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Original Article

Regulation of the mouse αB-crystallin and MKBP/HspB2 promoter activities by shared and gene specific intergenic elements: the importance of context dependency

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ABSTRACT The closely linked (863 bp), divergently arranged mouse *myotonic dystrophy kinase binding protein (Mkbp)/HspB2* and *small heat shock protein (shsp)/αB-crystallin* genes have different patterns of tissue-specific expression. We showed previously that an intergenic enhancing region (-436/-257 relative to *αB-crystallin* transcription start site) selectively activates the *αB*-crystallin promoter in an orientation-dependent manner (Swamynathan, S.K. and J. Piatigorsky 2002. J. Biol. Chem. 277:49700-6). Here we show that *cis*-elements αBE1 (-420/-396) and αBE3 (-320/-300) functionally interact with glucocorticoid receptor (GR) and Sp1, respectively, both *in vitro* and *in vivo*. αBE1:GR regulates both the HspB2 and αB-crystallin promoters, while αBE3:Sp1 selectively regulates the αB-crystallin promoter, as judged by mutagenesis and co-transfection tests. Enhancer blocking assays indicate that the -836/-622 fragment can act as a negative regulator in transfection tests, raising the possibility that it contributes to the differential expression of the proximal HspB2 promoter and distal αB-crystallin promoter. Finally, experiments utilizing transiently transfected cells and transgenic mice show that two conserved E-box elements (-726/-721 and -702/-697) bind nuclear proteins and differentially regulate the HspB2 and αB-crystallin promoters in a tissue-specific manner. Taken together, our results indicate that the linked, differentially expressed *HspB2* and *αB-crystallin* genes have evolved shared and promoter-preferred *cis*-control elements within the intergenic sequence. The context-dependency of *cis*-elements provides multiple opportunities for evolutionary novelty by small sequence changes.

KEY WORDS: *crystallin, gene regulation, promoter activity, enhancer, development*

Introduction

Precise tissue-specific gene regulation via *trans*-acting factors binding to DNA regulatory elements in locus control regions, enhancers and promoters plays a central role in normal development (Wray, 2003). While promoters are adjacent to the transcriptional initiation site of their respective genes, locus control regions and enhancers may be located within introns, at distant positions on the chromosome, or even on separate chromosomes from the genes they regulate (Lee *et al.*, 2006, Lee *et al.*, 2005, Spilianakis and Flavell, 2004, Spilianakis *et al.*, 2005). In addition to the challenge of understanding how distant DNA control elements regulate gene expression or differentially activate multiple genes, there is the problem of understanding how neighboring and potentially shared *cis*-control elements differentially regulate separate genes linked in a head-to-head arrangement. This is not a trivial issue since more than 10% of the human genes are closely linked (~1000 bp), divergently transcribed gene pairs (Adachi and Lieber, 2002, Doerwald, 2004, Labrador and Corces, 2002, Takai and Jones, 2004, Trinklein *et al.*, 2004). Bias towards bidirectional arrangement of genes appears to be the result of recent genome reorganizations and a unique feature of mammalian genomes (Koyanagi *et al.*, 2005). It has been suggested that the origin and evolution of bidirectional arrangement of genes may have its roots in the selective pressure imposed by the shared *cis* elements in the intergenic region on the expression patterns of the flanking genes (Adachi and Lieber, 2002, Koyanagi *et al.*, 2005, Takai and Jones, 2004, Trinklein *et al.*, 2004).

The *small heat shock protein (shsp)*/*αB-crystallin* and the related *myotonic dystrophy protein kinase binding protein (Mkbp)/*

Abbreviations used in this paper: DPE, downstream promoter element; GR, glucocorticoid receptor; mkbp, myotonic dystrophy kinase binding protein; NRE, negative regulatory element; shsp, small heat shock protein.

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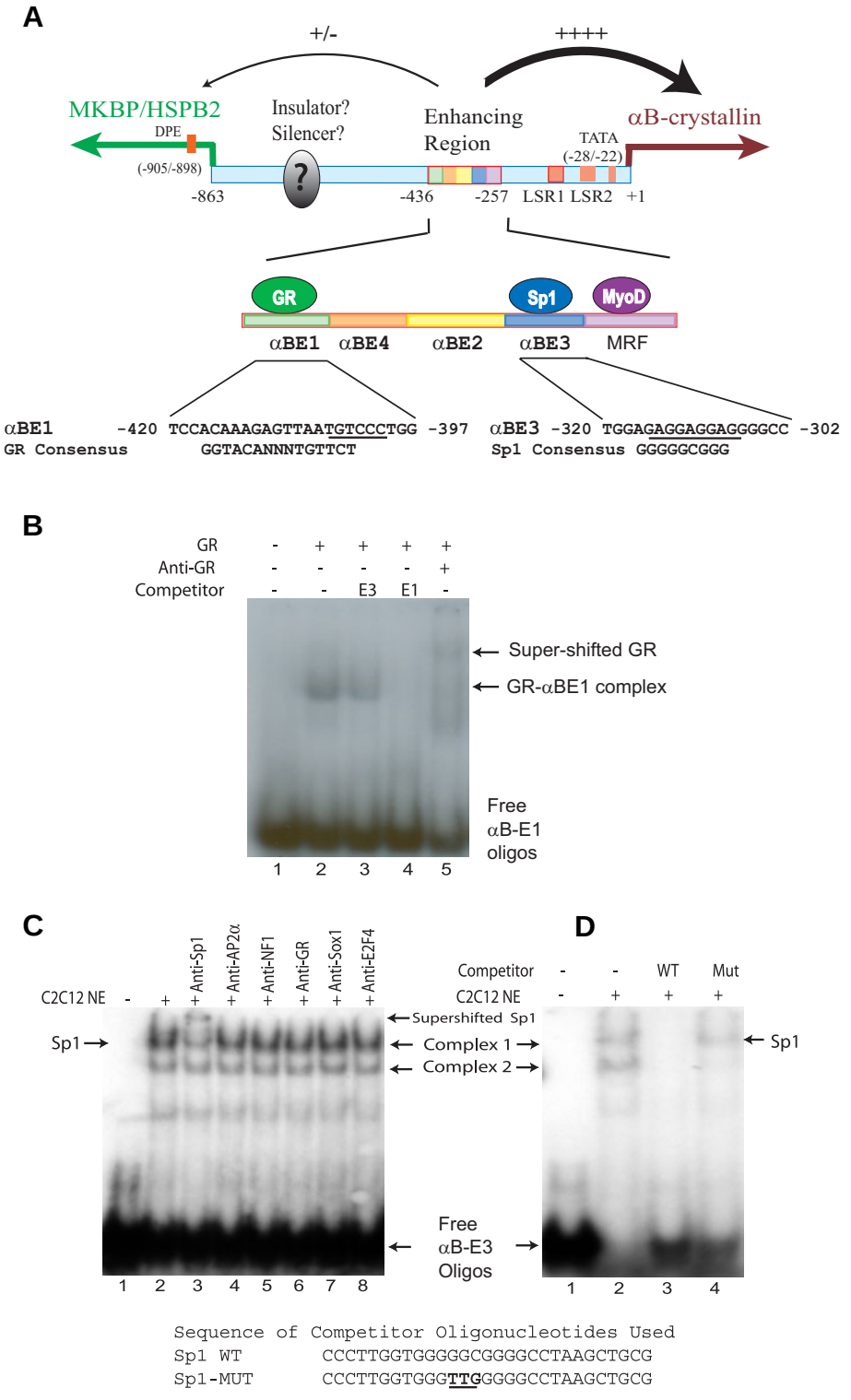


HspB2 genes provide a useful model of how shared intergenic sequences regulate the expression of divergently linked genes. For simplicity we refer to these genes as α*B-crystallin* and *HspB2*. The αB-crystallin protein is one of several lens crystallins - the abundant, water-soluble, cytoplasmic proteins that contribute to the transparent and refractive properties of the ocular lens - found in all vertebrates (de Jong *et al.*, 1989, Horwitz, 2003, Wistow and Piatigorsky, 1988). In mammals, at least ten members of the shsp family are expressed either in a constitutive or stress-inducible manner in different tissues (de Jong *et al.*, 1998, Franck *et al.*, 2004, Kappe *et al.*, 2003). HspB2 is a novel member of the shsp family that binds and activates myotonic dystrophy protein kinase (Iwaki *et al.*, 1997, Suzuki *et al.*, 1998). In mice, α*B-crystallin* and *HspB2* are arranged divergently with their transcription start sites separated by 863 bp (Iwaki *et al.*, 1997, Suzuki *et al.*, 1998) (Fig. 1A).

The expression patterns of the α*B-crystallin* and *HspB2* genes differ despite the presence of a small intergenic sequence containing gene regulatory elements that can be potentially shared. The α*B-crystallin* gene is inducible by physiological stress (Klemenz *et al.*, 1991) and is expressed abundantly in the lens and heart, moderately in the diaphragm, intestine and skeletal muscle and at low levels in kidney, spleen, liver, brain and retina (Bhat and Nagineni, 1989, Dubin *et al.*, 1989, Haynes *et al.*, 1996, Xi *et al.*, 2003). By contrast, the *HspB2* gene is neither stress inducible nor expressed in the lens and is expressed at low levels in the

Fig.1. Interaction of GR and Sp1 with elements αBE1 and αBE3, respectively. (A) *Schematic showing the known* cis *elements in the intergenic region of the mouse* αB-crystallin *and* HspB2 *genes. The -436/-257 intergenic enhancing region is expanded to show the organization of different* cis *elements. LSR1 and LSR2, lens specificity region 1 and 2, respectively. DPE, downstream promoter element. Sequence of elements αBE1 and αBE3, along with the consensus recognition sites of GR and Sp1 respectively, are shown. The glucocorticoid response element half site within the element αBE1 and the GAG trinucleotide repeats known to interact with transcription factors GR and Sp1 respectively are underlined.* **(B)** *Interaction of purified GR with αBE1. Double-stranded radiolabeled αBE1 oligonucleotides were incubated with (lanes 2-5) or without (lane1) purified GR. Specific double-stranded oligonucleotide competitors were included as indicated in lanes 3 and 4. The free αBE1 oligonucleotides, mobility shifted αBE1+GR complex (lanes 2 and 3) and super-shifted αBE1+GR+Anti-GR complex (lane 5) are indicated by arrows.* **(C)** *Interaction of Sp1 with labeled element αBE3. Double-stranded labeled αBE3 oligonucleotides were incubated with or without C2C12 nuclear extracts in the presence of different antibodies as shown. The slow-moving complex 1, which was super-shifted in the presence of anti-Sp1 antibody (lane 3), is indicated.* **(D)** *Double-stranded labeled αBE3 oligonucleotides incubated with C2C12 nuclear extracts in the presence of 100 molar excess of double-stranded competitors, Sp1 recognition site containing wild type oligonucleotides (WT) or mutant oligonucleotides not capable of binding Sp1 (Mut).*

skeletal muscle, heart and intestine (Iwaki *et al.*, 1997, Suzuki *et al.*, 1998, Swamynathan and Piatigorsky, 2002). A number of DNA regulatory elements have been identified for the mouse *αB-crystallin* gene. These include two *cis* elements (LSR1 and LSR2) conferring lens-specificity that interact with Pax6, Maf and RXR in the proximal promoter (Cvekl *et al.*, 2004, Gopal-Srivastava *et al.*, 1996, Gopal-Srivastava *et al.*, 1998, Gopal-Srivastava *et al.*, 1995, Gopal-Srivastava and Piatigorsky, 1994, Haynes *et al.*, 1997, Yang *et al.*, 2004) and an upstream enhancing region (-436/-258) containing at least 5 distinct *cis* elements in the intergenic sequence that increases αB-crystallin promoter activity in the lens and other tissues (especially heart and skeletal muscle) (Gopal-Srivastava *et al.*, 1995, Gopal-Srivastava and Piatigorsky, 1993, Swamynathan and Piatigorsky, 2002). No comparable studies have been performed delineating the *cis*-elements regulating *HspB2* gene expression. Phylogenetic footprint analysis by sequence alignment indicated that LSR1, LSR2 and the 5 *cis*-regulatory elements within the enhancer are conserved in mammals (Doerwald, 2004) (Fig. 1A).

We showed earlier that the intergenic enhancing region preferentially augments αB-crystallin promoter activity and is orientation-dependent in its natural context (Swamynathan and Piatigorsky, 2002), both traits consistent with the differences in the endogenous expression patterns of the *αB-crystallin* and *HspB2* genes. In addition, we suggested the existence of an insulator between the enhancing region and HspB2 promoter on the basis of sequence analysis and previously published transgenic mouse results (Swamynathan and Piatigorsky, 2002). In the present investigation we identify one *cis*-control element (αBE3, a Sp1-responsive site) within the enhancing region and two upstream E-boxes in the intergenic region that favor αB-crystallin promoter activity, another *cis*-control element (αBE1, a glucocorticoid responsive site) in the enhancing region that equally affects the activity of the two promoters and a sequence between the enhancing region and HspB2 promoter that acts as a negative regulatory element when tested in transfected cells in culture. These results provide new molecular insights suggesting that the differential use of shared *cis*-control elements contributes to the differences in the expression of the divergently transcribed *αB-crystallin* and *HspB2* genes in the mouse.

Results

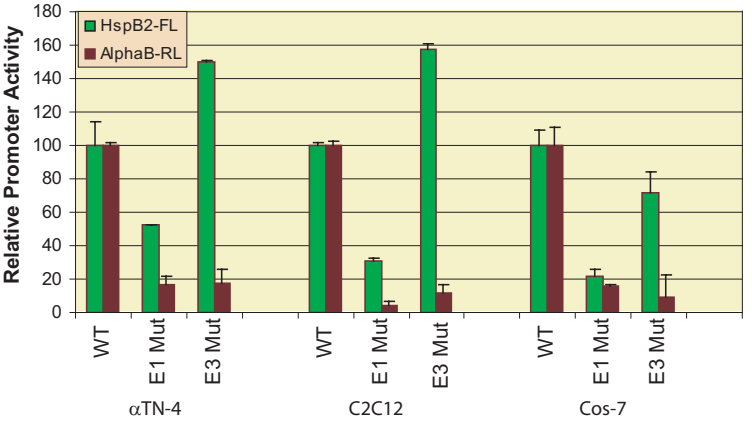
Candidate transcription factors for interaction with αBE1 and αBE3 within the enhancing region

Although 5 regulatory motifs (5' αBE1, αBE4, αBE2, αBE3 and MRF 3') have been delineated in the αB-crystallin enhancing region by DNase footprinting and functional studies (Gopal-Srivastava *et al.*, 1995, Gopal-Srivastava *et al.*, 2000, Gopal-Srivastava and Piatigorsky, 1993, Haynes *et al.*, 1995), few of the cognate transcription factors are known. We thus screened the TRANSFAC database of transcription factor recognition sites (http://www.cbil.upenn.edu/tess) (Schug, 2003) and identified the glucocorticoid receptor (GR) and Sp1 as candidate transcription factors for binding to sequences within αBE1 and αBE3, respectively. Even though glucocorticoid response elements (GRE) typically consist of a palindrome that can interact with a hormone bound GR dimer, GRE half sites also are effective in several promoters (Schoneveld *et al.*, 2004). The nucleotide sequence within αBE1 consists of a GRE half site, to which a GR monomer may bind (Segard-Maurel *et al.*, 1996) (Fig. 1A). Similarly, even though the hexanucleotide core GGGCGG is considered critical in the consensus Sp1 recognition site 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3', variations such as three repeats of the trinucleotide GAG present within αBE3 can bind Sp1 with high affinity (Marco *et al.*, 2003) (Fig. 1A). Based on these considerations, we pursued investigation of GR and Sp1 as candidates for interacting with elements αBE1 and αBE3, respectively.

Interaction of GR and Sp1 with αBE1 and αBE3

Interaction of GR with αBE1 was tested directly by incubating partially purified GR expressed in insect cells with radioactively labeled αBE1 oligonucleotides. GR formed a nucleotide sequence-specific complex with αBE1, which could be competed by non-labeled double-stranded αBE1 but not αBE3 oligonucleotide (Fig. 1B). Incubation of anti-GR antibody in the reaction mix resulted in a super-shift of the GR complex, consistent with GR interacting with the enhancing element αBE1 (Fig. 1B).

In electrophoretic mobility shift assays using labeled oligonucleotide αBE3 incubated with nuclear extracts from C2C12 muscle cells, 2 distinct complexes were observed (Fig. 1C). Anti-Sp1 antibody super shifted complex 1, consistent with Sp1 being a component of this complex. Treatment with other available

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Wild Type αBE1:-426 TAGGACTCCACAAAGAGTTAATGTCCCTGGGG
Mutant αBE1:   -426 TAGGACAGTCAGTCCTCACTCAACTTGACTGG

Wild Type αBE3:-326 CGAGACTGGAGAGGAGGAGGGGCCCACCAG
Mutant αBE3:   -326 CGAGACTCATATCTGACTACCATGCACCAG
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Fig. 2. Transient transfection assays showing the effect of mutations in elements αBE1 or αBE3 on αB-crystallin and HspB2 promoter activities. *Wild type, αBE1 mutant or αBE3 mutant pFL-HspB2 αB-RL plasmids were transfected into αTN-4, C2C12 or Cos-7 cells. αB-crystallin and HspB2 promoter activities measured using the αBE1 mutant or αBE3 mutant plasmids are shown relative to that obtained using the wild type plasmid. Nucleotide sequence of wild type and mutant elements αBE1 and αBE3 is shown below. Note that the endogenous expression level of αB-crystallin is about 30-100 fold higher than that of HspB2 in different tissues. Here, each mutant promoter activity is shown relative to the respective wild type promoter activity, which is set to 100.*

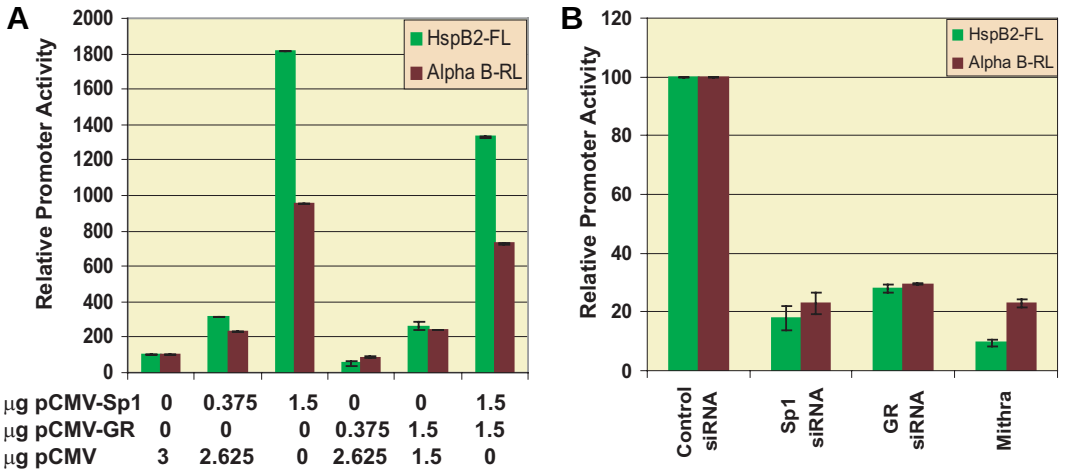

Fig. 3. Transient co-transfection assays showing the effect of modulating the intracellular levels of Sp1 or GR on αB-crystallin and HspB2 promoter activities. (A) *Effect of over-expression of Sp1 or GR. αB-crystallin and HspB2 promoter activities obtained upon co-transfection with plasmids pFL-HspB2 αB-RL and increasing amounts of pCMV-Sp1 or pCMV-GR in Cos-7 cells are shown relative to those obtained in co-transfections with empty vector pCMV.* **(B)** *Effect of suppression of Sp1 or GR. αB-crystallin and HspB2 promoter activities obtained upon co-transfection with plasmid pFL-HspB2 αB-RL and specific siRNAs to Sp1 or GR in Cos-7 cells are shown relative to those obtained in co-transfections with control siRNAs. Effect of treatment with mithramycin-A (mithra), a specific inhibitor of Sp1, on αB-crystallin and HspB2 promoter activities also is shown. Note that the endogenous expression level of αB-crystallin is about 30-100 fold higher than that of HspB2 in different tissues. Here, each promoter activity is shown relative to the respective basal promoter activity, which is set to 100.*

antibodies to different transcription factors (AP2-α, NF1, GR, Sox1 and E2F4) did not affect the formation of complex 1 (Fig. 1C). In competition experiments, inclusion of 100 molar excess of non-labeled Sp1 consensus double-stranded oligonucleotides in the reaction mix abolished the formation of complexes 1 and 2 (Fig. 1D). When similar amounts of oligonucleotides mutated in the Sp1 binding site were used for competition, only complex 2 was abolished (Fig. 1D). From these results, we conclude that the complex 1 includes Sp1, while complex 2 does not.

Mutational analysis of αBE1 and αBE3

In order to test the functional roles of αBE1 and αBE3 on the αB-crystallin and HspB2 promoters, we introduced specific mutations in elements αBE1 or αBE3 by recombinant PCR-mediated ligation and cloned the mutated fragments in between the firefly and *Renilla* luciferase reporter genes in the bidirectional dual reporter vector (Swamynathan and Piatigorsky, 2002). In this vector, the expression of the divergently arranged firefly and *Renilla* luciferase reporter genes is controlled by the HspB2 and αB-crystallin promoters respectively, mimicking the endogenous organization of the *HspB2* and *αB-crystallin* genes. Transient transfection analysis using αTN-4, C2C12 or Cos-7 cells showed that the mutation in αBE1 reduced the αB-crystallin and HspB2 promoter activities by 6 to 10-fold and 2 to 5-fold, respectively (Fig. 2). Upon mutagenesis of αBE3, αB-crystallin promoter activity was reduced by 6 to 10-fold, while HspB2 promoter activity was marginally increased in αTN-4 and C2C12 cells (by 1.5- and 1.58-fold, respectively) and marginally decreased in Cos-7 cells (to 72% of the WT) (Fig. 2). Therefore, αBE1 activates the flanking promoters in a bidirectional manner, while αBE3 activates the αB-crystallin promoter in a unidirectional manner with a moderate, variable and cell type dependent influence on HspB2 promoter.

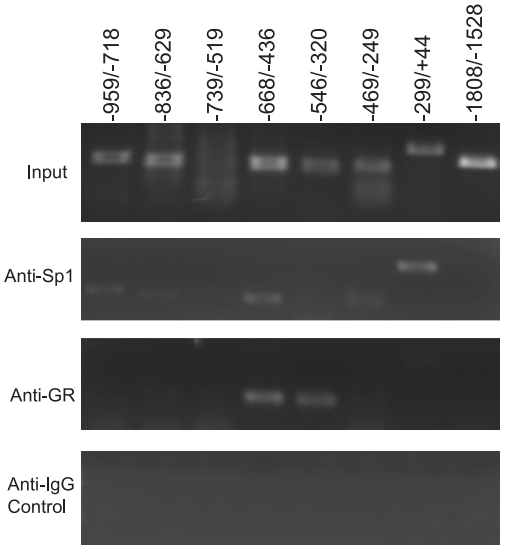

Fig. 4. Identification of *in vivo* association of Sp1 and GR with the intergenic fragment by chromatin immunoprecipitation (ChIP). *Cross-linked chromatin isolated from C2C12 cells was immunoprecipitated with anti-Sp1, anti-GR or negative control anti-IgG antibodies. After cross links were removed and the associated chromosomal DNA fragments purified, PCR was performed with specific primer pairs. PCR products resulting from different starting materials, (i) input DNA, (ii) anti-Sp1 antibody immunoprecipitated DNA, (iii) anti-GR antibody immunoprecipitated DNA and (iv) negative control anti-IgG immunoprecipitated DNA used as target are shown. Nucleotide position of each amplified fragment is shown on top.*

Effects of modulating the intracellular levels of Sp1 and GR on αB-crystallin and HspB2 promoter activities

The effect of increasing the intracellular levels of Sp1 and GR on αB-crystallin and HspB2 promoter activities was studied by co-transfection experiments with plasmids expressing Sp1 or GR. Cos-7 cells were used for these experiments because they support a relatively lower level of αB-crystallin promoter activity than the transfected αTN-4 and C2C12 cells, thus obviating a high background difficulty. αB-crystallin and HspB2 promoter activities were upregulated 10 and 18-fold respectively, by pCMV-Sp1 and 3 and 2.5-fold respectively, by pCMV-GR (Fig. 3A). There was no synergistic upregulation when pCMV-Sp1 and pCMV-GR were used

together in co-transfection experiments (Fig. 3A). The effect of decreasing the intracellular levels of Sp1 and GR on αB-crystallin and HspB2 promoter activities was studied using specific siRNAs. Compared to control siRNAs, co-transfection of Sp1- or GR-specific siRNAs resulted in approximately 5-fold reductions in αB-crystallin and HspB2 promoter activities in Cos-7 cells (Fig. 3B). In other tests, when transfected cells were treated with mithramycin-A, a specific inhibitor of transcription factor Sp1 (Christensen *et al.*, 2004), αB-crystallin and HspB2 promoter activities decreased by 5 and 10-fold, respectively (Fig. 3B).

In vivo *interactions of Sp1 and GR with the intergenic region*

Mutations in the Sp1 binding element αBE3 selectively lowered αB-crystallin promoter activity (Fig. 2). However, co-transfection experiments demonstrated that the activities of both the αB-crystallin and HspB2 promoters are elevated in response to Sp1 (Fig. 3). A likely explanation for this apparent discrepancy is that there are additional Sp1 binding sites within the intergenic region. Consistent with the idea that Sp1 influences the activity of αB-crystallin and HspB2 promoters at multiple binding sites, several potential Sp1-binding sites were identified within the intergenic region (-860/-853, -820/-800, -748/-738, -399/-388, -250/-240 and -21/-10) by the transcription element search software (URL: http://www.cbil.upenn.edu/tess) (Schug, 2003). Evidence that functional Sp1 binding sites occur at these sites was obtained by scanning the intergenic region by ChIP analysis. Sp1 was associated with the following fragments along the intergenic region: -959/-718, -836/-629, -668/-436, -469/-249 and -299/+44. By contrast, GR was associated only with the -668/-436 and -546/-320 fragments (Fig. 4). The precise location of individual Sp1- or GR-binding *cis*-elements within these fragments remains to be confirmed by other methods. Association of Sp1 with numerous sites explains why both the promoters are responsive to Sp1 even though the Sp1- binding site αBE3 selectively activates the αB-crystallin promoter.

Features of the nucleotide sequence between the αB-crystallin enhancing region and the HspB2 promoter

A discrete AT- rich stretch immediately upstream of the enhancing region (-636/-436 fragment, 42 % GC content) is followed by a GC-rich stretch proximal to the HspB2 promoter (-836/-636, 62 % GC content) (Fig. 5A). Within the GC-rich -836/-636 fragment, there are 14 repeats of CCCTC (or one nucleotide variants of this motif) that show extensive similarities with binding sites for CTCF (Chung *et al.*, 1997, Farrell *et al.*, 2002, Filippova *et al.*, 1996, Ohlsson *et al.*, 2001, Vostrov and Quitschke, 1997), the insulator-binding zinc finger protein (Bell and Felsenfeld, 1999, Bell *et al.*, 1999, Ohlsson *et al.*, 2001) (Fig. 5B). Insulators are position-dependent enhancer blockers that restrict the effects of an enhancer to a specific promoter in the vicinity of multiple promoters. Interaction with the zinc-finger protein CTCF is both necessary and sufficient for insulators to work (Bell and Felsenfeld, 1999, Bell *et al.*, 1999, Bell *et al.*, 2001). We thus tested the -836/-519 sequence for the presence of insulator activity by an enhancer blocking assay. An insulator located between the αB-crystallin enhancing region and the HspB2 promoter would contribute to the selective influence of the enhancing region on the αB-crystallin promoter.

Enhancer blocking assays reveal a potential NRE in the -836/-622 sequence

Enhancer blocking assays using stable transfection of linear fragments of DNA integrated into the genome are useful for identifying insulator elements (Chung *et al.*, 1997, Chung *et al.*, 1993, Recillas-Targa *et al.*, 1999). In the present experiments we cloned overlapping fragments from the -836/-519 intergenic sequence 3‘ or 5‘ to the *globin* enhancer in a plasmid (pNI CD) containing the *neomycin resistance* gene driven by the globin promoter (Fig. 5C) and tested for the number of colonies resistant to G418.

The -836/-519 fragment inserted between the globin enhancer and promoter blocked the effect of the globin enhancer on promoter activity in an orientation-independent manner (Fig. 5C). The 3-4 fold reduction due to the -836/-519 fragment was comparable to the 4-5 fold reduction achieved with the known *globin* FII insulator in pNI CD (Chung *et al.*, 1997), which was used as a positive control in these tests. When smaller overlapping sequences were tested, only the -836/-622 fragment was an effective insulator. Neither the -785/-572 nor the -739/-519 fragment

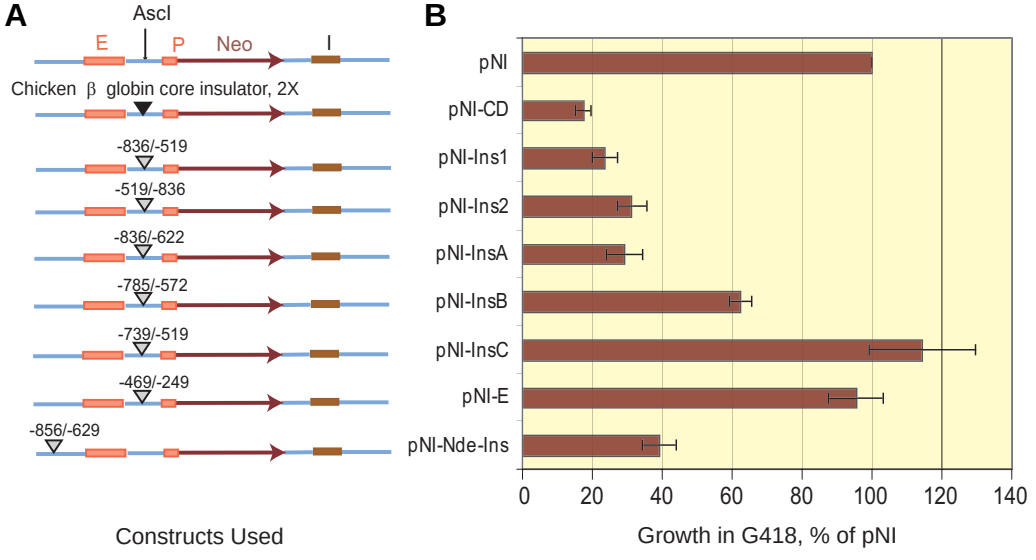

Fig. 5. Identification of a negative regulatory element (NRE) in between the αB-crystallin enhancing region and the HspB2 promoter. *Enhancer blocking assays identify an NRE in -836/-622 bp region.* **(A)** *A schematic showing the different fragments tested. pNI in which the globin promoter and enhancer drive the expression of the* neomycin resistance *gene is the base vector. Filled in triangle represents the chicken β-globin core insulator; empty triangles represent different fragments tested from the intergenic region, as shown.* **(B)** *The average percentage of G418-resistant colonies obtained with different constructs relative to that obtained with pNI.*

showed insulator activity, consistent with the core insulator activity residing within the -836/-739 fragment. The -469/-249 enhancing region fragment, used as a negative control for insulator function, did not affect globin promoter activity. Surprisingly however, the -856/-629 fragment cloned 5' to the enhancer-promoter-*neomycin resistance* gene cassette (pNI-Nde-856/-629) also reduced globin promoter activity (Fig. 5). Since insulators are position-dependent while negative regulatory elements (NREs) are not, these results suggest that the -836/-739 fragment harbors a NRE as well as, if not instead of an insulator.

Interaction of nuclear factors with the NRE

In order to identify the nucleotides within the -836/-628 fragment interacting with nuclear factors, we performed DNase I

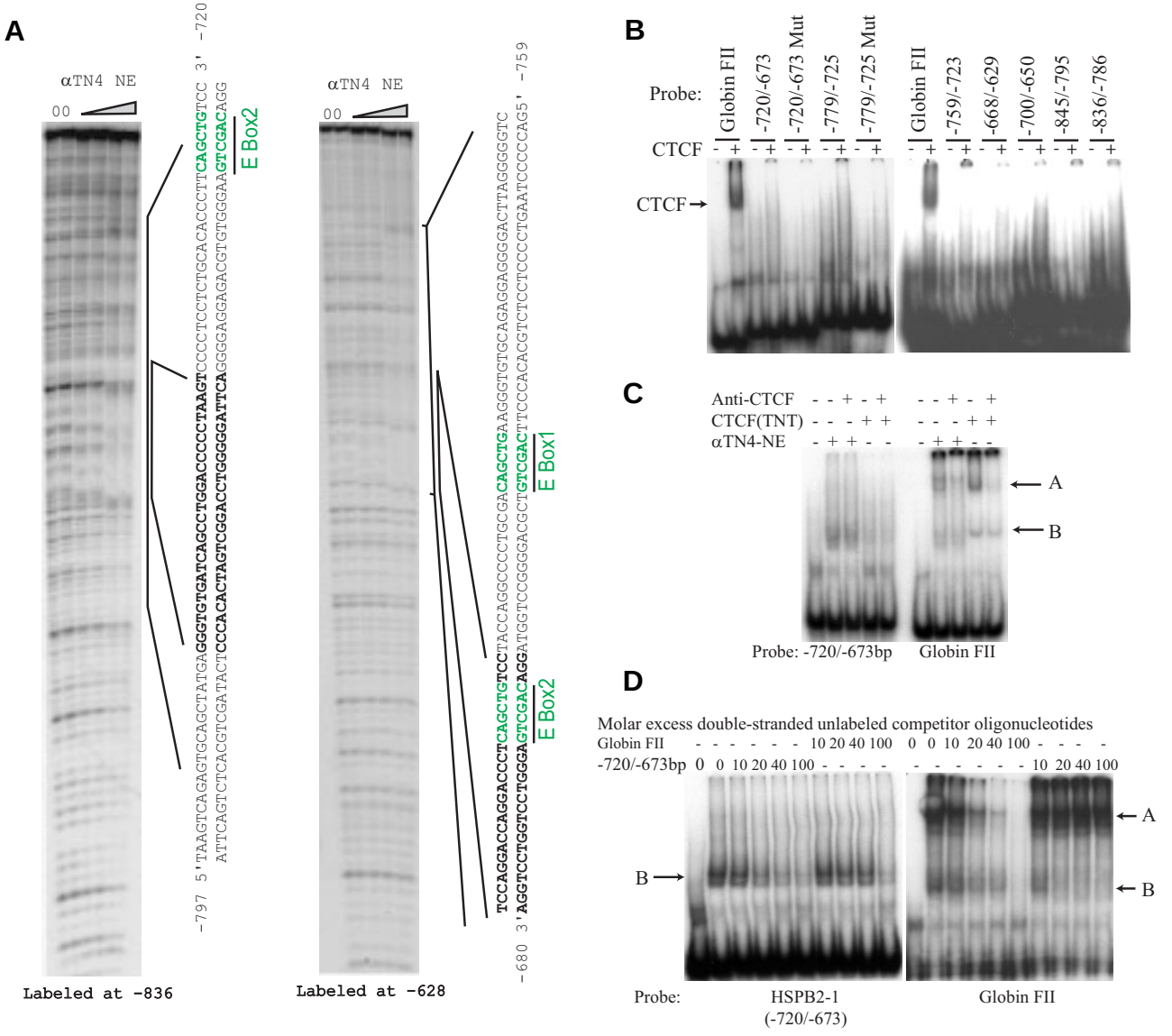

Fig. 6. Interaction of nuclear proteins with the -836/-628 fragment. (A) *DNase I footprint analysis of the -836/-628 fragment with nuclear extracts from lens epithelial αTN-4 cells. Results obtained with the DNA fragment labeled at the -836 end (left panel) and at -628 end (right panel) are shown. Sequence of protected nucleotides deduced from a sequencing reaction run in parallel is shown by the side. The sequence of the two conserved E-box elements is indicated.* **(B)** *The insulator protein CTCF does not interact with different oligonucleotides from the -836/-629 fragment. Electrophoretic mobility shift assays with partially purified CTCF expressed as 6 X histidine fusion protein in* E. coli *incubated with different labeled oligonucleotides (see Materials and Methods for their sequence). While the positive control globin FII oligonucleotides were efficiently bound by CTCF, none of the tested oligonucleotides from the -836/-629 region showed similar binding.* **(C)** *Electrophoretic mobility shift assays with αTN-4 nuclear extracts or CTCF produced* in vitro *by coupled transcription and translation reactions using rabbit reticulocyte lysate and anti-CTCF antibody as shown. Note that the formation of complex A with globin FII oligonucleotides is abolished in the presence of anti-CTCF antibody, indicating that CTCF is responsible for this complex. Comparable complex A was not formed by the -720/-673 oligonucleotide.* **(D)** *Competition with increasing amounts of unlabeled double-stranded oligonucleotides as shown using either labeled -720/-673 oligonucleotides (left panel) or labeled globin FII oligonucleotides (right panel). Note that the complex B in both panels is competed by both globin FII and -720/-673 oligonucleotides, while the complex A due to CTCF is competed only by globin FII oligonucleotides and not by -720/-673 oligonucleotides.*

footprint analysis. Extended regions of protection after DNase I digestion were present between sequences -797 and -680, within which there were shorter stretches of stronger protection (Fig.6A). The DNase I-protected regions corresponded to the most conserved nucleotides in this region including the two E-box elements identified by sequence comparison across different species (Doerwald, 2004), consistent with these sequences having an important role to play in the regulation of the flanking genes. Furthermore, the protected sequences correlated with the putative CTCF-binding sites, raising the possibility that the protein factor responsible for protection from DNase I may be CTCF.

In order to test whether CTCF interacts with the -797/-680 fragment we performed electrophoretic mobility shift assays (EMSAs) with histidine-tagged CTCF protein expressed in *E. coli* and with various end-labeled oligonucleotides from the -845/-629 fragment. While the positive control oligonucleotides comprising the *globin* FII insulator bound CTCF, none of the oligonucleotides from the -845/-629 intergenic region did (Fig. 6B). EMSAs performed with αTN4 mouse lens epithelial cell nuclear extracts or with CTCF produced *in vitro* by coupled transcription and translation reactions showed that the control *globin* FII insulator bound CTCF, while the -720/-673 intergenic fragment did not (Fig. 6C). We obtained similar results with other oligonucleotides from the -845/-629 fragment (data not shown). In competition experiments, complex B was abolished with increasing molar excess of the unlabeled -720/-673 fragment or of the *globin* FII insulator (Fig. 6D). However, the formation of complex A (CTCF) was competed only by the non-labeled *globin* FII insulator but not by the -720/-673 oligonucleotide. Based on these results, we conclude that CTCF has little if any, affinity for the -720/-673 oligonucleotide (Fig. 6D). Taken together, these results are consistent with the -836/-629 intergenic fragment being a negative regulatory element (NRE) rather than a CTCF-binding insulator.

Regulatory activity of two E-box elements

Our DNase I footprint showed that a pair of well conserved E-box elements (-726/-721 and -702/-697) (Doerwald, 2004) contained within the putative NRE bind nuclear proteins (see Fig. 6A). Clusters of E-box elements are known to be associated with negative (Naghavi *et al.*, 2001, Neuman *et al.*, 1993, Tesmer *et al.*, 1993, Yan *et al.*, 2001, Yoshida *et al.*, 2001) and/or positive regulatory activity (Calomme *et al.*, 2002, Kraner *et al.*, 1998, Nguyen *et al.*, 2003). In order to test if any regulatory activity is associated with these putative E-box binding sites, we introduced point mutations in either E-box1 (-726/-721) or E-box2 (-702/-697), or both (Fig. 7A) and then performed functional tests using transient transfection assays and transgenic mice. Mutations in either of the E-box elements reduced the αB-crystallin promoter activity near basal levels without affecting the low level of HspB2 promoter activity in transfected C2C12 cells (Fig. 7B). Unexpectedly, the αB-crystallin promoter activity was partially regained when both E-box elements were mutated (Fig. 7B). Similar results were obtained in αTN-4 and Cos-7 cells (results not shown). In transgenic mice, both αB-crystallin and HspB2 promoter activities were reduced in lens, heart, skeletal muscle and lung, although to differing extents, as a result of mutations in either or both of the E-box elements (Fig. 7C). In liver, however, the mutant transgenes were more active than their wild type counterpart and there were small and variable effects in kidney and spleen (Fig. 7C). In spite of the proximity of the E-box elements to the HspB2 promoter, the αB-crystallin promoter activity was proportionally more affected than the HspB2 promoter in these mutants (Fig. 5C). Taken together the data suggest that E-box1 (-726/-721) and E-box2 (702/-697) can have variable positive or negative, tissue-specific effects on αB-crystallin and HspB2 promoter activities.

Discussion

We showed previously that the intergenic enhancing region between the *αB-crystallin* and *HspB2* genes preferentially influences the αB-crystallin promoter and that the αB-crystallin promoter is 30-100 fold more active than the HspB2 promoter in different tissues (Swamynathan and Piatigorsky, 2002). The previous data also showed, unexpectedly, that the intergenic enhancing region is orientation-dependent within its normal context, resulting in strong preferential activation of the αB-crystallin promoter over the HspB2 promoter (Swamynathan and Piatigorsky, 2002). Here we report on four different intergenic control elements that contribute to the regulation of the linked, divergently transcribed mouse *αB-crystallin* and *HspB2* genes. Among the *cis*- elements within the enhancing region, αBE1 activates both the αB-crystallin and HspB2 promoters equally, while αBE3 selectively activates the αB-crystallin promoter. Two conserved E-box elements located upstream of the enhancing region affect both the promoters, albeit the αB-crystallin promoter preferentially, in a tissue-specific fashion. Finally, the fragment located between the E-box elements and the HspB2 promoter has negative regulatory potential when tested with foreign promoters in transfected cells.

The *cis*- element αBE1 (-420/-396) binds GR and presumably contributes to the widespread expression of the *αB-crystallin* gene (Bhat and Nagineni, 1989, Dubin *et al.*, 1989). This sequence is contained within the -426/-257 enhancing region that is responsible for αB-crystallin promoter activity in all tissues but lens and cornea during development of transgenic mice (Gopal-Srivastava *et al.*, 1995, Gopal-Srivastava *et al.*, 2000, Gopal-Srivastava and Piatigorsky, 1993, Haynes *et al.*, 1996). αBE1 is also within the -465/-398 fragment identified previously to be critical for glucocorticoid responsiveness of the *αB-crystallin* gene (Scheier *et al.*, 1996). Deletion of this part of the enhancing region virtually abolishes the ability of the αB-crystallin promoter to function in all tissues but lens and cornea in transgenic mice (Gopal-Srivastava *et al.*, 2000). The existence of a functional GR element within the enhancing region is consistent with the presence of functional GR in rat, mouse and human lens epithelial cells (Gupta and Wagner, 2003, James *et al.*, 2003) and with the accumulation of αB-crystallin mRNA over a period of several days in cultured non-lens cells treated with the synthetic glucocorticoid, dexamethasone (Nedellec *et al.*, 2002, Scheier *et al.*, 1996). It is noteworthy that the αBE1 element has a bidirectional positive influence on the flanking αB-crystallin and the HspB2 promoters. Whether αBE1, located within the orientation-dependent enhancing region (Swamynathan and Piatigorsky, 2002), is orientation-dependent remains to be tested.

The significant reduction of αB-crystallin promoter activity and a modest, variable and cell type dependent elevation of HspB2 promoter activity upon mutagenesis of αBE3 indicate that αBE3 selectively activates the αB-crystallin promoter, consistent with

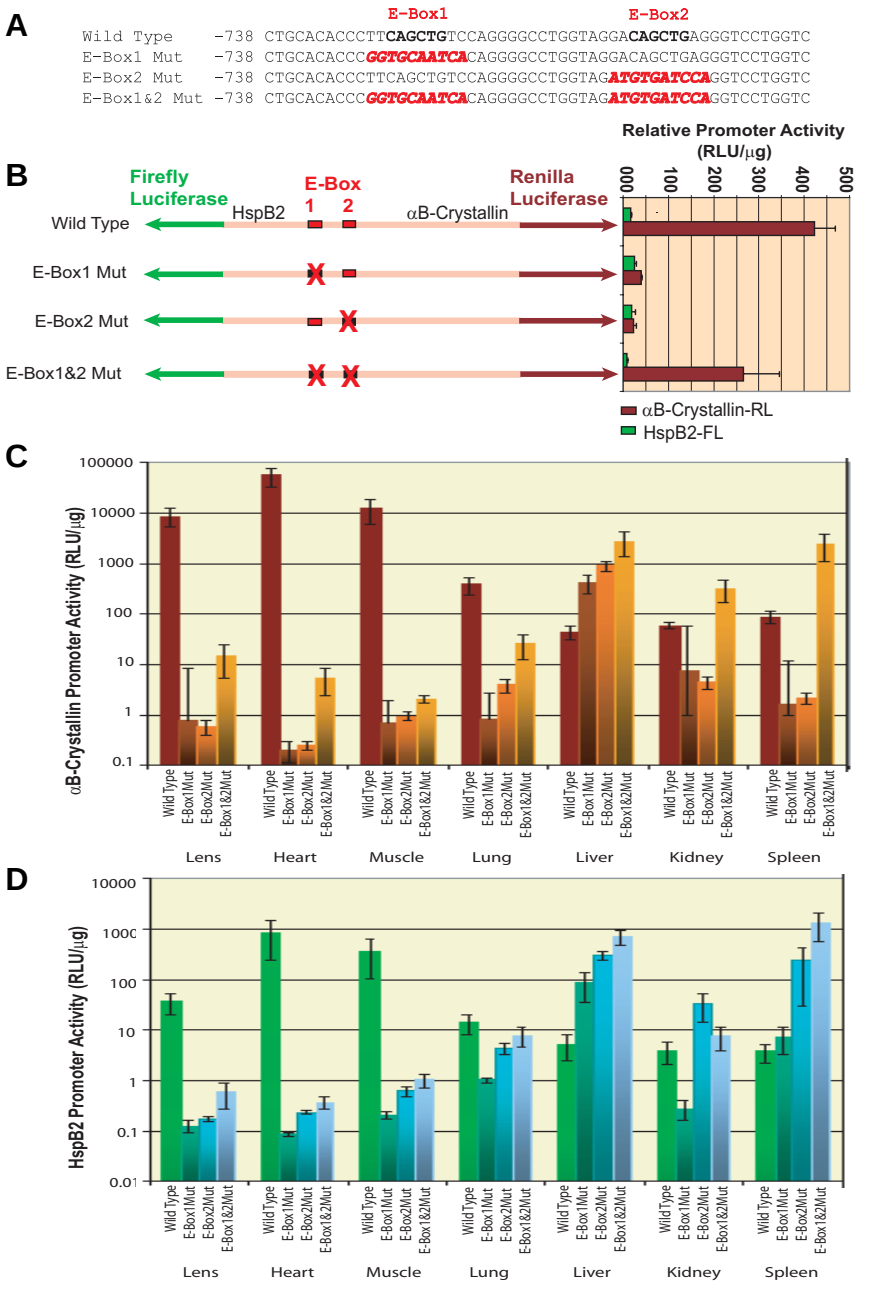

Fig. 7. Influence of the -726/-721 and -702/-697 E-box elements on αB-crystallin and HspB2 promoter activities in transfected cells and transgenic mice. (A) *Sequence of wild type (shown in bold) and mutated (shown in red italics) E-box elements.* **(B)** *Schematic representation of the bidirectional dual luciferase reporter vectors used is shown on the left. Relative αB-crystallin and HspB2 promoter activities in C2C12 cells transfected with different plasmids as indicated, presented as relative luciferase units per microgram protein (RLU/μg) in the cell extract used for the assay, is shown on the right.* **(C)** *αB-crystallin promoter-driven Renilla luciferase reporter gene activities in the lens, heart, skeletal muscle, lung, liver, kidney and spleen of transgenic mice generated with the wild type or mutated E-box element containing intergenic region, presented as relative luciferase units per microgram protein (RLU/μg) in the extract used.* **(D)** *HspB2 promoter-driven Firefly luciferase reporter gene activities in the lens, heart, skeletal muscle, lung, liver, kidney and spleen of transgenic mice generated with the wild type or mutated E-box element containing intergenic region, presented as relative luciferase units per microgram protein (RLU/μg) in the extract used. Note that logarithmic scale is used for presentation of data in (C,D).*

the preferential and orientation-dependent influence of the intergenic enhancing region on αB-crystallin promoter (Swamynathan and Piatigorsky, 2002). However, HspB2 promoter activity was activated 18-fold by pCMV-Sp1 and reduced by about 5-fold by anti-Sp1 siRNAs in cotransfection experiments, suggesting that the HspB2 promoter is activated by Sp1. The strong positive influence of Sp1 in cotransfection assays and moderate and variable effect of mutagenesis of the Sp1 binding element αBE3 on HspB2 promoter indicate that Sp1 may modulate the HspB2 promoter activity through additional Sp1 recognition sites present close to the HspB2 promoter (-860/-853, -820/-800 and -748/-738).

Ability of E-box elements to activate (Calomme *et al.*, 2002, Esumi *et al.*, 2004, Kraner *et al.*, 1998, Nguyen *et al.*, 2003) or suppress (Garami and Gardner, 1996, Naghavi *et al.*, 2001, Neuman *et al.*, 1993, Tesmer *et al.*, 1993, Yan *et al.*, 2001, Yoshida *et al.*, 2001) promoter activity is well documented. Indeed, our mutagenesis experiments using transgenic mice also indicate that the two E-box elements at positions -726/-721 and -702/-697 in the *αB-crystallin/HspB2* intergenic region can increase or reduce promoter activity of the two genes in a tissue-specific fashion. Despite the close proximity of these E-box elements to the HspB2 promoter, their activation effect appears stronger on the distant αB-crystallin promoter than on the HspB2 promoter due largely to the low strength of the latter relative to the former. Further studies are necessary to determine whether these E-box elements interact with myogenic regulatory factors, as might be expected considering their role in numerous muscle active E-box containing promoters (Rescan, 2001).

We suggested previously on the basis of sequence analysis and transgenic mouse results that the -836/-622 intergenic sequence might insulate the HspB2 promoter from the enhancing region (Swamynathan and Piatigorsky, 2002). Our present results indicate that the -836/-622 sequence acts as a negative regulatory element (NRE) rather than a classical insulator as judged by its ability to repress globin promoter activity in a position-independent fashion in transfected cells. This interpretation is consistent with the fact that the candidate CTCF binding sites in this region neither match the phylogenetic footprints among different mammals (Doerwald, 2004) nor bind CTCF (see Fig. 7). It thus appears that the similarity of these sequences to CTCF binding sites is fortuitous. The possibility that this putative NRE preferentially reduces the activity of its neighboring HspB2 promoter warrants further investigation.

In addition to being a remarkable example of the numerous adaptations that have evolved to coordinate the differential expression of closely linked, divergently arranged genes, the mouse *αB-crystallin/HspB2* intergenic region provides additional insight into the context dependency of regulatory elements. A previous example of such context dependency of *cis*-control elements within the *αB-crystallin/HspB2* intergenic region was demonstrated by the orientation-dependence of the enhancing region in its natural environment (Swamynathan and Piatigorsky, 2002) and its orientation-independence when tested in a foreign environment (Dubin *et al.*, 1991). Another example consistent with context-dependency is the striking change in tissue-specific activity of the mouse αB-crystallin promoter that contains a two nucleotide mutation in an apparently neutral stretch at the 3' end of the enhancing region (Li *et al.*, 2007). Despite that the mutated region can be deleted without eliminating lens promoter activity (Gopal-Srivastava *et al.*, 2000), lens promoter activity is virtually abolished by the mutation when it is present in the promoter fragment (Li *et al.*, 2007). Similarly, in the present study, site-specific mutations within the two E-box elements in the intergenic region markedly reduce promoter strength in lens and muscle of transgenic mice, yet the activity of truncated promoters lacking these E-boxes remains intact in these tissues (Gopal-Srivastava *et al.*, 2000, Swamynathan and Piatigorsky, 2002). It thus appears that these E-box elements may have a significant regulatory function within the natural promoter, but lens-specific activity of a truncated promoter function can compensate for their absence. It is also possible that the mutant E-box elements have simply gained a negative function by acquiring the ability to bind an inappropriate protein. The selective action of the Sp1: αBE3 site within the enhancing region on the αB-crystallin promoter may also be an example of context dependent specialization of Sp1 regulatory function. The putative NRE within the αB-*crystallin/HspB2* intergenic region represents yet another example of context dependent gene regulatory sequences. This DNA region contains multiple sites similar to the CTCF binding sites characteristic of insulators, yet we show here that it does not bind CTCF, suggesting that it is a negative regulator rather than an insulator. The absence of CTCF binding in this region supports the suggestion that DNA-binding zinc finger proteins (such as CTCF) are optimized for specificity by context dependency rather than affinity (Havranek *et al.*, 2004). It remains to be shown whether or not the putative NRE does indeed have a negative regulatory role within its natural context *in vivo*.

In conclusion, the potentially striking consequences of site-specific mutations on context- dependent utilization of gene regulatory elements have important implications for evolution. One implication is the provision of a powerful mechanism for evolving new patterns of gene expression by reassigning functional values of regulatory elements that result in novel evolutionary changes and/or new protein functions by a gene sharing mechanism (see (Piatigorsky, 2007)). The context dependency of regulatory sequences augments an already dynamic view of the genome during evolution whereby the mutation of any nucleotide may significantly change gene regulation in a quantitative and qualitative manner, even if the altered nucleotide appears to be neutral by deletion experiments or by being poorly conserved in homologous regions of other species. These far-reaching consequences of strong context-dependency of gene regulatory sequences add considerable interest into unraveling further the interrelationships of the *cis*-control elements within the complex αB-*crystallin/HspB2* intergenic region.

Materials and Methods

Cell culture, transfections, dual luciferase assays

Human erythroleukemia K562 cells from Dr. Felsenfeld, NIDDK, NIH, were cultured in IMEM containing 10 % fetal bovine serum (FBS), antibiotics penicillin and streptomycin and antifungal gentamycin. Murine lens epithelial αTN4 (Yamada *et al.*, 1990), myoblast C2C12 (Yamada *et al.*, 1990) and rabbit lens epithelial N/N1003 cells (Reddan *et al.*, 1986) were maintained in Dulbeccos Modified Eagles Medium (DMEM) containing 10% FBS, antifungal gentamycin, antibiotics penicillin and streptomycin. Plasmids pCMV-Sp1 and pCMV-GR were purchased from Open Biosystems, Huntsville, AL. The sequence of siRNAs against GR and Sp1 purchased from Ambion Inc. (Austin, TX) is as follows: Sp1 siRNA (sense strand): GGUGAACUUGACCUCACAGtt, Sp1 siRNA (antisense strand): CUGUGAGGUCAAGUUCACCtg, GR siRNA (sense strand): GGUUUCUGCGUCUUCACCCtt, GR siRNA (antisense strand): GGGUGAAGACGCAGAAACCtt. Different siRNAs were transfected along with the test plasmids in co-transfection assays. All cells were grown in humidified chamber containing air with 5% CO_2 at 37° C. 5 X 10^5 cells in mid-log phase of growth in 100 mm dishes were transfected with 1 μg of plasmids using 3 μl of FuGENE-6 (Roche Molecular Biochemicals, Indianapolis, IN). 10 ng of pCMV-Gal plasmid was included in all transfections in order to normalize the efficiency of transfection across treatments. After 2 days, cells were lysed with 500 μl of passive lysis buffer (Promega, Madison, WI). Luciferase activities in 100 μg (or normalized amount based on β-galactosidase assays) of lysate were measured using appropriate reagents from Promega and a Tropix TR717 microplate luminometer (Applied Biosystems, Foster City, CA). β-galactosidase assays were performed as described previously, using chlorophenol red-β-D-galactopyranoside (CPRG) (Boehringer Mannheim, Indianapolis, IN) as the substrate (Swamynathan and Piatigorsky, 2002).

Mutagenesis and construction of reporter vectors

Unless otherwise noted, all nucleotide positions used here are relative to *αB-crystallin* transcription start site. Construction and use of the bidirectional dual luciferase vector pFL-HSPB2 αB-RL in which the firefly and Renilla luciferase reporter genes are under the control of intergenic HspB2 and αB-crystallin promoters respectively, has been described earlier (Swamynathan and Piatigorsky, 2002). Mutations in elements αBE1, αBE3, E-box 1 and E-box2 were introduced by recombinant PCR mediated ligation as described below using oligonucleotides synthesized by Integrated DNA Technologies, Coralville, IA. The intergenic fragments upstream of the elements αBE1, αBE3, E-box 1 or E-box 2 were amplified using:

upstream primer 8703(-959): GACTGCTGTTGCGACTAGTAGC and downstream primer:

E1 Mut-2 (5' to 3'):

AGTCAAGTTGAGTGAGGACTGACTGTCCTAGAGGAGAGCAGAACTAG

E3 Mut-2:

CATGGTAGTCAGATATGAGTCTCGCATGCCAGGGGAATTGAG

E-box 1 Mut-2:

TGATTGCACCGGGTGTGCAGAGGAGGGGACTTAG, or

E-box 2 Mut-2: **TGGATCACAT**CTACCAGGCCCCTGCGACAGCTGA, respectively.

Similarly, the intergenic fragment downstream of the elements αBE1, αBE3, E-box 1 or E-box 2 was amplified using:

downstream primer 8704 (+42): GGCTAGATGAATGCAGAGTC and upstream primer:

E1 Mut-1 (5' to 3'):

AGTCAGTCCTCACTCAACTTGACTGGCTAAGCCTAGGAAGATTCCAG

E3 Mut-1:
TCATATCTGACTACCATGCACCAGCAGCTGCTTGGGATTCCA
E-box 1 Mut-1: **GGTGCAATCA**GCAGGGGCCTGGTAGGACAGCT,or
E-box 2 Mut-1: **ATGTGATCCA**GGTCCTGGTCCTGGAGTGAGCT,
respecively.

The bold and underlined 5' half of primers E1 Mut-1, E3 Mut-1, E-box 1 Mut-1 and E-box 2 Mut-2 contain mutations in elements αBE1, αBE3, E-box 1 and E-box 2 and are complementary to the 5' half of primers E1 Mut-2, E3 Mut-2, E-box 1 Mut-2 and E-box 2 Mut-2, respectively. The upstream and downstream PCR products containing complementary mutations were then purified, mixed together, denatured, annealed and ligated by means of PCR using primers 8703 and 8704, to generate the -959/+42 bp fragment with specific mutations in element αBE1, αBE3, E-box 1 or E-box 2. The fragments so produced were cloned into the SmaI site of pRLFL-Null vector described previously (Swamynathan and Piatigorsky, 2002), the orientation of the intergenic fragment was tested by restriction fragment analysis and the resultant plasmids were labeled as pFL-HspB2 αB-RL E1 Mut, E3 Mut, E-box 1 Mut or E-box 2 Mut, respectively. Desired mutations were confirmed by sequencing each of the final plasmid constructs.

Generation and analysis of transgenic mice

Studies using transgenic mice were performed in accordance with the guidelines set forth by the Animal Care and Use Committee at the NEI. Plasmids pFL-HspB2 αB-RL E-box 1 Mut or pFL-HspB2 αB-RL E-box 2 Mut were digested with BamHI to release the fragment containing the *firefly* and *Renilla luciferase* genes under the control of the intergenic region. These fragments were eluted from agarose gels and used to generate the transgenic mice at the National Eye Institute's Transgenic Mouse Facility as described previously (Wawrousek *et al.*, 1990). All analyses with the transgenic mice were done with approximately 8-week old mice. About 50 mg of each tissue analyzed was homogenized in 500 μl of ice-cold 1X passive lysis buffer (Promega, Madison WI) on ice using a hand-held Kontes homogenizer for 30 to 45 sec. The lysate was cleared by centrifugation at 12000 rpm for 15 min at 4°C. Protein concentration in the supernatant fractions was estimated using the Bicinchoninic acid (Pierce, Rockford, IL) method. 50 μg of lysate from each tissue was subjected to dual luciferase assays as above.

Identification of candidate transcription factors

Candidate transcription factors for elements αBE1 and αBE3 were predicted using the transcription element search software on the web available online at http://www.cbil.upenn.edu/tess and the transcription factor database TRANSFAC available at http://www.gene-regulation.com.

Enhancer blocking assays

Enhancer blocking assays were performed as described previously (Chung *et al.*, 1997, Chung *et al.*, 1993). Briefly, 1 μg of linearized purified DNA was transfected using FuGENE-6 into 1 X 10^7 human erythroleukemia K562 cells grown in suspension. After 24 hours of recovery, cells were plated in 150 mm dishes in IMEM soft agar containing 10 % FBS, antibiotics penicillin and streptomycin and geneticin at 750 μg/ml. Colonies were counted after 3 weeks of selection and the colony number was normalized to that obtained with the pNI fragment containing no insert in between the enhancer and promoter.

Expression and Purification of CTCF

Full length mouse CTCF cDNA (a kind gift of Dr. Felsenfeld, NIH) was cloned in expression vector pTrcHis (Invitrogen Life Technologies, Carlsbad, CA). *E. coli* cells containing pTrcHis:CTCF vector in mid-log phase of growth were induced to express CTCF with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 hours. The cells were then lysed and the expressed CTCF tagged with 6 residues of histidine was purified using the Ni-NTA purification system, following the protocol suggested by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA).

DNA-Protein Interactions

Sequence of oligonucleotides used in electrophoretic mobility shift assays (EMSA) is given below with mutations shown bold and underlined.
Globin FII:
CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA
-720/-673:
CGCAGGGGCCTGGTAGGACAGCTGAGGGTCCTGGTCCTGGAGTGAGCT
-720/-673 Mut:
CGCA**A**G**T**GCCTGGTAGGACAGCTGAG**AT**TCCTGGTCCTGGAGTGAGCT
-779/-725:
TATGAGGGTGTGATCAGCCTGGACCCCCTAAGTCCCCTCCTCTGCACACCCTTC
-779/-725 Mut:
TATGAG**TC**TGTGATCAGCCTGGAC**AC**T**C**TAAGTCC**AA**TCCTCTGCACAC**TA**TTC
-759/-723: GGACCCCCTAAGTCCCCTCCTCTGCACACCCTTCAC
-668/-629:
CTCTGCAGAGGGCAAGGAGAGGACTAGTTGGGCCTTCACC
-700/-650:
GCTGAGGGTCCTGGTCCTGGAATGAGCTCCTGCTCTGCAGAGGGCAAGGAG
-845/-795:
CCACCCAAAATAGTGCAGAGCCTCTGGGGGTGGGGGAGGGCTGGGAGCCT
-836/-786:
ATAGTGCAGAGCCTCTGGGGGTGGGGGAGGGCTGGGAGCCTAAGTCTAGAG
αBE1: -426 TAGGACTCCACAAAGAGTTAATGTCCCTGGGGCTAA
αBE3: -326 CGAGACTGGAGAGGAGGAGGGGCCCACCAG,
Sp1-WT: CCCTTGGTGGGGGCGGGGCCTAAGCTGCG and
Sp1-MUT: CCCTTGGTGGG**TTG**GGGGCCTAAGCTGCG.

Gel shift assays were performed by incubating 10 μg of nuclear extract or 100 ng of partially purified GR (Sigma Biochemicals, St. Louis, MO) or CTCF protein with ~ 0.1 ng double-stranded oligonucleotides end-labeled with γ ^{32}P-ATP by polynucleotide kinase in 20 μl reaction mix containing 20 mM HEPES (pH 7.9), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 20 μg double-stranded poly (dIdC) for 30 minutes at room temperature. For super-shift assays, nuclear extracts were incubated with corresponding antibody for 2 hours at room temperature before addition of the labeled oligonucleotide and further incubation for 30 minutes at room temperature. 10 μl of the reaction mix was loaded on 6 % acrylamide gel in 0.5X TBE buffer and electrophoresed at 60 volts for 2 hours. The gels were then dried and exposed to X-ray autoradiogram.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed following the EZ-Chip protocol suggested by Upstate USA Inc., (Charlottesville, VA) using C2C12 cells. DNA-bound proteins were cross-linked with DNA, chromatin was purified and sonicated to generate 200–1000-bp DNA fragments and immunoprecipitated with antibodies against Sp1, GR or IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using protein-A sepharose beads (Amersham Biosciences, Piscataway, NJ). The DNA–protein cross-links were reversed, DNA was purified, dissolved in TE buffer and used as a template for PCR amplification. PCR was carried out for 20 cycles using different primer pairs shown below:

-959 GACTGCTGTTGCGACTAGTAGC and
-718 CGACAGTGAAGGGTGTGCAGA,
-836 ATAGTGCAGAGCCTCTGG and
-629 ACTGGTGAAGGCCCAACT,
-739 TCTGCACACCCTTCACTGT and
-519 TTGTGGATGCTATGTGGCTCAT,
-668 CTCTGCAGAGGGCAAGGAGAGGAC and
-436 GAACTAGGTGTCTGACTG,
-546 TGTTCTATGAGCCACATAGC and
-320 GCCTGGTCTCGCATGCCAGGGGAA,
-469 ATCAGCTCAGGGTTCCAGT and
-249 ATCCTTGTTCTCTGGAGCTA,

-299 CAGCAGCTGCTTGGGATTCCG and
+44 CAGGGCTAGATGAATGCAGAGTC,
-1808 ATAGAGCAGCTCGAACCCGCCA and
-1528 CTCTATCACGGCTACTATGT,
+3744 GAACATGGCTTCATCTCCAG and
+4025 AGCTTCAGCACTAGTCACAG.

DNA samples from sonicated nuclear lysate that underwent reverse cross-link and purification were used as input. DNA sample immunoprecipitated by antibodies against IgG was used as negative control. PCR products were separated on 1 % agarose gel and visualized by staining with ethidium bromide.

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