
A: T-REx cells were co-transfected with pcDNA4TO LacZ and αB-crystallin or Hsp27 expression constructs or their empty vectors. Cells were heat shocked for 1 h at 45°C and harvested during recovery at 37°C. Values shown are relative to those obtained in cells co-transfected with pcDNA4TO LacZ and the empty expression vector after 6 h of recovery. B: T-REx cells were co-transfected with the pcDNA4TO LacZ construct and the αB-crystallin expression construct or the empty vector. Where indicated (preHS) cells were subjected to a pre-heat shock (30 min 45°C) 6 h before heat shock. After heat shock (1 hr 45°C) cells were harvested after 6 h of recovery at 37°C. Activities shown here are relative to activities found in non-heat shocked cells transfected in parallel and are the average of two experiments, error bars indicate the standard deviation.

The kinase inhibitor 2-aminopurine blocks translational thermotolerance

The data presented above suggest that both αB-crystallin and Hsp27 are capable of conferring translational thermotolerance equivalent to that found in thermotolerant cells. However, it must be remembered that during the heat shock applied to measure thermotolerance, the synthesis of endogenous heat shock proteins is also activated. It is therefore possible that the translational thermotolerance provided by one of these two small heat shock proteins requires the cooperation of endogenously synthesized heat shock proteins. To test this, we sought a means of inhibiting the synthesis of endogenous heat shock proteins. It has previous been shown that 2-aminopurine inhibits the synthesis of Hsp70 (51), a finding in agreement with the fact that one of the kinases inhibited by 2-aminopurine, PKR, is required for the heat shock response (52,53). We therefore tested the effect of 2-aminopurine. As 2-aminopurine inhibits eIF2α kinases (40,41), and as phosphorylation of eIF2α is thought to be at least partially responsible for inhibition of protein initiation during stress (31,32), treatment with 2-aminopurine should result in an increase in the rate of β-galactosidase synthesis if the synthesis of endogenous Hsps is not required for the sHsp translational tolerance. As expected, in control cells, 2-aminopurine enhanced the rate of accumulation of β-galactosidase presumably due to dephosphorylation of eIF2α (Fig. 9A). Cells recovering from a heat shock are known to have a higher rate of translation than control cells, an effect that has been attributed to inhibition of PKR (54). However, when 2-aminopurine was added to cells which had been allowed to recover for 6 h from a mild heat shock, an

inhibition of translation was found (Fig. 9A). In these cells the level of Hsp70 was still increasing and this increase is inhibited by 2-aminopurine (fig. 9B).

When 2-aminopurine was added just before a heat shock and remained present during recovery, β-galactosidase synthesis was blocked completely, irrespective of the presence of αB-crystallin (Fig. 10A). In these cells 2-aminopurine blocked the further accumulation of both Hsp70 and Hsp27, confirming that 2-aminopurine blocks the heat shock response (Fig. 10B). These results strongly suggest that the synthesis of the endogenous heat shock proteins is required for the translational thermotolerance seen in cells overexpressing αB-crystallin.

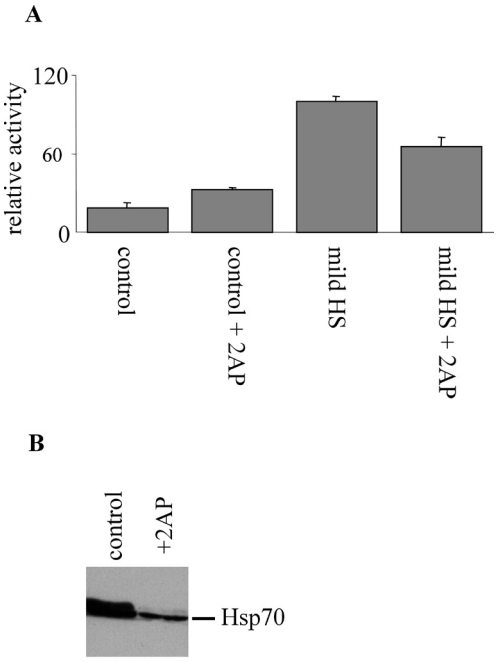

Figure 9. Effect of 2-aminopurine on translation.
A: T-REx cells were transfected with pcDNA4TO LacZ and where indicated (mild HS) subjected to a heat shock for 30 min at 45°C. 3 h after the heat shock LacZ was induced for 3 h, 2-aminopurine (2-AP) was added 6 h after the heat shock and cells were harvested 6 h later. Bars show the values relative to those obtained in cells subjected to a mild heat shock, error bars indicate the standard deviation. B: Equal amounts of extracts from control cells and from cells treated with 2-aminopurine (2-AP) were assayed on a Western blot for Hsp70 expression.

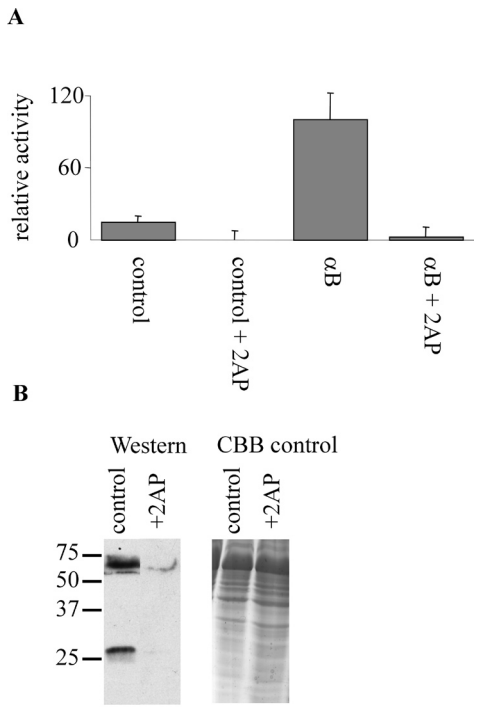

Figure 10. Effect of 2-aminopurine on the protection of translation by αB-crystallin.
A: T-REx cells were transfected with pcDNA4TO LacZ and αB-crystallin expression construct or the empty vector. 2-aminopurine was added directly before the heat shock and cells were harvested after 6 h of recovery at 37°C. Bars show the activities relative to those found in αB crystallin overexpressing cells in the absence of 2-aminopurine, error bars indicate standard deviations. B: Control heat shocked cells (control) and heat shocked cells treated with 2-aminopurine (2-AP) were harvested after 6 h of recovery at 37°C and extracts were assayed on Western blot for Hsp70 and Hsp27 expression levels. A Coomassie blue stained gel of the same samples is shown as the loading control.

Discussion

We have shown here that, during recovery from a heat shock, overexpression of αB-crystallin or Hsp27 increases expression from a luciferase reporter gene producing a cap-dependent transcript but not from a luciferase reporter gene producing a cap-independent transcript, while overexpression of Hsp70 increases expression from both constructs. For sHsps it has been shown that they cannot refold luciferase under these conditions (55)

and their effect must thus be on de novo synthesis. The effect of Hsp70 could in principle be due to refolding of previously synthesized luciferase. However, the leakage of the Hsp70 promoter used in these reporter constructs is very low as the level of luciferase in non heat shocked cells is only about 10 % of that found in cells after recovery from a heat shock in the absence of an overexpressed Hsp (data not shown). The increase in luciferase activity in the presence of Hsp70 must thus also be due to de novo synthesis. The Hsp70 isoform Hsp72 has been shown to be associated with polysomes after a heat shock (27) and the effect of Hsp70 could well be on elongation as well as on initiation. In the case of sHsps, only cap-dependent synthesis increases, making it very unlikely that these proteins affect elongation. The most likely interpretation of our data is that sHsps protect one of the steps in cap-dependent initiation. This suggestion seems at first glance to be in contrast to the data of Cuesta et al., who showed that Hsp27 forms an insoluble complex with eIF4G (38). However, the experiments reported by Cuesta et al. were performed at the heat shock temperature, while we examined the effect of Hsp27 in cells recovering from a heat shock. Our data, combined with the data of Cuesta et al., would suggest a model in which Hsp27 (or αB-crystallin) interacts with and stabilizes eIF4G during the heat shock, resulting in increased availability of eIF4G during recovery from a heat shock. Such a model is attractive, as it would also be in accordance with the presumed role of the sHsps as chaperones of non-native proteins. For αB-crystallin there is indeed a correlation between protective activity and chaperone activity, as for this protein phosphorylation abolishes its ability to confer translational thermotolerance and phosphorylation also decreases its chaperone activity (17,18). However, we find that mimicking phosphorylation of Hsp27 does not affect its ability to provide translational thermotolerance, yet it has been clearly shown that such phosphorylation mutants lack in vitro chaperone activity (16). Conversely, mutants of Hsp27 that cannot be phosphorylated retain full chaperone activity but are inactive in our translation tolerance assay. Thus for Hsp27 there is no correlation between chaperone activity and translational thermotolerance. Either αB-crystallin and Hsp27 differ in the mechanism by which they confer translational thermotolerance - they could for example target different components of the cap-binding complex - or, if their mechanism of action is the same, then the establishment of translational tolerance by sHsps does not require chaperone activity.

The heat shock response is elicited by proteotoxic stress. The nature of the cellular damage sensors is still unclear but one recently elucidated mechanism involves the activation of the dsRNA activated protein kinase PKR through activation of RAX/PACT (56,57). In the absence of PKR, Hsp70 is not induced (53). Hsp70 induction is also blocked by 2-aminopurine (ref 51 and Fig. 10B), an effect possibly mediated by inhibition of PKR. To our surprise we found that treatment with 2-aminopurine abolished the protective effect of overexpressing αB-crystallin. The most likely explanation is that the protective effect of αB-crystallin requires the synthesis of endogenous heat shock proteins. Translational thermotolerance would then be a cooperative effect of different heat shock proteins. Present studies are directed at determining which heat shock proteins are required in addition to αB-crystallin or Hsp27.

The translational response to a heat shock is complex. Initially translation is strongly inhibited, while upon recovery from a heat shock translation is enhanced (see for example Fig. 9A). PKR has been suggested to be the controlling factor in both effects: its activation upon stress would cause phosphorylation of eIF2α and thus inhibition of translation initiation; its inhibition during recovery would lead to dephosphorylation of eIF2α and thus to enhanced translation initiation. The presumed inhibition of PKR during heat shock recovery has been suggested to be due to enhanced transcription of Alu repeats and inhibition of PKR by those transcripts (54,58). Indeed, this mechanism has been suggested to be an evolutionary advantage of maintaining SINEs (59). Our, and other, data strongly call this interpretation into question. Activation of PKR is required for synthesis of heat shock proteins and thus for the recovery of the cells; inhibition of PKR during the heat shock and during recovery from the heat shock blocks rather than stimulates protein synthesis. The mechanism of the stimulation of protein synthesis in recovering cells thus remains obscure. If dephosphorylation of eIF2α is involved, then in recovering cells, eIF2α is not a PKR substrate. Alternatively GADD34 upregulation in stressed cells might result in a higher rate of dephosphorylation of eIF2α than phosphorylation by PKR (60).

Hsp27 and αB-crystallin are constitutively expressed in a number of tissues. Our finding that these proteins provide these tissues with translational thermotolerance, albeit in cooperation with other heat shock proteins, is thus of physiological importance. We have further shown that the translational thermotolerance provided by the sHsps is specific for cap-dependent mRNAs. The sHsps thus can shift the translation pattern by maintaining translation of cap-dependent mRNAs during stress. It is becoming more and more apparent that shifts in the activity of general initiation factors have a major effect on the translational efficiency of specific genes (32). It would be of considerable interest to determine whether there is also a subtle interaction between sHsps and the translation initiation in the absence of stress. The level of sHsps could then be one of the determinants of the translational pattern of a cell.

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How does αB-crystallin protect cap-dependent translation after heat shock?

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Chapter 6

How does αB-crystallin protect cap-dependent translation after heat shock?

Abstract. Overexpression of αB-crystallin or Hsp27 has been shown to protect cap-dependent but not cap-independent translation initiation against heat stress. We show here that overexpression of αB-crystallin targeted to the nucleus also protects cap-dependent translation initiation. This protection might be a result of the chaperoning by αB-crystallin of factors that would otherwise be inactivated by a heat shock. To test whether reduced availability of such factors is the cause for inhibition of cap-dependent translation, we tested whether mere overexpression of translation factors eIF4E, eIF4G or PABP and of the two nuclear cap-binding proteins CBP80 and/or CBP20 could increase translation during recovery from a heat shock. Furthermore we tested whether overexpression of these factors could increase the protection mediated by overexpression of αB-crystallin. Neither overexpression of eIF4E, eIF4G, PABP nor of CBP80 and/or 20 showed an increase in translation after heat shock; overexpression of eIF4E, eIF4G and CBP20 even tended to decrease translation after heat shock. The inhibition by eIF4E and CBP20 could be at least partially reversed by αB-crystallin while the inhibition by eIF4G could not.

Inhibition of translation initiation results in the accumulation in the cytoplasm of phase dense granules, the stress granules (SGs), containing almost all components of the 48S pre-initiation complex. We show here that the inclusion of EGFP-eIF4E in SGs is not prevented by αB-crystallin but that αB-crystallin does increase the mobility of cytoplasmic EGFP-eIF4E and promotes the disappearance of aggregates containing this protein.

Introduction

Cells in stress downregulate protein synthesis using various strategies. A common factor in various types of stress is the activation of eIF2α kinases. Phosphorylation of eIF2α inhibits eIF2B, the guanine nucleotide exchange factor responsible for regenerating eIF2-GTP. This results in a limited availability of eIF2-GTP-tRNA-Met and thus a reduced rate of translation initiation on all messengers (Pain 1996; Brostrom and Brostrom 1998). In addition, the eIF4E binding proteins are dephosphorylated and sequester eIF4E, thereby inhibiting the cap-binding complex (Vries et al. 1997; Patel et al. 2002). The reduced availability of eIF4E in stressed cells could explain why translation initiation via an internal ribosome entry site (IRES) is more resistant to inhibition by stress than cap-dependent translation initiation. Finally, during a heat shock, eIF4G, the scaffold protein, has been shown to be inhibited by interaction with Hsp27 (Cuesta et al. 2000). As eIF4G is required for both IRES and cap-dependent translation initiation, the inhibition of this factor would inhibit all translation initiation. The fate of the polyA binding protein (PABP) during stress has not been examined. This protein contributes to the efficiency of translation initiation by mediating circularization of the mRNA. It has recently been shown that PABP is cleaved during apoptosis, as is eIF4G (Gingras, Raught, and Sonenberg 1999; Bushell et al. 2000; Marissen et al. 2004), and is thus also a target for regulation.

Inhibition of translation initiation results in the accumulation in the cytoplasm of phase dense granules, the stress granules (SGs). In these SGs stalled translation initiation complexes containing almost all components of the 48S pre-initiation complex, but not the 60S ribosomal subunit, are found. A number of other proteins are also localized in the SGs. For example, the RNA-binding proteins TIA-1 and TIAR as well as the endoribonuclease G3BP are found in SGs. These proteins appear to play a role in the formation of SGs (Kedersha et al. 1999; Kedersha et al. 2000; Tourriere et al. 2003).

The assembly of SGs is a highly dynamic process; untranslated mRNAs are thought to be sorted and processed in SGs for either reinitiation, degradation or packaging into nonpolysomal mRNP complexes (Kedersha et al. 2000). The fact that proteins that stabilize (HuR) or destabilize (tristetraprolin) mRNA molecules have been found in SGs as well supports this theory. Accumulation of proteins and mRNA in SGs is irreversible when cells are lethally stressed, but SG formation is reversible in cells recovering from sublethal stresses (Kedersha et al. 1999; Kedersha et al. 2000). To disaggregate SGs, Hsp70 and ATP are necessary (Anderson and Kedersha 2002). Small heat shock proteins may also play a role as at least Hsp27 has been detected in SGs (Nover, Scharf, and Neumann 1983; Nover and Scharf 1984; Kedersha et al. 2000).

We have previously shown that the sHsps Hsp27 and αB-crystallin protect cap-dependent but not cap-independent translation initiation against a heat stress (Doerwald et al. 2003). The mechanism of this protection is unknown. It could involve assembly and disassembly of the SGs; it could also be due to protection of one or more components of the translation initiation complex. The first possibility is indicated by the localization of sHsps in SGs; the second possibility would be in line with the known chaperoning activity of the sHsps. A complicating factor is that when cells are heat shocked or stressed with arsenite, both αB-crystallin and Hsp27 translocate to the nucleus (Geum, Son, and Kim 2002 and chapter 4 of this thesis). Thus, the protective effect of the sHsps could be exerted in the nucleus rather than the cytoplasm. If so, this still allows the possibility that the sHsps protect translation initiation factors as eIF4E and eIF4G have been reported to traffic to the nucleus as well (Dostie et al. 2000; Dostie, Lejbkowicz, and Sonenberg 2000; McKendrick et al. 2001). It, however, extends the possibility of protection to nuclear proteins. With respect to translation initiation, obvious candidates would be the two nuclear cap-binding proteins CBP80 and CBP20. In the nucleus these two cap-binding proteins form a nuclear cap-binding complex (CBC) that is known to be important for nuclear export of mRNA (Izaurralde et al. 1995). In the cytoplasm the CBC is replaced by eIF4E via an unknown mechanism (Fortes et al. 2000). Ishigaki et al. (2001) suggested that the CBC could be used in the first round of translation after which it would be replaced by eIF4E. In vitro CBC can replace eIF4E in initiation of translation although with lesser activity than eIF4E (Fortes et al. 2000) and it could possibly do so in vivo as well.

Here we have tested whether overexpression of eIF4E, eIF4G, CBP20, CBP80, CBP20 + CBP80, or PABP can rescue translation initiation in heat shocked cells in the absence of αB-crystallin. We have also tested whether overexpression of these factors enhances the protective effect of αB-crystallin. The idea behind these

experiments was that if the inhibition of cap-dependent translation is due to the lack of a factor, then overexpression of that factor could rescue this process. Additionally or alternatively, if the protection by αB-crystallin is due to chaperoning of a factor, then the increased availability of that factor should lead to a higher level of protection by αB-crystallin. We have also looked at the effect of αB-crystallin on the formation and dynamic nature of SGs, using EGFP-eIF4E as a marker. We show here that SG formation is not prevented by αB-crystallin but that αB-crystallin does increase the cytoplasmic mobility of EGFP-4E and promotes the disappearance of aggregates containing this protein.

Materials and methods

Cell culture

C2 cells (mouse myoblast cells) were cultured in Dulbecco's modified Eagle's medium (Gibco) with penicillin and streptomycin (Roche) and supplemented with 20% fetal calf serum (PAA laboratories) to prevent differentiation of these cells. T-REx αB cells [T-REx HeLa cells (Invitrogen) stably transfected with an expression construct for αB-crystallin (pcDNA4-TO-αB) under the control of the tetracycline repressor] were cultured in Minimum essential medium Eagle (Biowhittaker) with glutamax (Gibco), 10% fetal calf serum, penicillin, streptomycin and blasticydin (Invitrogen).

Transfections

C2 cells were transfected using lipofectAMINE plus (Invitrogen). Approximately $6.5x10^4$ cells were plated in Dulbecco's modified Eagle's medium with penicillin and streptomycin and 10% foetal calf serum in 6-well plates. After 24 h cells were transfected with a total of 1 μg DNA per well using 4 μl lipofectamine and 6 μl plus reagent. As a transfection control 0.1 μg of CMV-β-galactosidase was co-transfected; the other 0.9 μg of DNA was divided over the various constructs, pHsp-Cap-Luc or pHsp-IRES-Luc (0.2 μg) and expression vectors for αB-crystallin or Hsp27 (0.35 μg), for eIF4E, eIF4G or PABP (0.35 μg) or CBP80 + CBP20 (0.175 μg each) or the empty vectors as control. Forty-eight h after transfection cells were heat shocked for 30 min at 45°C and assayed for reporter gene activity during recovery at 37°C. Transfections were done in triplicate and repeated at least twice. Unless otherwise indicated results of representative experiments are shown.

For EGFP-eIF4E localization experiments C2 cells were transfected with 0.5 μg EGFP-eIF4E and 0.5 μg αB-crystallin expression construct or its empty vector per dish using lipofectAMINE. These cells were heat-shocked for 30 min at 45°C 48 h after transfection and fixed with paraformaldehyde either immediately after heat shock or during recovery at 37°C. Pictures were taken using a Zeiss Axiovert 135 TV microscope.

For the fluorescence recovery after photobleaching (FRAP) experiments, approximately $2.5x10^5$ T-REx -αB cells were plated in Minimum essential medium Eagle with glutamax, 10% fetal calf serum, penicillin and streptomycin in 10 mm glass dishes (HBST 3522; Willco Wells) and transfected after 24 h with 0.2 μg pEGFP-eIF4E DNA and 0.8 μg of pBluescript DNA as carrier using 3 μl of Fugene reagent (Roche) per dish. Where indicated, after 24 h 1 μg/ml doxycyclin was added to the medium for 24 h to induce αB-crystallin expression before the heat shock (1 h at 45°C).

Reporter assays

Cells were harvested by vigorously shacking in 200 µl reporter lysis mix (25 mM Bicine pH 7.5, 0.05% Tween-20 and 0.05% Tween-80) per well. For the β-galactosidase assay, galacton (Tropix) was diluted 1:100 in 100 mM phosphate buffer pH 8.1, 5 mM MgCl2, of this dilution 200 µl was added to 20 µl of the cell lysate. After 30 minutes incubation at room temperature, 300 µl of light emission accelerator (Tropix) was added. For the luciferase assay also 20 µl of the cell lysate was used. Immediately before measurement, 100 µl of luciferase reagent (Promega) was added to the lysate. Measurements were performed on a Lumat LB 9507 luminometer for 10 seconds.

Calculation of results

In all experiments reported here the β-galactosidase reporter gene driven by the CMV promoter was used as transfection control. Usually values are corrected for the transfection efficiency (luciferase activity divided by the β-galactoside activity) and expressed as percent of the yield of the luciferase reporter in the control experiment as indicated in the figure legend. In some cases we found that overexpression of a protein significantly enhanced the yield of the β-galactosidase reporter, suggesting that that protein enhanced the level of expression of β-galactosidase during the culture period prior to the heat shock. In those cases the β-galactosidase and luciferase activities are both shown.

Stress granule formation

C2 or T-REx -αB cells were transfected with the constructs indicated as described above. Where indicated, after 24 h 1 µg/ml doxycyclin was added to the medium of the T-REx-αB cells for 24 h to induce αB-crystallin expression before the heat shock (1 h at 45°C). Cells were either fixed at various times after heat shock and recovery and photographed using a Zeiss Axiovert 135 TV microscope or living cells were photographed directly after heat shock and during recovery using a Zeiss LSM510 meta microscope. FRAP experiments were performed on these cells during recovery at 37 °C using a Zeiss LSM510 meta microscope. Excitation wave length was 488 nm and the Band Pass filter was 500-550 nm. The region of interest was bleached for 4s using the 488 laser line at maximal intensity and recovery of fluorescence was measured during 200s after bleaching.

Staining for αB-crystallin was performed on cells fixed with 3% paraformaldehyde in PBS and then permeabilised with 0.2% Triton X-100, using a monoclonal antibody (raised against the complete chicken αB-crystallin; Riken Cell Bank nr. 1304) and a TRITC labeled secondary antibody (DAKO A/S, Copenhagen, Denmark).

Constructs

The pHsp-Cap-Luc, the pHsp-IRES-Luc, the αB-crystallin expression construct, the Hsp27 expression construct and the αBnls expression construct have been described previously (Doerwald et al. 2003; chapter 4). The pAD4G vector was a kind gift of R.E. Rhoads. The PQE30 CBP80 and pRSETB CBP20 prokaryotic expression vectors were a kind gift of M. Ohno. The CBP20 coding sequence of pRSETB CBP20 was recloned into the pAD vector using XbaI and HindIII sites. The CBP80 coding sequence of PQE30 CBP80 was recloned BamHI blunt and HindIII into the pAD vector digested with XbaI and HindIII, where the XbaI site was blunted. The eIF4E coding region from the pTCEEC 4E construct (a kind gift of R.E. Rhoads) was amplified using PCR with primers located

at the ATG (TCTAGATCTAAGATGGCGA CTGTCG) and in the 3'UTR (AAGCTTCC CAAATCTCGATTGCTTGA) and this PCR fragment was ligated into a pGem-T Easy vector (Promega). After verification of the sequence, the insert was cloned BglII and EcoRI into the pSVmp10 vector that was digested with BamHI and EcoRI and then the coding region was recloned HindIII and EcoRI blunt into pGL3 control vector (Promega) digested with HindIII and XbaI, where the XbaI site was blunted. To create the pEGFP-eIF4E construct, the eIF4E coding region in the pGemT-easy vector was cloned in frame 3' of the EGFP coding region into the pEGFP-C1 vector using BglII and SacII sites.

Results

A role for nuclear αB-crystallin in protection of translation?

To test whether αB-crystallin targeted to the nucleus protects translation after heat shock, a construct was made in which αB-crystallin is fused to the nuclear localization signal of FGF-2 (αBnls). Immunohistochemical staining of cells transfected with the αBnls expression construct showed copious accumulation of αB-crystallin in the nucleus (data not shown and chapter 4 of this thesis). This αBnls expression construct was co-transfected into C2 cells with the two constructs designed to test for cap-dependent or cap-independent translation: pHsp-Cap-Luc, a construct with the Hsp70 promoter driving luciferase reporter gene expression, for cap-dependent translation, and pHsp-IRES-Luc, a construct with the 5' non-coding region of the FGF-2 mRNA inserted between the Hsp70 promoter and the luciferase coding region, for IRES-dependent translation (Doerwald et al. 2003). Expression of αBnls stimulated the luciferase expression from the cap-Luc mRNA about three fold (Figure 1). The yield of luciferase from the IRES-dependent construct, however, was decreased by half when αBnls was expressed (Figure 1). Nuclear αB-crystallin thus clearly protected the cap-dependent initiation of translation but not IRES-dependent translation after heat shock. Why nuclear αB-crystallin should lower the level of luciferase expression from the IRES-dependent construct, while cytoplasmic αB-crystallin has no effect there on, is not clear. Possibly, the differential behaviour of eIF4E and eIF4G, both factors that can be found not only in the cytoplasm, but also in the nucleoplasm (Dostie et al. 2000; Dostie, Lejbkowicz, and Sonenberg 2000; McKendrick et al. 2001), after a heat shock may be responsible for this (see below and discussion).

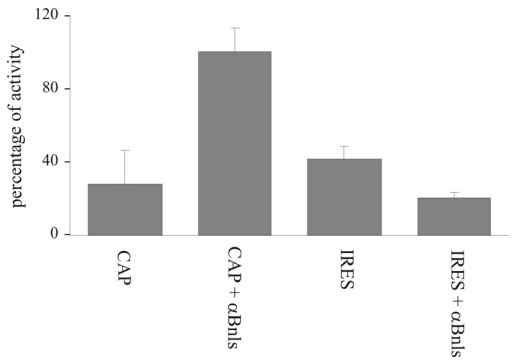


Fig 1: The effect of overexpressing nuclear αB-crystallin on cap- and IRES-dependent translation during recovery from a heat shock.
C2 cells co-transfected with the pHsp-Cap-Luc or pHsp-IRES-Luc constructs and the nuclear αB-crystallin (αBnls) expression construct or the empty expression vector were heat shocked for 30 min at 45°C. Reporter gene activity was measured after 6 h of recovery at 37°C. Values shown are relative to those obtained in cells transfected with pHsp-Cap-Luc and the αBnls expression construct; error bars indicate the standard deviation.

Can translation initiation after heat shock be rescued by overexpression of factors involved in translation initiation?

The nuclear cap-binding proteins

When the expression constructs for CBP80, CBP20 or the empty vector were co-transfected with the pHsp-Cap-Luc and CMV-β-galactosidase reporter constructs into C2 cells, the levels of β-galactosidase, a measure of the transfection efficiency, were very similar. However, when CBP80 + CBP20 (CBC) were co-expressed, β-galactosidase values were more then doubled (Figure 2A), strongly suggesting that the CBC complex is limiting for the expression of this reporter gene under normal conditions. Quantitative RT-PCR experiments using HeLa cells confirmed that β-galactosidase mRNA levels were increased when CBP80 + CBP20 were co-expressed (data not shown). After a heat shock, the luciferase yields from the pHsp-Cap-Luc reporter gene were not significantly different when the CBP80 or CBP80 + CBP20 were overexpressed as compared to the yield in the absence of added CBP (Figure 2B). CBC is thus not limiting after a heat shock, possibly because far fewer mRNAs need to be transported from the nucleus.

However, overexpression of CBP20 alone led to a decrease in luciferase expression in heat shocked cells (Figure 2B). This inhibitory effect was reversed by overexpression of αB-crystallin, leading to an apparently higher level of protection by αB-crystallin in the presence of CBP20 (Figure 2C). In the case of CBP80 or CBP20 + CBP80, the relative protection by αB-crystallin did not differ from that found in the absence of the CBPs. It is thus unlikely that the protection of translation by αB-crystallin is due to changes in the availability of CBP20 and/or CBP80.

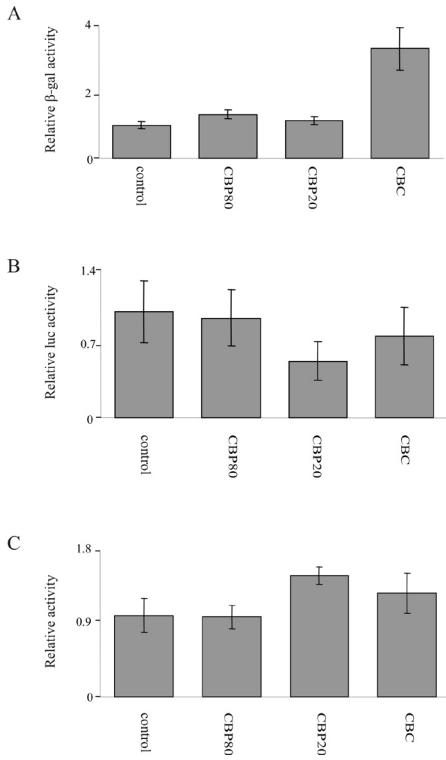


Figure 2: The effect of overexpressing the nuclear cap-binding proteins on cap-dependent translation after heat shock.
C2 cells were transfected with the pHsp-Cap-Luc construct, the expression construct for αB-crystallin or its empty vector and expression constructs for the CBP80 and/or CBP20 or their empty vector as a control. Cells were heat shocked for 30 min at 45°C and reporter gene activity was measured after 6 h of recovery at 37°C.
A: β-galactosidase activities in cells overexpressing CBP80 and/or CBP20 are given relative to the β-galactosidase activity of cells transfected with only the pHsp-Cap-Luc construct. B: Luciferase activities in cells overexpressing CBP80 and/or CBP20 are given relative to the luciferase activity in control cells. C: The ratio of luciferase activity after a 6 h recovery from a heat shock in the absence or presence of αB-crystallin in C2 cells also transfected with pHsp-Cap-Luc and expression vectors for CBP20, CBP80, or the CBC or their empty vector as a control. As only values from cells transfected with the same CBP construct(s), but differing in the level of αB-crystallin, are compared, the values shown were corrected for transfection efficiency on the basis of the β-galactosidase levels. The relative activity of the controls was set at 1 and the activities shown are the average of four experiments, error bars indicate the standard deviation.

The translation initiation factors eIF4E and eIF4G and the polyA binding protein

Overexpression of the polyA binding protein PABP had no effect on the heat shock induced expression of luciferase from the pHsp-Cap-Luc reporter either in the absence or in the presence of αB-crystallin (Figure 3). Hence, overexpression of PABP cannot rescue translation initiation after a heat shock.

Overexpression of the cap-binding protein eIF4E might be expected to affect the level of overall translation initiation in normal cells. However, unlike CBC, added eIF4E in C2 cells had no significant effect on the expression of the CMV-β-galactosidase construct (data not shown). Apparently, this factor is not limiting for translation initiation in these cells under normal, non-stressed, conditions. In heat shocked cells, overexpression of eIF4E did not rescue translation, in fact the yield of luciferase was less in the presence of added eIF4E than in its absence (Figure 3). The inhibitory effect of overexpression of eIF4E was partially reversed by αB-crystallin (Figure 3). The effect of overexpressing the EGFP-eIF4E fusion protein was similar to that of overexpression of eIF4E, although less pronounced, suggesting that fusing EGFP to the N-terminal region might have a slight inhibitory effect on this protein.

The effect of overexpressing eIF4G on translation in non-stressed cells, as assayed by the yield of β-galactosidase from the CMV-β-galactosidase construct, was dependent on the cell type: overexpression of eIF4G in C2 cells had no effect while in HeLa cells it resulted in an increase in β-galactosidase level (data not shown). This increase in β-galactosidase level in HeLa cells corresponded with an increase in β-galactosidase mRNA as assayed by quantitative RT-PCR (data not shown). In heat shocked C2 cells containing an increased level of eIF4G, the yield of luciferase from pHsp-Cap-Luc was not significantly different from that in control cells (Figure 3). However, αB-crystallin did not protect translation when eIF4G was overexpressed (Figure 3). Similar results were obtained in HeLa cells and with Hsp27 (data not shown). Apparently, overexpression of eIF4G abolishes the protective effect of sHsps on cap-dependent translation initiation.

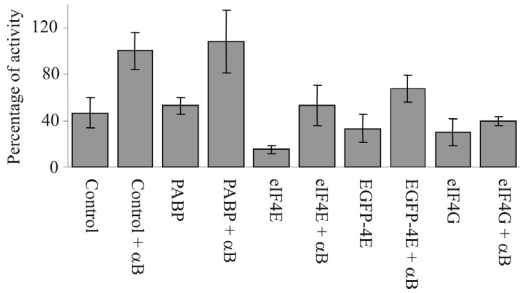


Fig 3: The effect of overexpressing PABP, eIF4E or eIF4G on cap-dependent translation after heat shock.
C2 cells were transfected with the pHsp-Cap-Luc construct, the expression construct for αB-crystallin or its empty vector and expression constructs for the poly A binding protein PABP, the cap-binding protein eIF4E, the EGFP-eIF4E fusion protein, the scaffolding protein eIF4G or their empty vector as a control. Cells were heat shocked for 30 min at 45°C and reporter gene activity was measured after 6 h of recovery at 37°C. Values shown are relative to those obtained in cells transfected with pHsp-Cap-Luc and the αB-crystallin expression construct, error bars indicate the standard deviation.

Effect of overexpression of αB-crystallin on stress granule formation after heat shock.

To determine whether αB-crystallin affects the localization or the mobility of a translation initiation factor in heat shocked cells, we fused eIF4E with EGFP (EGFP-eIF4E). As shown in figure 3, this fusion protein is at least partially active. To minimize the potentially inhibitory effect of transfected eIF4E, the EGFP-eIF4E expression construct was transfected in tracing rather than overexpressing amounts.

In heat shocked C2 cells EGFP-eIF4E was found to be predominantly localized in cytoplasmic granules, presumably SGs, directly after the heat shock (Figure 4A). In a few cells, larger aggregates were found (Figure 4B). In cells overexpressing αB-crystallin no difference in the localization of EGFP-eIF4E was seen. In HeLa cells similar results were obtained (Figure 5A and B), although in these cells larger aggregates of EGFP-eIF4E were more often found in the presence of αB-crystallin (note that HeLa cells stably transfected with an αB-crystallin expression construct under the control of the tetracyclin repressor were used in these and following experiments). HeLa cells that had been pre-heat shocked contained mostly aggregates; only a few of those cells showed the typical localization of EGFP-eIF4E in SGs (Figure 5B). Again, pre-heat shocked cells overexpressing αB-crystallin were somewhat more likely to have aggregates than cells lacking αB-crystallin. Staining of these HeLa cells for αB-crystallin did not provide unequivocal evidence for an association of αB-crystallin with the SGs or EGFP-eIF4E aggregates (Figure 5A). In some cells, αB-crystallin was not detected in the SGs or associated with the aggregates, while in other cells there appeared to be a co-localization. Whether by direct association or not, αB-crystallin appears to promote first of all the coalescence of the EGFP-eIF4E containing granules into larger aggregates (Figure 5B) and then the disappearance of these aggregates. After 24 hours of recovery aggregates could no longer be detected in αB-crystallin expressing cells, while they were still present in control cells (Figure 5C).

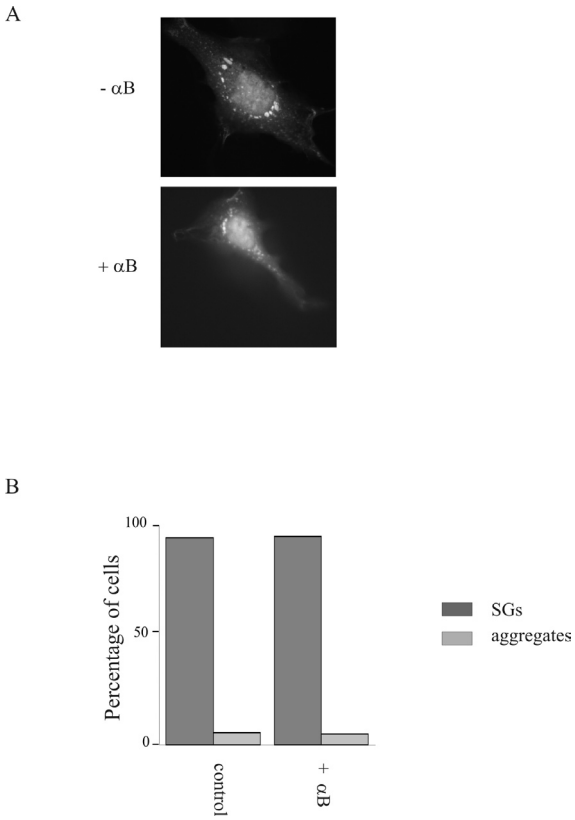


Fig 4: Stress granule formation during recovery from a heat shock in control C2 cells and C2 cells overexpressing αB-crystallin.
C2 cells transfected with the expression construct for αB-crystallin and the EGFP-eIF4E construct were heat shocked for 60 min at 45°C. Cells were fixed immediately after heat shock. A: Representative photographs of C2 cells with or without overexpression of αB-crystallin immediately after heat shock. B: The percentage of C2 cells containing EGFP-eIF4E aggregates resembling SGs or larger aggregates immediately after heat shock in the absence or presence of αB-crystallin.

As the data presented above suggested that αB-crystallin might increase the mobility of EGFP-eIF4E we measured the fluorescence recovery after photobleaching (FRAP) of EGFP-eIF4E in SGs and aggregates as well as of the cytoplasmic EGFP-eIF4E. As shown in figure 6A, during the first six hours of recovery from a heat shock, EGFP-eIF4E containing granules or aggregates recovered about 30% of the initial fluorescence, irrespective of the presence of αB-crystallin. With time, the extent of recovery decreased in control cells (Figure 6B). αB-crystallin expressing cells were not examined at later times as in these

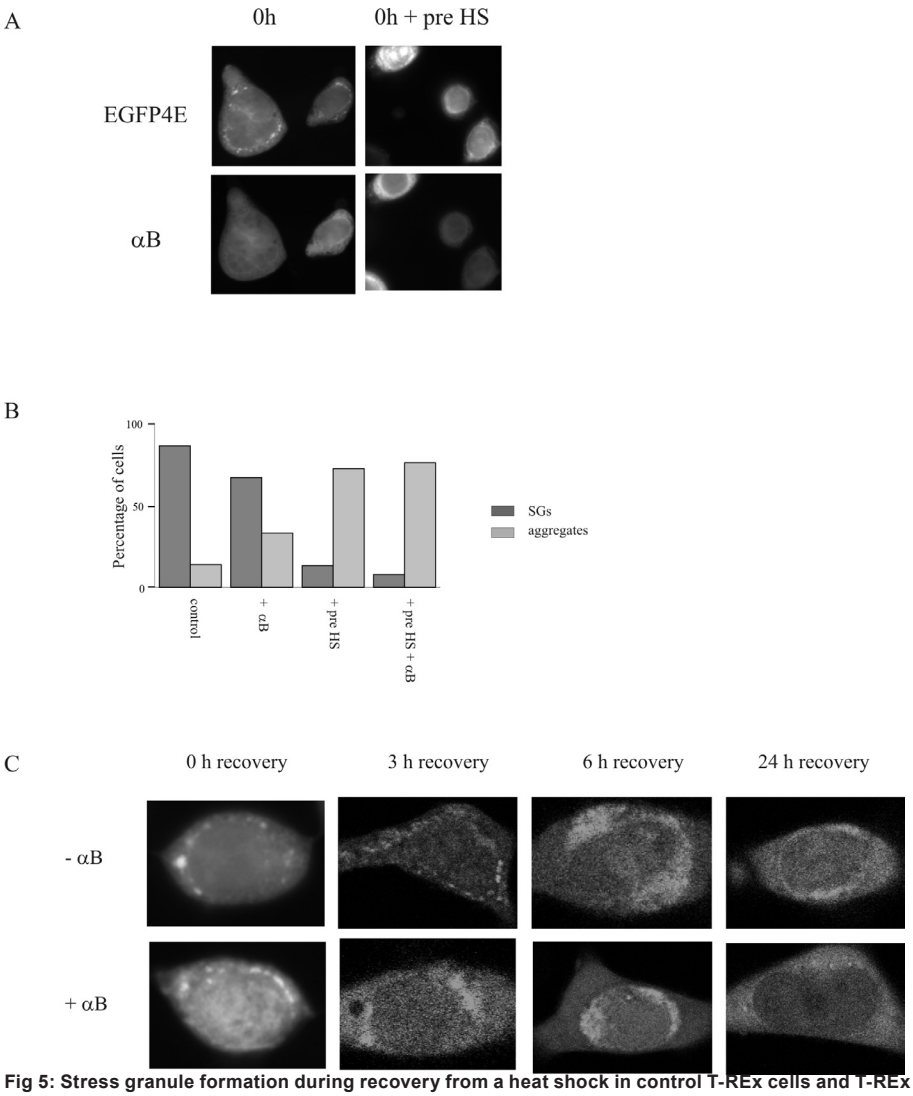


Fig 5: Stress granule formation during recovery from a heat shock in control T-REx cells and T-REx cells overexpressing αB-crystallin.

T-REx cells stably transfected with αB-crystallin were transfected with the EGFP-eIF4E construct and αB-crystallin expression was induced for 24 h prior to heat shock. Where indicated a pre-heat shock was performed for 30 min at 45°C 6 h prior to the heat shock. Cells were either fixed and then photographed (A and B) or photographed live (C) immediately after heat shock (60 min 45°C) or during recovery at 37°C at the times indicated. A: Representative photographs of T-REx cells overexpressing αB-crystallin immediately after heat shock. Localization of both EGFP-eIF4E and αB-crystallin is shown. B: The percentage of T-REx cells containing EGFP-eIF4E aggregates resembling SGs or larger aggregates immediately after heat shock in cells overexpressing αB-crystallin and in control cells. C: Representative photographs showing EGFP-eIF4E aggregates in control cells (-αB) and in T-REx cells overexpressing αB-crystallin (+αB) after heat shock and during recovery at 37 °C (0-24 h).

cells the aggregates disappear. To obtain at least a rough estimate of the kinetics of the recovery, the rate of the recovery of fluorescence during the first five seconds after bleaching was calculated. In cells expressing αB-crystallin the rate of increase in fluorescence of SGs or aggregates was very variable (Figure 7) but on the average not significantly faster than in cells lacking αB-crystallin. In contrast, the fluorescence in the cytoplasm of αB-crystallin expressing cells recovered faster (Figure 7) and to a larger extent (about 45% versus about 35%; see Figure 6C) than in cells without

αB-crystallin. Surprisingly, even after 24 hrs of recovery from the heat shock, the rate and extent to which EGFP-eIF4E fluorescence recovered after bleaching of the cytoplasm of αB-crystallin expressing cells was still lower than in cells that had not been exposed to a heat shock, showing that these cells had not yet fully recovered.

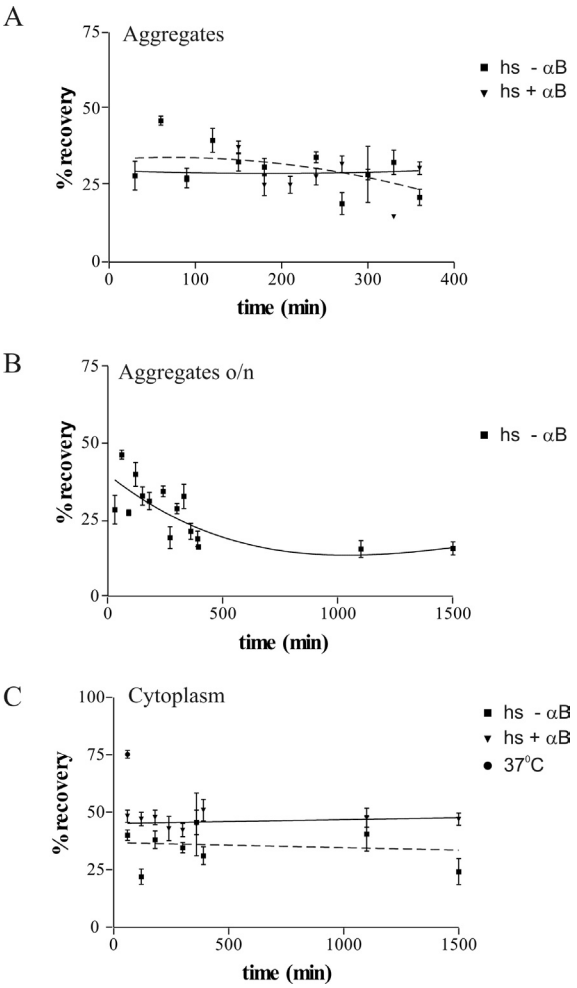
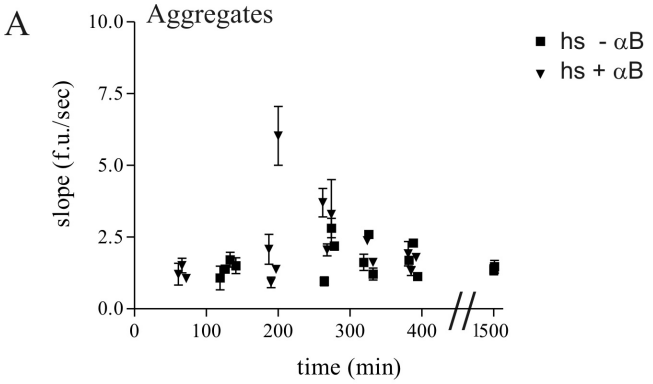


Fig 6: FRAP experiments on EGFP-4E in T-REx-αB cells after heat shock.
T-REx-αB cells, with or without prior induction of αB-crystallin expression, were heat shocked for 60 min at 45°C. FRAP experiments were done on EGFP-eIF4E aggregates and cytoplasm in these cells, graphs show the recovery of the fluorescence in the bleached areas with time. A: Recovery of fluorescence in bleached EGFP-eIF4E aggregates during the first 6 h of recovery from a heat shock with (▼) and without (□) αB-crystallin overexpression. B: Recovery of fluorescence in EGFP-4E aggregates in cells without αB-crystallin overexpression during the first 24 h of recovery from a heat shock. C: Recovery of fluorescence in bleached areas of the cytoplasm with (▼) and without (□) αB-crystallin overexpression during the first 24 h of recovery from a heat shock. As a control also the recovery of fluorescence in bleached cytoplasm (•) of non heat-shocked cells is shown.

Discussion

The premise of the experiments presented in the first part of this chapter was that overexpression of a single factor could compensate for a stress related decrease in the activity of that factor, or, alternatively, that if αB-crystallin can protect a factor from partial inactivation, then overexpression of that factor in the presence of added αB-crystallin should result in increased availability of that factor and thus in an increase in cap dependent translation. Although these experiments did not pinpoint the site of protection of translation initiation by αB-crystallin, they did yield some interesting results. First of all, in non-stressed C2 cells, the nuclear cap binding complex is apparently limiting as overexpression of the two subunits of this complex caused a significant increase in expression of the CMV-β-galactosidase reporter construct. Secondly, in non-stressed C2 cells there appears to be an excess of eIF4E as overexpression of this protein did not increase expression of the CMV-β-galactosidase reporter construct. In C2 cells, eIF4G appears to be in excess as well. In HeLa cells, overexpression of eIF4G increased the level of β-galactosidase. This increase corresponded with an increase in β-galactosidase mRNA level in these cells, and therefore could indicate a difference in transfection efficiency. However, as major differences in the relative transfection efficiency of various DNA constructs in HeLa cells compared to C2 cells are unlikely, the increase in β-galactosidase mRNA could be a nuclear effect of eIF4G. eIF4G is known



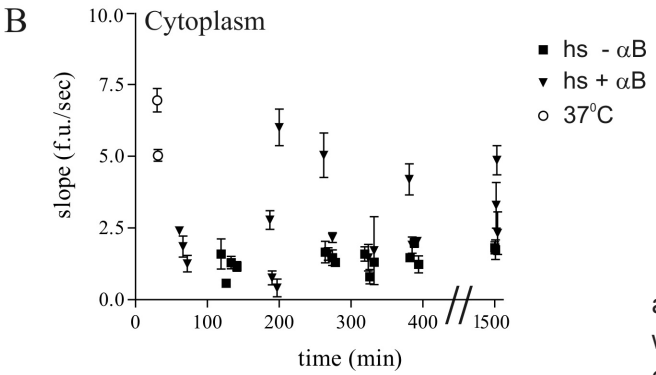


Fig 7: Rate of recovery of fluorescence in FRAP experiments.
T-REx-αB cells, with or without prior induction of αB-crystallin expression, were heat shocked for 60 min at 45°C. During recovery at 37°C, FRAP experiments were done on EGFP-eIF4E aggregates and cytoplasmic EGFP-eIF4E in these cells. Graphs show the rate of the recovery in fluorescence during the first five seconds after bleaching of EGFP-eIF4E aggregates (A) or cytoplasm (B) in T-Rex cells overexpressing αB-crystallin (▼) and control cells (□) and in cytoplasm of non-heat shocked cells (○).

to be present in the nucleus as well as in the cytoplasm (McKendrick et al. 2001) and eIF4G in the nucleus might increase the export of the mRNA from the nucleus to the cytoplasm and thus increase the β-galactosidase mRNA levels. This effect in the nucleus would then be dependent on the cell type. The effect of an increase in these factors on the level of the expression of the luciferase reporter gene after a heat shock was quite different from that seen in non-stressed cells. The CBC complex is no longer limiting, indeed overexpression of CBP20 even caused a decrease in luciferase levels. Similarly, overexpression of eIF4E, and to a lesser extent that of eIF4G, inhibited luciferase expression. The inhibition by eIF4E and CBP20 could be at least partially reversed by αB-crystallin while the inhibition by eIF4G could not. The explanation for these results is not clear. Possibly, these factors are binding a limiting component and thus quenching translation initiation by, for example, capturing this factor in SGs and thus depleting the cytoplasm of that factor. Both eIF4E and eIF4G form complexes with a number of other translation initiation factors, any of which could be limiting. It is somewhat more difficult to envisage how overexpression of CBP20 could inhibit the expression of luciferase from pHsp-Cap-Luc. It could possibly be due to the interaction between CBP20 and eIF4G or be a consequence of the role of the CBC complex in the first round of translation initiation. In this context it is also of interest to note that CBP20 is the target of regulation by growth factor stimulated signal transduction (Wilson et al. 1999).

It has been previously shown that the SGs are not static entities but that at least GFP tagged TIA-1 and PABP, both RNA binding proteins, rapidly shuttle in and out of these structures (Kedersha et al. 2000). We find a very similar mobility of EGFP-eIF4E. Surprisingly, the shuttling of EGFP-eIF4E in and out of SGs was not affected by αB-crystallin, while the mobility of EGFP-eIF4E in the cytoplasm was higher in the presence of αB-crystallin. In addition the extent of recovery of fluorescence after bleaching of EGFP-eIF4E in the cytoplasm was higher in the presence of αB-crystallin. From the cytoplasmic behaviour of EGFP-eIF4E one would predict that the fluorescence recovery of EGFP-eIF4E in the SGs would also be more rapid and more extensive in the presence of αB-crystallin. As this is not the case, the dynamics of association of EGFP-eIF4E with SGs is not dictated by its availability in the cytoplasm and must be determined by association with another SG component of which the behaviour is not affected by αB-crystallin. Our results thus suggest that EGFP-eIF4E associates with SGs as a larger complex.

Although αB-crystallin does not prevent the formation of SGs and does not change the association of EGFP-eIF4E with SGs, it does assist the dispersal of SGs and/or EGFP-eIF4E aggregates. In heat shocked cells lacking αB-crystallin such aggregates are still visible after 24 hours of recovery at 37°C, while in αB-crystallin overexpressing cells such aggregates are gone. Hsps have been shown to be involved in the dissolving or prevention of formation of inclusions or aggregates. In desmin-related myopathy, for example, overexpression of αB-crystallin decreased the amount of desmin aggregates (Hoffman 2003; Wang et al. 2003). Furthermore, αB-crystallin lowered the number of cells with glial fibrillary acidic protein (GFAP) inclusions found in several neuropathalogical conditions, by reorganizing the GFAP in these inclusions to its filamentous form (Koyama and Goldman 1999). Overexpression of Hsp70 and Hsp40 lessens inclusion body formation by the polyglutamine proteins which cause Huntington's disease (polyglutamine stretch in the huntingtin protein; Carmichael et al. 2000; Jana et al. 2000; Muchowski et al. 2000), spinal cerebellar ataxia (SCA3, polyglutamine stretch in ataxin-3; Chai et al. 1999; Schmidt et al. 2002) and spinal or bulbar muscular atrophy (SBMA; polyglutamine stretch in the androgen receptor; Kobayashi et al. 2000; Bailey et al. 2002; Adachi et al. 2003). This decrease in inclusion body formation is likely to be the combined effect of two processes: protein degradation and prevention of protein aggregation. Overexpression of chaperones generally enhances the function of the ubiquitin-proteasome pathway (for reviews see: Hayes and Dice 1996; Mathew and Morimoto 1998; Hartmann-Petersen, Seeger, and Gordon 2003). Furthermore, the Hsps function as chaperones, and could keep the polyQ proteins in soluble complexes.

The polyQ aggregates usually coalesce to form an aggresome (Kopito 2000; Waelter et al. 2001; Shimohata et al. 2002). The EGFP-eIF4E containing SGs or aggregates also coalesce, but to a structure quite distinct from that of an aggresome. At present we cannot rule out that the mechanism by which the disappearance of these structures is enhanced in αB-crystallin overexpressing cells involves degradation of EGFP-eIF4E. We however favour a model in which αB-crystallin, together with other Hsps, resolubilizes EGFP-eIF4E. Anderson and Kedersha described that for the dissolution of SGs during recovery

Hsp70 and ATP are needed (Anderson and Kedersha 2002). This is in agreement with our finding that the protection by αB-crystallin or Hsp27 of cap-dependent translation after heat shock is blocked by treatment with 2-aminopurine (Doerwald et al. 2003), a kinase inhibitor that also blocks the induction of Hsp70. We are presently investigating whether Hsp70 and/or other Hsps are also required for the αB-crystallin effect on the mobility of EGFP-eIF4E.

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Summary and Conclusion

Samenvatting en Conclusie

Chapter 7

Summary and conclusion

The DNA sequences of many genomes have been elucidated in the last years. The challenge facing us now is to predict first the genotype and then the phenotype from the genomic sequence. To discover the genes, first a comparison between the genome sequence and the sequences in EST-databases can be performed to locate coding sequences. Once the coding region is known, the amino acid sequence can be deduced from the triplet code. Even taking into account the possibility of alternative splicing variants, prediction of the protein product of a gene is simple compared to prediction of the expression pattern of that gene. This expression pattern is, of course, dependent on the cell type, the differentiation state and the stress state of the cell. To predict expression patterns, not only the regulation at the level of transcription (and post-transcriptional processing), but also that at the level of translation is important. To obtain more information about regulation of transcription, the promoter and its regulatory elements need to be mapped. “Promoter bashing”, as such experiments are nowadays somewhat derogatory known, may not be glamorous but is still essential. Only by detailed and systematic characterization of promoters and enhancers can the “code” of the sequence elements that together set the expression pattern of a gene be elucidated.

The importance of the sequence elements and their cognate factors in determining the level of transcription by the common transcription machinery from the basal promoter is well recognized. The role that the translation machinery plays in regulating the level of expression has received less attention. The efficiency of translation of an mRNA is dependent on the activity of the translation factors present. Different translation factors can be limiting depending on the cell type, and the activity of the different translation factors is subject to extensive and complex regulation. Depending on the spectrum of active translation factors, different mRNA cohorts will be translated. An extreme example is seen in cells recovering from a heat shock where cap-dependent translation is inhibited but cap-independent translation still proceeds.

Promoter studies

The first step in the regulation of expression of a gene is at the transcriptional level, i.e. the frequency with which a promoter is used. Promoters of all genes are regulated by regulatory elements; transcription factors bound to these elements can either activate or repress transcription of a gene. Transcription factors bind to a specific DNA sequence and, as many consensus binding sequences for transcription factors are already known, a search for known consensus binding sites can be performed in the sequence ‘around’ the promoter of that gene. This approach was taken in the experiments described in chapter 2, where the regulatory elements of the rat βB2-crystallin promoter were studied. The consensus regulatory elements that were found directly upstream of the βB2-crystallin promoter were a Maf responsive element (MARE) located at -131/-119 and two Sox binding elements located at -164/-159 and

-145/-150 relative to the rat βB2-crystallin transcription start site. When the role of these sequence elements in the regulation of the promoter activity was determined, however, the MARE sequence was found not to be important for the regulation of expression of the βB2-crystallin gene in lens cells. Furthermore, we found that only one of the two Sox binding sites was required for full activity of the βB2-crystallin promoter in lens cells. This shows the importance of confirming in silico predictions by deletion mapping of promoter regions.

Predicting transcription factor binding sites from consensus sequences requires prior knowledge of those consensus sequences. An alternative approach to determine which sequence elements are likely to be important in gene regulation is to investigate the evolutionary conservation of the sequence elements. To do this, an alignment should be made of the sequences upstream of the gene of interest of many different species. The assumption is that the conserved regions in these alignments will be important for (the regulation of) the promoter activity of that gene, since non-informative sequences would diverge freely during evolution. This can lead to a confirmation of the importance of known regulatory sites, but also to the finding of new sites. In chapter 3 we described such a study for the head-to-head located genes encoding the small heat shock proteins αB-crystallin and HspB2. The stress inducible αB-crystallin gene is highly expressed constitutively in both lens and muscle, while the HspB2 gene is not stress inducible and its product is found only in muscle.

Sequences of the intergenic region between the genes coding for αB-crystallin and HspB2 were determined for many mammals representing a broad range of mammalian taxa, including marsupials (opossum) and monotremes (platypus). The sequence of the same region in an avian genome (chicken) was determined as well; the duck sequence was already known. These sequences were then aligned to search for conserved regulatory elements. In mammals we found a remarkable sequence conservation of almost all known regulatory elements that were described for the mouse αB-crystallin promoter, i.e. the two lens specific regions (LSR1 and LSR2), the two heat shock elements (HSE) and the αB elements in the upstream enhancer region. On the HspB2 side of the intergenic region the sequence similarity was less pronounced. The sequence of the platypus intergenic region showed some significant differences; it lacks the Pax-6 binding site in the LSR2, and in the LSR1 only the Pax-6 site is conserved. Furthermore, it has only one HSE. The chicken and duck intergenic region sequences lack the LSR1 and both HSEs and their αB-elements have insertions that separate conserved sequence blocks. The αB-elements could however still be detected in phylogenetic footprints.

In spite of the differences in the platypus intergenic region, we found that the platypus αB-crystallin promoter is still active in lens and that one HSE is sufficient for a heat shock response. Therefore, we propose that the primordial mammalian αB-crystallin promoter had two LSRs and two HSEs and that the loss of one of the two LSRs and one of the two HSEs is secondary. This indicates some redundancy in regulatory elements.

The results in chapters 2 and 3 show that it is important to perform functional studies on promoter regions to map the sequences regulating gene expression. There are, however, limitations to these studies; for example, different cell types can have different expression patterns of

a gene, therefore, conclusions can only be drawn for regulation of transcription of the gene in the cell type used. Furthermore, changing the ratio between the promoter and the transcription factors in the cell, as a consequence of transfecting DNA constructs in the cell, might influence the activation of transcription. Another limitation is that transfection studies are not useful in detecting insulators since the chromatin structure is important for the activity of an insulator and this is not likely to be mimicked in transient transfection assays. Transfection studies are thus essential but not sufficient to map the elements involved in regulation of transcription.

Regulation of gene expression at the level of translation.

Although regulation of gene expression at the level of the promoter is the first and usually main step in regulation, regulation of expression can also be found at the level of translation. A good example of this is the regulation of translation after stress. When cells are heat shocked, general cap-dependent translation is inhibited, while cap-independent translation can still take place. During and after a heat shock the expression of heat shock proteins (Hsps) is increased. These Hsps are thought to have a protective effect on the cells in stress: when cells express a full set of Hsps due to an earlier heat shock, they are more resistant to a second heat shock. This phenomenon is called thermotolerance. Overexpression of a single Hsp (for example Hsp70, Hsp27 or αB-crystallin) can already induce thermotolerance. In this thesis we focused on translation during recovery from a heat shock and on translational thermotolerance provided by overexpression of the small heat shock proteins αB-crystallin and Hsp27. In chapter 5 we showed that overexpression of αB-crystallin or Hsp27 by transient transfections resulted in a protection of cap-dependent but not cap-independent translation after heat shock. In contrast, overexpression of Hsp70 protected both cap-dependent and cap-independent translation after heat shock. Our data cannot distinguish between a protection by Hsp70 at the level of initiation or, as has been shown in 1994 by Beck and De Maio (J. Biol. Chem. 269 p.21803-21811), at the level of elongation, or both. In any case, there is a difference in the mechanism of protection mediated by the small and the large Hsps. Furthermore, although both αB-crystallin and Hsp27 protect cap-dependent translation, they do not necessarily do so by the same mechanism. We showed in chapter 5 that for αB-crystallin protection correlates with chaperone function, as phosphorylation mimics that are poor chaperones are also poor protectors of cap-dependent translation. For Hsp27, however, a phosphorylation mutant that does not function as a chaperone, did protect translation after heat shock as well, if not better, than wild type Hsp27. This could indicate either that chaperone activity is not necessary for protection of cap-dependent translation after heat shock mediated by sHsps or that these two sHsps differ in their mechanism of protection.

The initial steps in a search for the mechanism of protection of translation initiation by the sHsps are described in chapter 6. Inhibition of translation during and after heat shock is thought to be due to a decrease in the activity of one or more translation initiation factors (eIFs). One possibility is that sHsps directly prevent inactivation of a factor. If so, mere overexpression of that factor might rescue

translation initiation after a heat shock. In addition or alternatively, overexpression of that factor in the presence of a sHsps could result in increased availability of that factor during recovery from the heat shock resulting in increased cap-dependent translation initiation. In chapter 6 the effect of overexpression of a number of factors in the presence or absence of αB-crystallin on translation initiation after a heat shock is tested. In the selection of factors to be tested, the results presented in chapter 4 were taken into account. In that chapter we described that αB-crystallin and Hsp27 translocate to the nucleus under normal and stress conditions. However, during stress more αB-crystallin and Hsp27 is present in the nucleus than under normal conditions. Overexpression of αB-crystallin targeted to the nucleus (αBnls) protected cap-dependent translation to the same extent as cytoplasmic αB-crystallin. However, unlike the cytoplasmic αB-crystallin, overexpression of αB(nls)-crystallin decreased the cap-independent translation. The protective function of αB-crystallin or Hsp27 could thus be exerted on nuclear proteins such as the subunits of the cap binding complex.

Mere overexpression of some translation factors (eIF4E, eIF4G and PABP) or nuclear cap binding proteins (CBP20 and CBP80) did not increase translation after heat shock. Furthermore, overexpression of any one of these factors together with αB-crystallin did not increase the protective effect of αB-crystallin. Therefore, the protection mediated by αB-crystallin is not due to a direct prevention of the inactivation of one of these factors.

After heat shock, stalled translation initiation complexes are known to be localized in stress granules (SGs). We showed that αB-crystallin could not prevent SG formation and did not change the association of EGFP-4E with SGs. αB-crystallin does, however, increase the mobility of EGFP-4E in the cytoplasm and speeds up the dispersal of these SGs after heat shock. A more rapid dispersal of SGs could be a result of increased protein degradation since overexpression of chaperones generally enhances the function of the ubiquitin-proteasome pathway. We cannot rule out that this is the mechanism by which the more rapid disappearance of the SGs is mediated by αB-crystallin. However, we favor a model in which αB-crystallin, probably together with other Hsps, resolubilizes the aggregates.

A possible explanation for protection of cap-dependent translation after heat shock by sHsps is the increased availability of eIF4E directly after heat shock together with a more rapid rate of dispersal of SGs and thus an increase in the availability of translation factors and mRNAs that are trapped in these SGs. Since eIF4E is only necessary for cap-dependent and not for cap-independent translation initiation, the increased availability of this factor also explains the selective effect on cap-dependent translation. The mechanism of the increased availability of eIF4E after a heat shock in the presence of αB-crystallin is not clear. It could be a direct effect of a chaperone-like activity of αB-crystallin. However, one would then predict that overexpression of eIF4E together with αB-crystallin would increase the apparent level of protection of cap-dependent translation by αB-crystallin and this is not the case. More likely then, the protection is the indirect consequence of an interaction of αB-crystallin with another, as yet unknown, cellular component.

In chapter 5 we showed that addition of 2-amino purine to the cells abolished

the protective effect of overexpressing αB-crystallin. Addition of 2-amino purine to the cells is known to block Hsp70 induction; in this chapter we showed that not only the Hsp70 induction, but also the Hsp27 induction was blocked in cells treated with 2-amino purine when compared to control cells. Therefore, we proposed that for protection of translation after heat shock mediated by αB-crystallin the synthesis of other Hsps is required as well. Translational thermotolerance would then be a cooperative effect of different heat shock proteins.

Protection of cap-dependent translation mediated by overexpression of αB-crystallin or Hsp27 (in cooperation with other Hsps) is of physiological relevance since these sHsps are constitutively expressed in a number of tissues. The presence of αB-crystallin or Hsp27 in these tissues thus provides them with translational thermotolerance. The fact that only cap-dependent and not cap-independent translation is protected during heat shock indicates that these sHsps can shift the translation pattern during stress by maintaining translation of cap-dependent mRNA's. Shifts in the activity of general translation initiation factors have a major effect on translational efficiency of specific mRNAs and thus on the amount of gene product. Possibly under normal conditions αB-crystallin and Hsp27 also have a subtle interaction with translation initiation factors. The level of sHsps in a cell could then be one of the determinants of the translational pattern of the cell.

Samenvatting en conclusie

In de laatste jaren zijn de DNA-volgorden van vele genomen geheel of gedeeltelijk opgehelderd. De opgave is nu om in deze volgorden eerst de genen te identificeren (het genotype) en vervolgens uit de volgorden het expressiepatroon van deze genen af te leiden (het fenotype). Door de volgorde van het genomisch DNA te vergelijken met volgorden in de EST databanken kunnen coderende volgorden gelokaliseerd worden. Met behulp van de triplet-code kan dan de coderende DNA-volgorde vertaald worden in de aminozuurvolgorde van het eiwit. Zelfs wanneer rekening wordt gehouden met alternatieve splice varianten is het voorspellen van het eiwit dat wordt gecodeerd door een bepaald gen nog makkelijk vergeleken met het voorspellen van het expressiepatroon van dat gen. Wanneer men de DNA-volgorde van een gen en het omliggende DNA kent, wil dit nog niet zeggen dat ook het expressiepatroon van dit gen bekend is. De expressie van een gen wordt bepaald door een aantal factoren: hoe vaak wordt het getranscribeerd, hoe efficient wordt het primaire RNA-product verwerkt en hoe vaak wordt het rijpe mRNA in het cytoplasma vertaald. Deze processen zijn afhankelijk van vele eiwit-factoren die in de cel aanwezig moeten of kunnen zijn. Sommige factoren stimuleren de expressie terwijl andere factoren juist remmend werken. Welke factoren in de cel actief en/of aanwezig zijn is afhankelijk van het celtype, het differentiatiestadium en de omgeving van de cel. Het is dus (nog) niet mogelijk om alleen vanuit de opgehelderde DNA-volgorde een expressiepatroon te voorspellen.

Om er achter te komen hoe de transcriptie van een gen gereguleerd is, moeten de regulatoire elementen experimenteel worden gekarteerd. Hieruit blijkt welke transcriptiefactoren van belang zijn voor de transcriptie van het gen in een bepaald celtype. Alleen door het systematisch en gedetailleerd in kaart brengen van de promoter en de enhancers van een gen, kan opgehelderd worden hoe de regulatoire elementen in het DNA samen het transcriptiepatroon van het gen bepalen.

Het belang van promoterstudies is alom bekend, er wordt echter veel minder aandacht besteed aan het effect van vertaling van het mRNA op het expressiepatroon van een gen. Toch is de eiwitsynthese evenzo van belang voor regulatie van gen-expressie; de aanwezigheid van het transcript van een gen wil namelijk niet direct zeggen dat het ook daadwerkelijk vertaald gaat worden en ook de efficiëntie van de eiwitsynthese is van belang voor het expressieniveau. De efficiëntie van de eiwitsynthese wordt beïnvloed door de aanwezigheid en activiteit van translatiefactoren; verschillende translatiefactoren kunnen beperkend zijn in verschillende celtypes en de activiteit van translatiefactoren is afhankelijk van complexe regulatie mechanismen. Welke mRNA's in welke mate worden vertaald is afhankelijk van het spectrum van actieve translatiefactoren. Na een hitte stress wordt bijvoorbeeld de cap-afhankelijke vertaling veel sterker geremd dan de cap-onafhankelijke vertaling. Om meer inzicht te krijgen in het expressiepatroon van een gen zullen dus zowel studies aan de factoren die bepalend zijn voor de promoteractiviteit als aan die die de efficiëntie van de eiwitsynthese bepalen, moeten worden verricht.

Promoterstudies

De eerste stap in de regulatie van de expressie van een gen is op het niveau van transcriptie, dat wil zeggen de mate van gebruik van de promoter. De activiteit van de promoter wordt gereguleerd door binding van transcriptiefactoren aan bepaalde volgorden (transcriptiefactor bindende elementen) in het omringende DNA. Transcriptiefactoren gebonden aan dergelijke elementen kunnen vervolgens de transcriptie van een gen stimuleren of remmen. Voor veel transcriptiefactoren is de consensusvolgorde waaraan zij kunnen binden al bekend. Om meer informatie te krijgen over de regulatie van transcriptie van een bepaald gen kan dus rondom de promoter van dat gen gezocht worden naar consensusvolgorden voor transcriptiefactoren. Dit soort onderzoek is uitgevoerd in de experimenten die beschreven staan in hoofdstuk 2. Dit hoofdstuk beschrijft de studie van de regulerende elementen van de βB2-crystalline promoter van de rat. De consensusvolgorden die direct bovenstrooms van de βB2-crystalline promoter gevonden zijn, zijn een MAF responsive element (MARE) op -131/-119 en twee Sox bindende elementen op -164/-159 en -145/-150 relatief ten opzichte van de transcriptie startplaats van het βB2-crystalline gen. Wanneer de rol van deze elementen in de regulatie van de promoteractiviteit wordt bepaald, blijkt echter dat de MARE in lenscellen niet nodig is voor promoteractiviteit. Tevens is slechts één van de twee gevonden Sox-bindende elementen rond deze promoter vereist in lens cellen. Dit geeft aan dat het van belang is om de gevonden mogelijke transcriptie factor bindings plaatsen ook te testen op functionaliteit door middel van deletiestudies van de promoter.

Het is ook mogelijk om op basis van evolutionaire conservering van DNA-volgorden het belang van transcriptiefactor bindingsplaatsen te bepalen. Hiervoor is het noodzakelijk om de DNA-volgorde rondom de promoter van het betreffende gen te bepalen voor vele verschillende diersoorten. Deze volgorden worden dan onder elkaar gezet en vergeleken. Gebieden in deze vergelijking die goed geconserveerd zijn, zijn waarschijnlijk gebieden die van belang zijn voor de (regulatie van) de promoter. Gebieden die niet informatief zijn zullen namelijk zonder nadelige gevolgen kunnen muteren en deze gebieden zullen dus gedurende de evolutie minder behouden blijven. Op deze manier kan het belang van bekende elementen bevestigd worden, maar ook nieuwe elementen kunnen zo gevonden worden. In hoofdstuk 3 wordt zo'n studie beschreven voor de kop-aan-kop gelegen genen die coderen voor de kleine heat shock eiwitten αB-crystalline en HspB2. Het αB-crystalline gen is stress-induceerbaar en komt constitutief ondermeer hoog tot expressie in lens en spierweefsel, het HspB2 gen is niet stress-induceerbaar en het HspB2 eiwit komt alleen voor in spierweefsel.

De DNA-volgorden van de intergene regio tussen de genen coderend voor αB-crystalline en HspB2 zijn bepaald voor zoogdieren die representatief zijn voor een breed scala van zoogdiertaxa, inclusief de buideldieren (opossum) en de monotremen (vogelbekdier). Tevens is de volgorde van deze regio bepaald in het genoom van een vogel (kip); de volgorde bij de eend was reeds bekend. Om geconserveerde elementen te vinden zijn al deze volgorden vergeleken. De meeste regulerende elementen van de αB-crystalline promoter zoals die beschreven zijn voor de muis, zijn sterk geconserveerd bij de zoogdieren, dit

zijn de lensspecifieke regio's (LSR1 en 2), de heat shock elementen (HSE) en de αB-elementen. De volgorde aan de HspB2 kant van deze intergene regio is minder duidelijk bewaard gebleven. De volgorde van de intergene regio van het vogelbekdier is op een aantal punten significant verschillend van die bij de andere zoogdieren: het mist het Pax-6 bindende gebied in de LSR2 en in de LSR1 is alleen het Pax-6 bindende gebied geconserveerd, tevens mist het een van de HSE's. Ook de volgorden van deze intergene regio van de kip en de eend wijken af van die in de zoogdieren, zij missen namelijk de LSR1 en beide HSE's en hebben inserties in de geconserveerde delen van de αB-elementen. De αB-elementen kunnen echter nog wel terug gevonden worden met behulp van fylogenetische "footprints".

Ondanks de verschillen met de volgorden van de andere zoogdieren, is de αB-crystalline promoter van het vogelbekdier actief in de lens en wordt de activiteit van deze promoter tijdens stress verhoogd. Waarschijnlijk had de evolutionaire voorouder van de zoogdieren twee LSR's en twee HSE's en is daarna het verlies van één van de LSR's en één van de HSE's in het vogelbekdier opgetreden. Sommige transcriptiefactor bindplaatsen zijn dus niet per se altijd van belang voor de regulatie van gen-expressie en deze kunnen dan tijdens de evolutie gemuteerd raken en verdwijnen.

De hoofdstukken 2 en 3 laten het belang zien van functionele studies aan promoter regio's om meer inzicht te krijgen in de volgorden die gen-expressie reguleren. Er zijn echter enkele beperkingen aan deze studies. Verschillende celtypes hebben bijvoorbeeld verschillende expressiepatronen van een gen, er kunnen dus alleen conclusies worden getrokken voor de transcriptie van het gen in het gebruikte celtype. Tevens kan een verandering in het aantal promoters of de hoeveelheid transcriptie factor die aanwezig is in de cel, als gevolg van transfectie van DNA-constructen in de cel, de activiteit van de promoter beïnvloeden. Een andere beperking is dat transfectie studies niet gebruikt kunnen worden om insulators te detecteren; dit komt omdat de chromatinestructuur van belang is voor de activiteit van een insulator en het is niet waarschijnlijk dat deze structuur tijdens transiënte transfectie wordt nagebootst. Transfectiestudies zijn dus essentieel om elementen in kaart te brengen die betrokken zijn bij de regulatie van transcriptie, maar deze studies zijn niet voldoende om een compleet beeld te krijgen van transcriptionele regulatie.

Regulatie van gen-expressie op het niveau van vertaling.

Regulatie van gen-expressie vindt voornamelijk plaats op het niveau van transcriptie, maar ook op het niveau van de eiwitsynthese kan de expressie van een gen gereguleerd worden. Een goed voorbeeld hiervan is de eiwitsynthese tijdens stress. Tijdens een hitteschok wordt de normale, cap-afhankelijke vertaling geremd, vertaling via cap-onafhankelijke mechanismen vindt echter nog steeds plaats. Ook worden tijdens stress de heat shock eiwitten (Hsp's) verhoogd tot expressie gebracht. Overexpressie van deze Hsp's zorgt voor een beschermend effect tijdens en na de stress. Wanneer cellen een volledige set Hsp's tot expressie brengen als gevolg van een eerdere hitteschok, dan zijn ze namelijk beter beschermd tegen een tweede hitteschok. Dit wordt thermotolerantie genoemd. Overexpressie van een enkele

Hsp (b.v. Hsp70, Hsp27 of αB-crystalline) kan een cel al thermotolerant maken. In dit proefschrift wordt translationele thermotolerantie en het effect van overexpressie van de sHsp's αB-crystalline of Hsp27 hierop bestudeerd.

In hoofdstuk 5 wordt aangetoond dat overexpressie van αB-crystalline en Hsp27 de cap-afhankelijke vertaling beschermt tegen een hitteschok, maar geen effect heeft op de cap-onafhankelijke vertaling. Overexpressie van Hsp70 beschermt zowel de cap-afhankelijke als de cap-onafhankelijke vertaling. In onze experimenten kunnen we echter geen onderscheid maken tussen bescherming van de initiatie van eiwitsynthese of, zoals in 1994 door Beck en De Maio (J. Biol. Chem. 269 p.21803-21811) is aangetoond, bescherming van de elongatie van de eiwitsynthese of bescherming van beide processen door Hsp70. Er is in ieder geval een verschil in het mechanisme van bescherming door de kleine en de grote Hsp's. Tevens is gebleken dat, hoewel zowel αB-crystalline als Hsp27 de cap-afhankelijke vertaling beschermen, dit niet noodzakelijkerwijs via hetzelfde mechanisme verloopt. Het effect van αB-crystalline correleert namelijk met de chaperonne-activiteit van dit eiwit; fosforylatiemutanten van αB-crystalline die slechte chaperonnes zijn, kunnen cap-afhankelijke vertaling niet beschermen, terwijl voor Hsp27 een mutant die geen chaperonne-activiteit heeft juist beter beschermt dan een mutant die wel nog chaperonne-activiteit bezit. Het is dus mogelijk dat αB-crystalline en Hsp27 een ander mechanisme van bescherming hebben of dat chaperonne-activiteit geen directe rol speelt bij deze bescherming.

De eerste stappen in het ophelderen van het mechanisme van de bescherming van initiatie van eiwitsynthese door deze sHsp's staan beschreven in hoofdstuk 6. Waarschijnlijk wordt de remming van de eiwitsynthese tijdens een hitteschok veroorzaakt door de inactivatie van een of meer translatiefactoren. Mogelijk beschermen sHsp's direct een factor tegen inactivatie. In dat geval zou overexpressie van die factor de initiatie van de eiwitsynthese na een hitteschok kunnen herstellen. Een andere mogelijkheid is dat overexpressie van een factor in de aanwezigheid van een sHsp een verhoging van de cap-afhankelijke vertaling tot gevolg kan hebben door een verhoging van de hoeveelheid van de (beschermde) factor. In hoofdstuk 6 is het effect van overexpressie van een aantal factoren op cap-afhankelijke vertaling na een hitteschok getest in aan- of afwezigheid van αB-crystalline. De selectie van de factoren is mede gebaseerd op de resultaten van hoofdstuk 4. Hier staat beschreven dat αB-crystalline en Hsp27 tijdens stress verhoogd in de kern voorkomen. Overexpressie van αB-crystalline in de kern resulteert, evenals die van cytoplasmatisch αB-crystalline, in de bescherming van cap-afhankelijke vertaling, maar, in tegenstelling tot cytoplasmatisch αB-crystalline, remt het de cap-onafhankelijke vertaling. Mogelijk beschermen de sHsp's de eiwitsynthese door middel van bescherming van kerneiwitten, zoals b.v. de subeenheden van het cap-bindend complex.

Wanneer de translatiefactoren eIF4E, eIF4G en PABP of de cap-bindende eiwitten CBP20 en/of 80 echter tot overexpressie worden gebracht resulteert dit niet in een activatie van de eiwitsynthese na de hitteschok. Wanneer αB-crystalline deze factoren zou beschermen tegen inactivatie, zou overexpressie van deze factoren samen met αB-crystalline een

verhoging van de bescherming van de eiwitsynthese door αB-crystalline moeten geven. Dit is echter niet het geval. Hieruit kan dus geconcludeerd worden dat αB-crystalline deze translatiefactoren niet direct beschermt tegen inactivatie.

Na hitte-schok bevinden zich 'geblokkeerde' translatie initiatie complexen in stress granula (SG's). In hoofdstuk 6 staat beschreven dat overexpressie van αB-crystalline het ontstaan van deze SG's niet kan voorkomen, en de associatie van EGFP-eIF4E met de SG's niet verandert. αB-crystalline verhoogt wel de bewegelijkheid van het EGFP-eIF4E in het cytoplasma en de snelheid waarmee SG's verdwijnen. Omdat van chaperonnes bekend is dat zij afbraak van niet goed gevouwen eiwitten via het ubiquitine-proteasome systeem stimuleren, zou het sneller verdwijnen van SG's na hitteschok een gevolg kunnen zijn van een verhoogde afbraak van SG's. Wij kunnen dit mechanisme niet uitsluiten maar zijn voorstander van een ander mechanisme waarin αB-crystalline, waarschijnlijk in samenwerking met andere Hsp's, de SG's weer oplost.

Een mogelijke verklaring voor de bescherming van cap-afhankelijke vertaling na hitteschok door sHsp's is dat ze niet alleen de hoeveelheid van het beschikbare eIF4E direct na een hitteschok verhogen maar ook, door het versneld oplossen van de SG's, de beschikbaarheid van de translatiefactoren en mRNA's die geblokkeerd waren in de SG's. Omdat eIF4E alleen nodig is voor cap-afhankelijke vertaling zou dit kunnen verklaren waarom alleen cap-afhankelijke en niet cap-onafhankelijke vertaling wordt beschermd door overexpressie van sHsp's. Het mechanisme van de bescherming van eIF4E is echter niet duidelijk. Het zou een direct chaperonne-achtig effect van αB-crystalline kunnen zijn, maar dan zou overexpressie van eIF4E samen met αB-crystalline tot een verhoogde bescherming van cap-afhankelijke vertaling na een hitteschok moeten leiden en dit is niet het geval. Het is waarschijnlijker dat de bescherming een indirect gevolg is van een interactie van αB-crystalline met een andere, nog onbekende, cellulaire component.

In hoofdstuk 5 is met behulp van 2-amino purine aangetoond dat voor de bescherming van de eiwitsynthese na een hitteschok waarschijnlijk ook de expressie van andere Hsp's noodzakelijk is. Toevoeging van 2-amino purine remt namelijk de bescherming van de cap-afhankelijke vertaling door αB-crystalline na een hitteschok maar ook de inductie van Hsp70 en Hsp27. Translationele thermotolerantie zou dan een gezamenlijk effect zijn van verschillende Hsp's.

Bescherming van cap-afhankelijke vertaling door middel van overexpressie van αB-crystalline of Hsp27 (in samenwerking met andere Hsp's) is van fysiologische betekenis omdat deze sHsp's constitutief tot expressie komen in verschillende weefsels. De aanwezigheid van αB-crystalline of Hsp27 in deze weefsels zorgt ervoor dat ze translationeel thermotolerant zijn. Het feit dat alleen cap-afhankelijke vertaling beschermd wordt tijdens een hitteschok geeft aan dat deze sHsp's het patroon van de eiwitsynthese tijdens stress kunnen veranderen door de vertaling van cap-afhankelijke mRNA's in stand te houden. Veranderingen in activiteit van translatie-initiatiefactoren hebben een groot effect op de efficiëntie van de vertaling van bepaalde mRNA's en dus op de hoeveelheid van het gen product dat in de cel aanwezig is. Mogelijk hebben αB-crystalline en Hsp27 ook onder normale omstandigheden een subtiele interactie met translatie-initiatiefactoren. De hoeveelheid sHsp's in de cel zou dan een van de factoren zijn die het patroon van eiwitsynthese van de cel bepalen.

Construction and Initial Characterization of Stable T-REx Cell Lines Inducible for (Mutant) αB-crystallin or Hsp27 Expression

Appendix

Appendix: Construction and initial characterization of stable T-REx cell lines inducible for (mutant) αB-crystallin or Hsp27 expression.

Ectopic expression of a protein is commonly used to study the effect of that protein on the cellular phenotype. Prolonged ectopic expression of a protein carries the danger, however, that cells respond by adapting their expression profile to the ectopic expression. For that reason, a number of regulated expression systems have been developed for eukaryotic cells. One of these systems makes use of the prokaryotic tetracycline repressor (TetR) and its cognate DNA target sequence, the tetracycline operator (TetO). In HeLa cells stably transfected with the tetracycline repressor gene (T-REx Hela cells, Invitrogen), expression of a promoter can be placed under control of the TetR. When a pCDNA4/TO expression construct is transfected in these cells, expression of this construct is inhibited by binding of the TetR to the promoter region in this vector (Fig. 1). When tetracycline (or doxycyclin, a more potent form of tetracycline) is added to the medium of these cells the TetR is inactivated and expression of the construct is activated.

Inducible expression constructs for αB-crystallin and Hsp27 and their (phosphorylation) mutants were made using the pCDNA4/TO vector (Invitrogen) as outlined in Table 1. The constructs were tested for expression in transient transfection assays and then used to make double stable transfected cell lines in the T-REx HeLa cell line. For each construct several clonal lines were obtained. sHsp expression in these new cell lines was tested by immunofluorescence and western blotting. The western blot of the different αB-crystallin wild type cell lines (Fig. 2A) and Hsp27 wild type cell lines (Fig. 2B) showed that the expression levels were different in each cell line. This was also true for the stable cell lines of the αB-crystallin or Hsp27 mutant constructs (data not shown). For wild type αB-crystallin the B9 cell line showed the highest expression level and was used in further experiments, for wild type Hsp27 the H4 cell line was used.

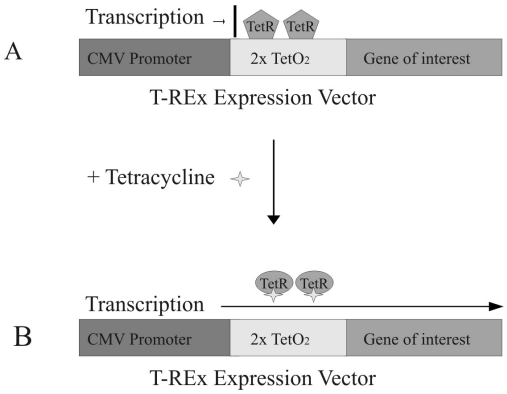

Fig 1: Schematic overview of the tetracycline induced expression system (T-REx)
A: Transcription is initially repressed by two TetR homodimers that bind to the TetO2 sequence. B: Tetracycline binds the TetR protein causing it to release the operator sequence. This derepresses the promoter, allowing transcription of the gene of interest to proceed. The picture was adapted from that in the Invitrogen catalogue.

Construct name	mutated sites	Constructed from	Sites used	pCDNA4/TO sites used
Hsp27 wt	-	pSVHaHsp27 wt	HindIII	HindIII
Hsp27 AS	S15A	pSVHaHsp27 AS		
Hsp27 SA	S90A	pSVHaHsp27 SA		
Hsp27 AA	S15,90A	pSVHaHsp27 AA		
αB wt	-	pET 16b αB wt	XbaI(blunt), XhoI	EcoRV, XhoI
αB S59D	S59D	pET 16b αB S59D		
αB SD	S19,45,59D	pET 16b αB SD		
αB SA	S19,45,59A	pET 16b αB SA		
αB RG	R120G	pET 16b αB RG		

Table 1: Clones constructed for making double stable T-REx cell lines.
Inducible clones for αB-crystallin (human) and Hsp27 (hamster) and their mutants were made by inserting the coding regions of αB-crystallin or Hsp27 into the pCDNA4/TO vector using the sites indicated in the Table. Clones were sequenced and expression of the sHsps was confirmed by transient transfection assays in T-REx cells and testing expression on western blot. The pSVHaHsp27 constructs were kindly provided by J. Landry, l'Université Laval, Quebec, Canada; the pET 16b αB-crystallin clones were kindly provided by P. Muchowski, University of Washington, Seattle, USA.

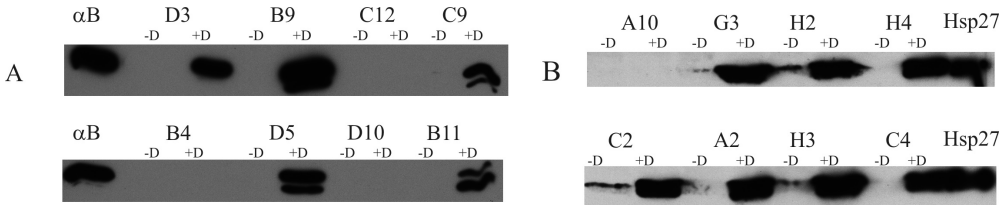

Fig 2: Expression levels of αB-crystallin or Hsp27 in different stable cell lines with and without induction of expression.
T-REx cells were plated in Minimum essential medium Eagle (EMEM, Biowhittaker) with glutamax (Gibco), 10% fetal calf serum, penicillin, streptomycin and blasticidin (Invitrogen) in 10 cm^2 dishes. Double stable cell lines were made by transfecting 1 µg of DNA per 10 cm^2 dish using 3 µl Fugene (Roche) according to the manufacturer's protocol in medium without fetal calf serum or antibiotics. After 4 h incubation, 1 ml of EMEM + glutamax + 20% fetal calf serum was added and cells were incubated at 37°C. After 24 h the medium was replaced with 2 ml of EMEM + glutamax + 10 % fetal calf serum + penicillin, streptomycin and blasticidin. One day later, cells were divided over six 80 cm^2 dishes. Cells were cultured in EMEM + glutamax + 10 % fetal calf serum + penicillin, streptomycin and blasticidin and stable transfected cells were selected by addition of 200 µg/ml zeocine (Invitrogen). After approximately 2 weeks, colonies were picked and plated into 96-well plates. Cultures were then scaled up from 96-well via 24-well, 6-well and T25 flasks to T75 flasks. The Figure shows a western blot showing the expression levels of αB-crystallin (A) or Hsp27 (B) in the stable cell lines with and without culture with doxycyclin (1 µg/ml) for 48 h.

The H4 cell line overexpressing Hsp27 was tested for the expression level of Hsp27 as a function of time of induction with doxycyclin. After 3 h of induction a weak signal of Hsp27 could be seen on a western blot, this signal was stronger when cells were induced for 6 h, while the expression level seen in cells induced for 24 h was the same as that found in cells induced for 48 h (Fig 3). Thus 24 h of induction suffices to obtain the maximal level of expression.

To determine whether overexpression of a (mutant) sHsp affects the growth rate of cells, the number of cell doublings during a three to four day period after different times of induction of expression was determined. Table 2A shows that no significant difference in growth after 4 days of Hsp27 (wt or mutants) expression was observed. However, 8 and 11 days of induction of the expression of the Hsp27 AS and SA mutants decreased cell growth, in particular

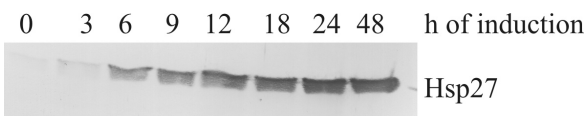

Fig 3: Western blot showing the increase in expression of Hsp27 in T-REx Hsp27 wt (H4) cell line during induction.
T-REx Hsp27 wt cells were plated in 10 cm^2 dishes and treated with 1 µg/ml of doxycyclin for 0-48 h. Equal amounts of cell lysate were separated on SDS-PAGE and Hsp27 was detected by western blotting.

of the Hsp27 AS expressing cell line. In contrast, induction of expression of the Hsp27 AA mutant had only a slight effect on cell growth, comparable to that of wt Hsp27. When the growth rate of stable cell lines of αB-crystallin and its mutants was tested, neither αB-crystallin nor the SA, the S59D or the RG mutant was found to have an effect (Table 2B,C). Cells overexpressing the αB-crystallin mutant mimicking fully phosphorylated αB-crystallin (αB SD, Table 2C) did cease growth. After 11 days of induction of expression a subpopulation of cells, which had apparently adapted to the expression of the αB-crystallin SD mutant, resumed growth. Although αB-crystallin expression does not adversely affect growth in these experiments, it should be noted that in our experience expression of the wt αB-crystallin tends to be lost during prolonged culture of the B9 line. Whether this is a consequence of a deleterious effect of prolonged expression of wt αB-crystallin or of the integration site of the expression construct remains to be tested.

The rate of cell division of (mutant) sHsp expressing cell lines was also determined by measuring the amount of BrdU incorporated in DNA during 24 h after replating cells grown in the presence of doxycyclin for 7 (αB-crystallin and mutants thereof, Fig 4A) or 14 days (Hsp27 and mutants thereof, Fig 4B).

A

	Hsp27 wt		Hsp27 SA		Hsp27 AS		Hsp27 AA	
days after induction	-D	+D	-D	+D	-D	+D	-D	+D
0-4d	0.6	0.6	0.6	0.6	0.7	0.7	0.6	0.6
4-8d	0.6	0.5	0.4	-0.1	0.5	-0.1	0.5	0.4
8-11d	0.7	0.5	0.9	0.4	0.6	0.1	0.8	0.6

B

	αB wt		αB SA		αB S59D	
days after induction	-D	+D	-D	+D	-D	+D
0-3 d	0.5	0.6	0.6	0.6	0.7	0.6
3-6 d	0.6	0.4	0.7	0.7	0.8	0.8
6-10 d	0.4	0.5	0.5	0.5	0.6	0.5
10-13 d	0.4	0.3	0.6	0.6	0.8	0.7
13-17 d	0.6	0.5	0.6	0.5	0.7	0.6
17-20 d	0.4	0.5	0.6	0.6	0.6	0.6

C

	αB SD		αB RG	
days after induction	-D	+D	-D	+D
4-7d	0.4	0.2	0.6	0.4
7-11d	0.4	-0.1	0.4	0.3
11-15d	0.6	0.1	0.5	0.4
15-19d	0.6	0.1	0.6	0.4
19-22d	0.4	0.1	0.4	0.4

Table 2: Cell doublings per day of stable T-REx cell lines cultured with or without doxycyclin.
Equal numbers of cells were plated on 6-well dishes with (+D) or without (-D) induction of sHsp expression by doxycyclin (1 µg/ml), the cells were grown for 3 or 4 days at 37°C. Cells were harvested and counted using a Beckman Ac.T8 coulter counter. The number of cell doublings per day was then calculated. Equal numbers of cells were then plated in a new 6-well dish and the procedure was repeated. A: Stable T-REx cell lines for Hsp27 wt, SA, AS and AA. B: Stable T-REx cell lines for αB-crystallin wt, SA and S59D. C: Stable T-REx cell lines for αB-crystallin SD and RG.

Only in the case of the Hsp27 SA expressing cell line was a significant decrease in BrdU incorporation found. The differences seen between the results presented in Table 2 and those in Figure 4 are presumably due to differences in the experimental protocol. Possibly, replating the cells induced one round of cell division.

When heat shocked cells are allowed to recover and are then subjected to a subsequent heat shock, they are more resistant to this second heat shock; this phenomenon is called thermotolerance. As thermotolerance can also be conferred by overexpression of Hsp70 or a sHsp, it is thought to be caused by the presence of Hsps of which the synthesis was induced by the first heat shock. Overexpression of Hsps results in protection not only against heat but also against other stresses and thus Hsps are general cytoprotective agents. Most experiments showing the acquisition of thermotolerance by the overexpression of a single Hsp used cell lines continuously expressing that Hsp. We therefore tested the effect of inducing expression of wt Hsp27 just 24 h before challenging the cells with a heat shock. Unexpectedly, expression of Hsp27 did not result in protection of the level of "metabolic activity" as measured by the cleavage of a tetrazolium salt to formazan (WST-1 assay; Roche) after heat shock (Fig. 5 and data not shown). However, also the control cells were barely affected by the heat shock.

More effect of a heat shock was seen in an experiment in which the effect of αB-crystallin expression on thermotolerance was measured. However, no significant differences were seen in the level of metabolic activity with or without induction of αB-crystallin expression (Fig. 6). This indicates that in these cells αB-crystallin alone did not confer thermotolerance with respect to this cellular parameter. Differences in the thermotolerance of metabolic activity

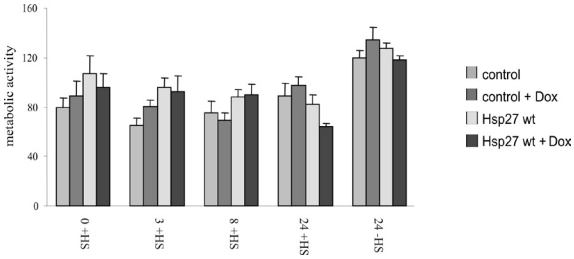

Fig 5: Cell survival after heat shock with and without induction of Hsp27 wt.
T-REx cells stably transfected with the Hsp27 wt construct or with the empty pCDNA4/TO vector as a control were induced for Hsp27 expression by addition of 1 µg/ml doxycyclin 24 h prior to heat shock (60' 46°C) and metabolic activity was measured using WST-1 assays (measurements were performed as described by manufacturer's protocol; Roche) before and after heat shock and recovery (0-24 h).

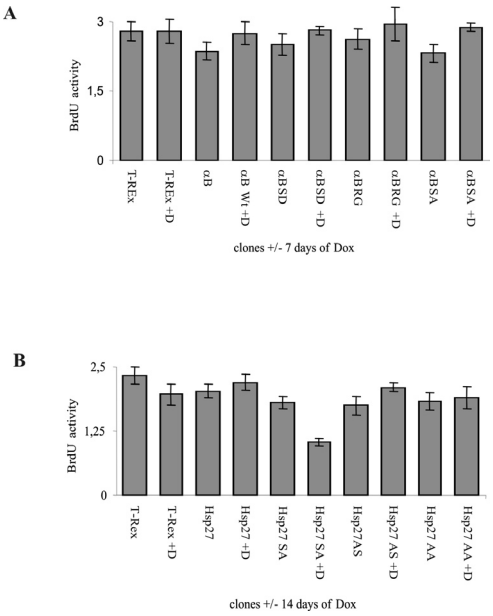

Fig 4: BrdU incorporation in the stable T-REx cell lines induced and uninduced for sHsp expression.
Cells were grown in the absence or presence of doxycyclin for 7 days (αB-crystallin clones; A) or for 14 days (Hsp27 clones; B). Equal amounts of cells were then plated in 6-well plates and after 24 h BrdU was added and incorporation assays were performed according to the manufacturer's protocol (Roche).

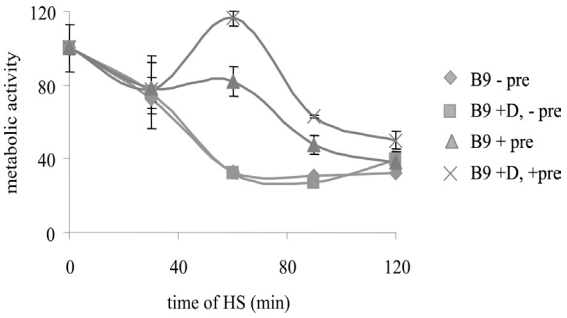

Fig 6: Cell survival after heat shock with and without induction of αB-crystallin expression.
T-REx cells stably transfected with the αB-crystallin wt construct were induced by doxycyclin 24 h prior to being challenged with a heat shock; control cells were uninduced. Furthermore, one set of induced and control cells was pre-heat shocked for 30' at 45°C 6 h before the challenge heat shock. Cells were then heat shocked for 0-120' at 45°C and the metabolic activity was measured using WST-1 assays 24 h after recovery from the heat shock. The WST-1 activity of control, non-heat shocked, cells was set at 100%, the WST-1 activity of heat shocked cells was calculated relative to that.

could be seen, however, when pre-heat shocked cells were compared with control cells. The pre-heat shock lowered the metabolic activity by 50% when compared to control cells (data not shown). When pre-heat shocked cells were heat shocked for 30-60' at 45°C, no significant decrease in metabolic activity could be seen, whereas the metabolic activity was significantly lower in heat shocked cells not treated with a pre-heat shock (Fig. 6). Thus pre-heat shocked cells are more thermotolerant. When the second heat shock was more severe (90-120' at 45°C), the metabolic activity decreased in pre-heat shocked cells as well. Induction of αB-crystallin expression in pre-heat shocked cells increased the residual metabolic activity when the second heat shock was performed for longer than 30'. This indicates that, although in these cells αB-crystallin alone cannot confer thermotolerance as measured by the metabolic activity assay, increased αB-crystallin expression in combination with induction of other Hsps, does increase the level of protection of the cells above that mediated by a pre-heat shock alone.

Although the WST-1 assay indicated a lack of metabolic activity in heat shocked cells, the cells were not dead, at least not after a heat shock regimen of 60' at 45ºC. For example, as detailed in chapters 5 and 6, ample reporter gene activity can be measured in cells recovering from a heat shock. Although WST-1 is commonly used in cell viability assays, it is more properly a measure of the state of oxidative stress as WST-1 is thought to be reduced at the cell surface in a reaction which involves superoxide.

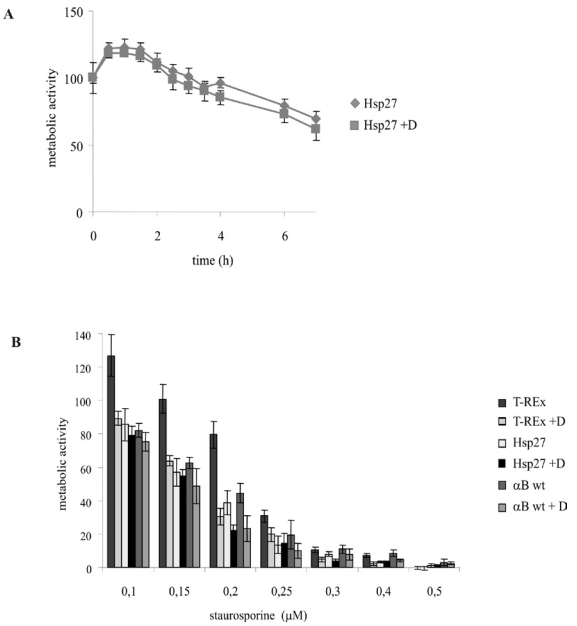

Fig 7: Cell survival after induction of apoptosis.
Stable T-REx cell lines for Hsp27 wt or αB-crystallin wt were induced for expression of the sHsp by addition of 1 μg/ml of doxycyclin 24 h prior to induction of apoptosis. Metabolic activity of cells induced for sHsp expression and of control cells was measured using the WST-1 reagent as described by the manufacturer's protocol (Roche). The metabolic activity of control cells was set at 100%, the metabolic activity of cells induced for apoptosis was calculated relative to that.
A: Induction of apoptosis in induced and control Hsp27 wt cells mediated by culturing with 1 μg/ml of anisomycin for 0-7 h. B: Apoptosis was induced for 18 h by addition of 0-0.5 μM of staurosporin with and without induction of αB-crystallin or Hsp27 expression.

Both Hsp27 and αB-crystallin have been implicated in protection of cells against apoptosis. Therefore we tested the effect of overexpression of αB-crystallin and Hsp27 in these stable T-REx cells on the sensitivity of the cells to apoptotic agents. Whether apoptosis was induced by anisomycin (Fig. 7A) or staurosporine (Fig. 7B), no increased resistance to apoptosis could be seen in cells overexpressing the sHsp when compared to control cells. In fact and unexpectedly, the presence of sHsps tended to decrease the resistance to these apoptotic agents.

Pre-heat shocked cells also did not show an increased resistance to apoptosis induced by anisomycin or staurosporine (Fig. 8). However, a pre-heat shock reversed the increased susceptibility of Hsp27 or αB-crystallin expressing cells to anisomycin or staurosporine. A possible explanation for the sensitivity of αB-crystallin expressing cells is that in these cells the level of Hsp70 is decreased significantly (John den Engelsman, unpublished data). Possibly the level of Hsp70 (or other Hsps) is too low for these cells to resist the induction of apoptosis. Induction of Hsp expression mediated by a pre-heat shock would then increase the Hsp levels and this could result in normal survival of the cells when compared to control cells. A similar change in Hsp expression profile could cause the increased sensitivity of Hsp27 cells to an apoptotic challenge, but this still needs to be determined. Hsp27 expression does not change the level of Hsp70 (John den Engelsman, unpublished results).

These results taken together show that prolonged induction of the expression of the Hsp27 AS and SA mutants as well as the αB-crystallin SD mutant significantly decreased

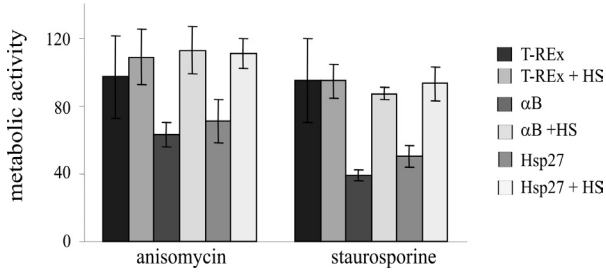

Fig 8: Survival of cells overexpressing αB-crystallin or Hsp27 after induction of apoptosis, with or without a pre-heat shock.
Stable T-REx cell lines for αB-crystallin or Hsp27 were induced for sHsp expression by addition of doxycyclin (1 μg/ml) for 24 h prior to induction of apoptosis. Where indicated (+HS) cells were pre-heat shocked for 30' at 45°C 4 h prior to induction of apoptosis by anisomycin (1 μg/ml, 4 h) or by staurosporin (0.25 μg/ml, 4 h). The metabolic activity of cells induced for apoptosis and control cells was measured using the WST-1 reagent as described by the manufacturer's protocol (Roche). The activity of cells uninduced for apoptosis was set at 100%, the activity of cells induced for apoptosis was calculated relative to that.

cell growth in these stable cell lines. Thus overexpression of these sHsps mutants is not favorable for the cell. Furthermore, overexpression of αB-crystallin or Hsp27 in T-REx cells did not result in protection against a heat shock or the apoptotic agents anisomycin and staurosporine. When these cells were pre-heat shocked however, protection was seen. Thus, in T-REx cells cytoprotection mediated by a pre-heat shock cannot be mimicked by overexpression of αB-crystallin or Hsp27 alone but requires the presence of other Hsps. These results are at odds with previously published work showing that sHsp can confer cytoprotection to cells. One possible explanation for this discrepancy is that prolonged culture of cells overexpressing a sHsps results in epigenetic changes, which in turn result in increased cytoprotection. This possibility can be tested by examining the thermotolerance and resistance to apoptotic insults of cells overexpressing sHsps cultured for a longer time.

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Curriculum vitae

Linda Doerwald is geboren op 26 juli 1975 te Emmerich (Duitsland). In 1993 behaalde zij het VWO diploma aan het Dukenburg college in Nijmegen, hierna begon zij haar studie Biologie aan de Katholieke Universiteit Nijmegen (KUN). Tijdens haar studie heeft ze twee hoofdvak stages gedaan aan de KUN, een bij de afdeling moleculaire biologie (dr. N.H. Lubsen) en een bij de afdeling Biochemie (prof. dr. W.J. van Venrooij). Het doctoraal diploma met de afstudeer richting “Fysiologisch biochemisch” behaalde zij in augustus 1998. Vanaf oktober 1998 tot februari 1999 heeft zij als junior onderzoeker gewerkt bij de afdeling Moleculaire Biologie van de KUN, waarin zij onder andere het practicum Biotechnologie II voor derde jaars biologie studenten heeft voorbereid, georganiseerd en begeleid. Van februari 1999 tot augustus 2003 was zij werkzaam als AiO op de afdeling Biochemie FNWI van de KUN. In deze tijd heeft zij het in dit proefschrift beschreven onderzoek verricht.Sinds Oktober 2004 is zij via CTO gedetacheerd bij Chiron als clinical data coordinator.

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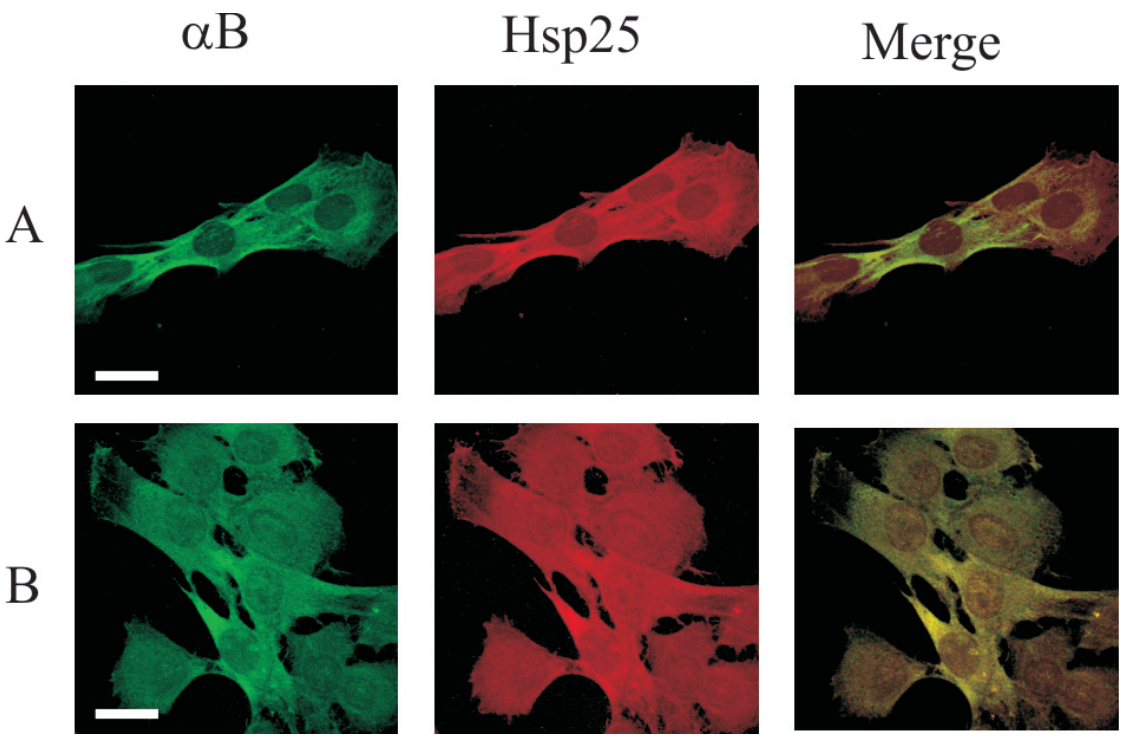
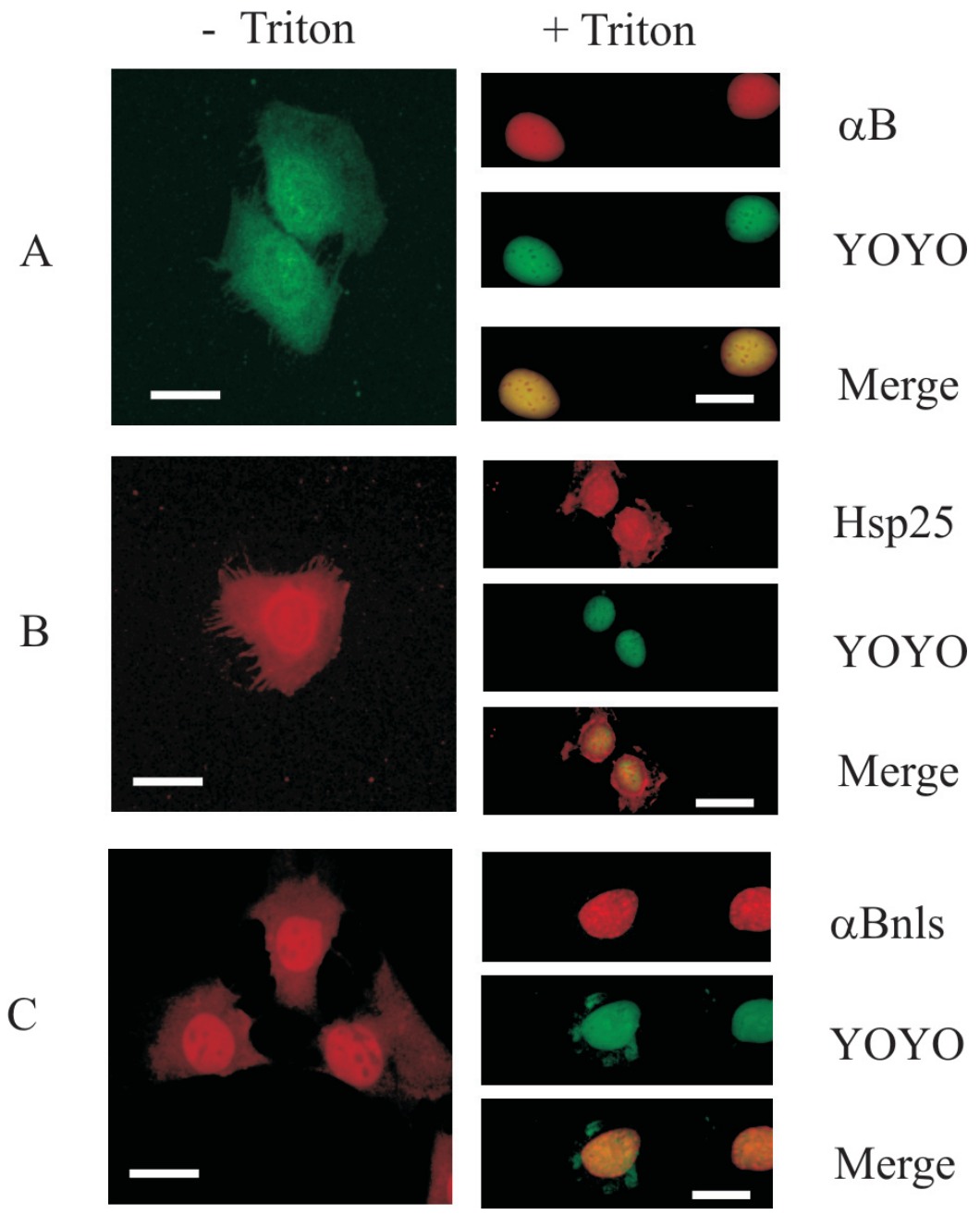
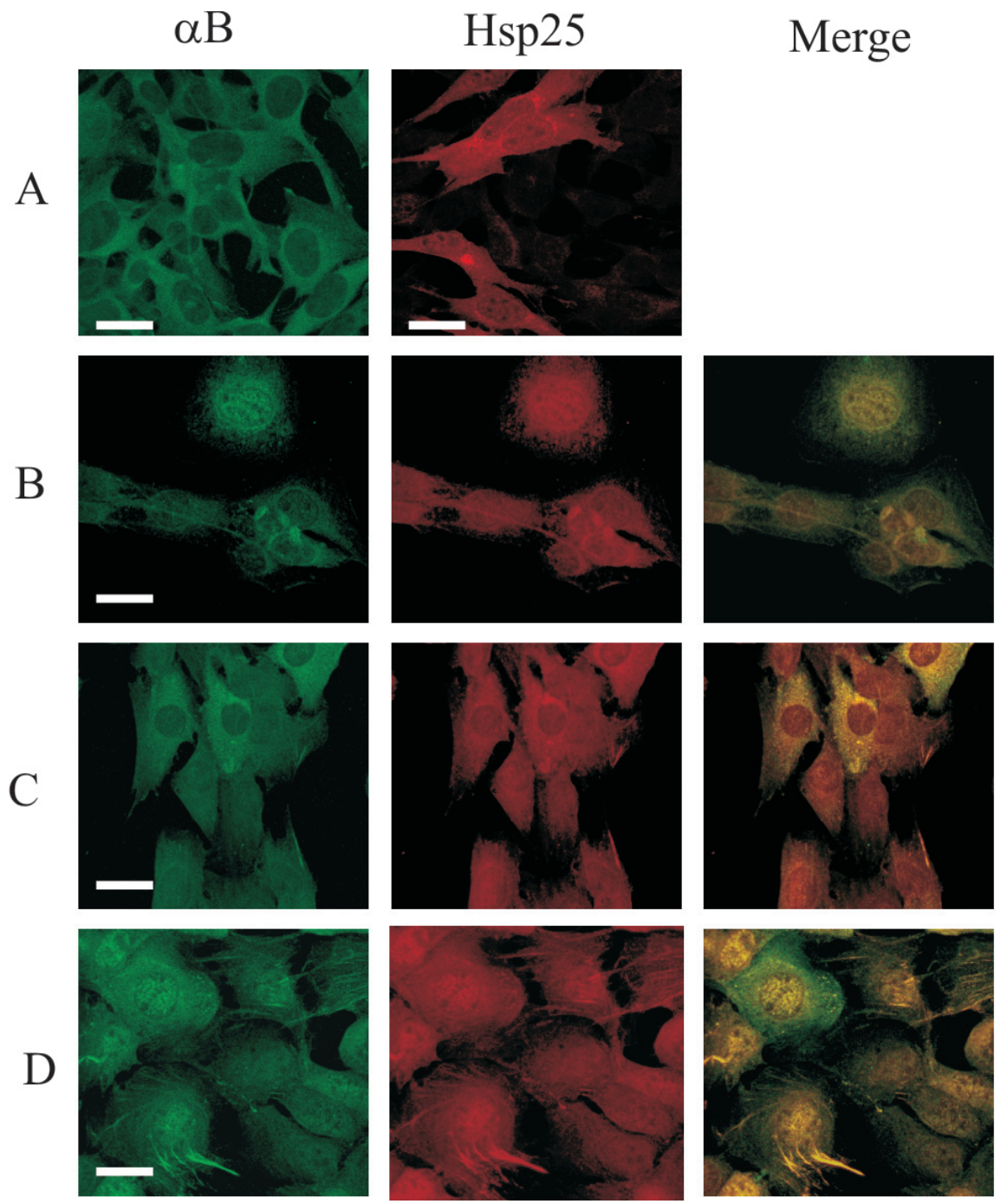


Chapter 4, Fig.1



Chapter 4, Fig. 2



Chapter 4, Fig. 3