J. Biochem. 129, 813–820 (2001)

Heat Shock Factor 2 Is Involved in the Upregulation of αB-Crystallin by High Extracellular Potassium[1]

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Received February 5, 2001; accepted March 2, 2001

αB-Crystallin, a member of the small heat shock protein (HSP) family, accumulates in reactive astrocytes in a variety of pathological conditions. We previously reported the upregulation of αB-crystallin in response to high extracellular potassium concentration. In the present study, we investigated the regulatory mechanism of αB-crystallin expression by KCl. When human glioma U-251MG cells were exposed to continuous KCl treatment, induction of αB-crystallin mRNA was observed after 8 h and persisted for a few days. Functional promoter analysis using deletion and mutation constructs revealed that the proximal heat shock element (HSE-P), which contributes to heat shock induction in HeLa cells, is essential for transcriptional activation of the αB-crystallin gene by KCl in U-251MG cells. Gel mobility shift and antibody supershift assays showed that KCl induces the HSE-binding activity of heat shock factor (HSF) 2, while heat shock induces that of HSF1. This is the first demonstration that HSF2 can be activated by KCl and is involved in the upregulation of αB-crystallin gene expression in glial cells.

Key words: αB-crystallin, glioma cells, heat shock factor (HSF), potassium ion (K^+), stress response.

αB-Crystallin is a member of the small heat shock protein (HSP) family, which includes hsp27, αA-crystallin, HSPB2/MKBP (*1*, *2*), HSPB3/HSPL27 (*3*), p20/hsp20 (*4*), and cvHSP/HSPB7 (*5*) in mammals, and which possesses molecular chaperone activity (reviewed in Ref. *6*). Several lines of evidence suggest that αB-crystallin binds to the cytoskeleton and modulates remodeling of the cytoskeletal network (*7–10*). Under non-stress conditions, αB-crystallin is expressed in various tissues, such as the eye lens, heart, skeletal muscle, kidney, lung, brain, and placenta (*11–14*). In the brain, αB-crystallin is preferentially expressed in glial cells, mainly in oligodendrocytes and a few astrocytes, but not in neurons (*12*). Although the level of expression in the normal brain is low, αB-crystallin accumulates in reactive astrocytes under various pathological conditions, such as Alexander's disease, Parkinson's disease, Huntington's disease, Alzheimer's disease, Creutzfeldt-Jakob disease, and Lewy Body disease (*11*, *15–18*), and in astrocytoma cells (*19*). It is likely that the induction of αB-crystallin is a common process in response to pathological stresses, which have yet to be identified, in the brain.

In cultured cells, αB-crystallin expression is induced by a variety of stimuli, including heat shock, KCl, oxidative stress, hypertonic stress, sodium arsenite, tumor necrosis factor α, and dexamethasone (*20–24*). Overexpression of αB-crystallin supports the survival of cells challenged with thermal and hypertonic stresses (*25*, *26*). We have previously shown that treatment of rat C6 glioma cells with a culture medium containing elevated potassium ions (K^+) increased αB-crystallin mRNA (*21*). Treatment of C6 cells with extremely augmented K^+ in the culture medium resulted in the cell death. However, an overexpression of αB-crystallin in C6 cells conferred a resistant phenotype against the insult by elevated extracellular K^+ (*21*).

Maintenance of the ionic composition of the neuronal intracellular and extracellular fluids is crucial to normal brain function. Extracellular K^+ concentration has been reported to increase under pathological conditions. For example, during cerebral ischemia, increases in extracellular K^+ concentration to 30–80 mM have been observed (*27*). Astrocytes accumulate much more K^+ than neurons at the same extracellular K^+ concentration (*28*). Astrocytes are also more efficient than neurons in regulating the intracellular fluid volume and pH when exposed to an unfavorable environment such as increased K^+ concentration, suggesting that astrocytes have specific properties that allow them to respond to K^+ treatment.

Stress-induced transcription requires activation of the heat shock factor (HSF) that binds to the heat shock element (HSE), which is characterized by multiple adjacent and inverse iterations of the pentanucleotide motif 5′-nGAAn-3′ (*29–31*). In the 5′-flanking region of the αB-crystallin gene, two putative HSEs occur, the proximal and the distal. A DNA fragment of 711 bp between −666 and +45 of the mouse αB-crystallin gene, containing both HSEs, is heat shock responsive (*20*). Furthermore, gel mobility shift assays have revealed that both HSEs can bind to HSF1 that has been activated by cadmium treatment (*32*). However, it has not been determined whether HSF1 binding to

[1] This work was supported by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan to A.I. (10680654).

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Abbreviations: CAT, chloramphenicol acetyltransferase; HS, heat shock; HSE, heat shock element; HSE-D, distal heat shock element; HSE-P, proximal heat shock element; HSF, heat shock factor; HSP, heat shock protein.

these HSEs, as demonstrated by gel mobility shift assays, is actually the functional determinant of the increased transcription of αB-crystallin in response to heat shock. Moreover, the regulatory mechanism of αB-crystallin induction in response to other stimuli, such as K^+, is unknown.

To determine the regulatory regions of the αB-crystallin gene involved in heat shock and KCl induction in cultured cells, we generated reporter constructs containing a series of 5′-truncated forms and mutations at HSEs of the αB-crystallin promoter. Transient transfection experiments revealed that the same HSE mediates transcriptional activation of the αB-crystallin gene by heat shock in HeLa cells and by KCl in U-251MG cells. Intriguingly, gel mobility shift experiments provide evidence that the HSE binds to HSF1 upon heat shock and HSF2 upon KCl induction in U-251MG.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-human HSF1 (αHSF1γ), anti-human HSF2 (αHSF2δ), and anti-mouse HSF4 (αHSF4t) antisera were generous gifts from Dr. Akira Nakai (Yamaguchi University) (*33*, *34*).

Culture and Treatment of Cells—U-251MG and HeLa cells were grown in Dulbecco's modified Eagle's medium (Nissui), containing 10% fetal bovine serum, 1 mM glutamine, and penicillin-streptomycin. Cells were exposed transiently to heat shock (HS) by immersion in a 44°C water bath, followed by recovery at 37°C for specified periods until harvest. Alternatively, cells were exposed continuously to a medium containing an additional 60 mM or 80 mM KCl until harvest.

Northern Blot Analysis—Northern blotting was performed as previously described (*35*). A human αB-crystallin cDNA probe was obtained as a 0.4-kb insert from pRF1 (*11*). Human hsp27 and hsp70 probes were obtained from StressGen. Probe DNA was labeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system (Amersham). Hybridization was performed with a ^{32}P-labeled probe in Church solution (*36*) overnight at 65°C. Autoradiography was carried out at −70°C using Fuji RX film with an intensifying screen. The intensity of the hybridization signals was measured with a BAS2000 image analyzer (Fuji).

Plasmid Construction—The human αB-crystallin promoter was fused to the chloramphenicol acetyltransferase (CAT) reporter gene to generate a series of pHXCAT plasmids (see Fig. 3). The construction of deletion mutants is described elsewhere (Nagano, T. *et al.*, in preparation). The introduction of mutations into HSEs was achieved using PCR as follows. To prepare pHXCAT14-Pm, two DNA fragments were first generated by PCR with two sets of primers, HSEPm1 (5′-CCATAGCACTAGCTTGACAAGACTGCATATATAAGG-3′: −55/−20, Δ*Hin*dIII) and U (5′-AAATCTAGATGAGTGTGAGGGGTCA-3′), and HSEPm2 (5′-CTTGTCAAGCTAGTGCTATGGTGATGTCAGGGGTTT-3′: −35/−70, Δ*Hin*dIII) and Reverse (5′-GAGCGGATAACAATTTCACACAGG-3′), using pHXCAT14 as a template. The sites of mutations are underlined. Next, the two fragments were annealed, extended by mutually primed synthesis, and amplified by PCR using primers U and Reverse. The amplified fragment was digested with *Hin*dIII and *Xba*I, then ligated into pHXCAT14 digested with *Hin*dIII and *Xba*I. pHXCAT12-Pm, pHXCAT12-Dm, and pHXCAT12-DmPm were prepared in a similar manner. The introduction of mutations at the proximal and distal HSEs resulted in disruption of the −42 *Hin*dIII and −418 *Bln*I sites, respectively. All mutations were confirmed by sequencing.

Transfection and Reporter Analysis—Approximately 24 h before transfection, cells were plated at 0.5–1.0 × 10^6 per dish (diameter 60 mm). Transfection was performed with the TransIT™-100 reagent (2 μl) and 1 μg of plasmid DNA according to the protocol of the manufacturer (PanVera). After 3 h, the medium was replaced with complete growth medium. After incubation at 37°C for 2 h, cells were exposed to the stress stimulus. The cells were incubated at 37°C for 24–48 h before harvest, collected by brief centrifugation, and resuspended in 100 μl of 250 mM Tris-HCl (pH 7.5). Extracts were prepared by freeze-thawing, incubated at 65°C for 10 min, and the cell debris removed by brief centrifugation. The lysate was used in the CAT assay, which was performed as previously described (*37*).

Preparation of Nuclear Extracts—Nuclear extracts were prepared by the method of Dignam *et al.*, with some modifications (*38*). Cells were harvested and centrifuged. The pellets were suspended in buffer A, containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% Nonidet P-40 (NP-40), and protease inhibitors (0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin), then homogenized. The homogenate was layered onto an equal volume of buffer B, containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 M sucrose, and the protease inhibitors, then centrifuged. The crude nuclei were suspended in buffer E, containing 20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, and the protease inhibitors, and gently mixed for 30 min at 4°C. After centrifugation, the supernatant was dialyzed with buffer D, containing 20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, and the protease inhibitors. The nuclear extracts were stored at −80°C. The concentration of protein in the extracts was measured using the Bradford assay (Bio-Rad).

Gel Mobility Shift Assay—A typical binding reaction was carried out in a 20-μl volume containing 2.5 μg of nuclear extracts, 50 fmol ^{32}P-labeled oligonucleotide probe, 1.25 μg of poly(dI-dC), and 10 μg of BSA, in 10 mM HEPES, 25 mM Tris-HCl, 50 mM KCl, 50 mM NaCl, 0.95 mM EDTA, 10% glycerol, 0.25 mM DTT, 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin, at room temperature for 20 min. The antibody supershift experiments were performed by incubating 1.0 μl of antiserum (1:10 dilution) in PBS with 2.5 μg of nuclear extracts, before the addition of the labeled probes. For the competition experiment, a 100-fold molar excess of the unlabeled oligonucleotide was added to each reaction. The competitors used as negative and positive controls were, respectively, an unrelated sequence corresponding to positions −322 to −299 on the human αB-crystallin gene (5′-CCCATCCTCCCCCCACCCCGCGTG-3′), and a typical HSE from human hsp70 (5′-TCGGCTGGAATATTCCCGACCTGGCAGCCGA-3′) (*39*). The reaction products were separated electrophoretically on 4% polyacrylamide gel for 2 h at 200 V and detected by autoradiography.

RESULTS

The Proximal HSE Is Responsible for Heat-Induced Acti-

vation of the αB-Crystallin Promoter in HeLa Cells—HeLa cells were exposed to heat shock at 44°C for 15 min, and the mRNA levels of αB-crystallin, hsp27, and hsp70 were measured by Northern blotting. After heat treatment, the level of αB-crystallin mRNA increased after 1 h, reached a maximal level after 4–8 h, then returned to the control level within 24 h (Fig. 1A). Similar heat shock responses for the hsp27 and hsp70 genes were observed, although both the increase and subsequent decrease of the hsp70 mRNA were faster than those of the αB-crystallin and hsp27 transcripts.

Two HSEs are present in the promoter region of the human αB-crystallin gene. We named these HSEs the proximal HSE (HSE-P, –53/–39) and the distal HSE (HSE-D, –418/–399), because of their locations relative to the transcription initiation site (Fig. 2). The proximal HSE is found immediately 5′ to the TATA box (–28/–23). To determine the regulatory regions of the αB-crystallin gene responsible for transcriptional activation by heat shock and by KCl, we generated CAT reporter constructs containing a series of 5′-truncated forms of the αB-crystallin promoter, which were used in transient transfection experiments (Fig. 3A). Furthermore, we introduced mutations at HSE-P and HSE-D in the CAT constructs to evaluate the role of the HSEs (Figs. 2 and 3B).

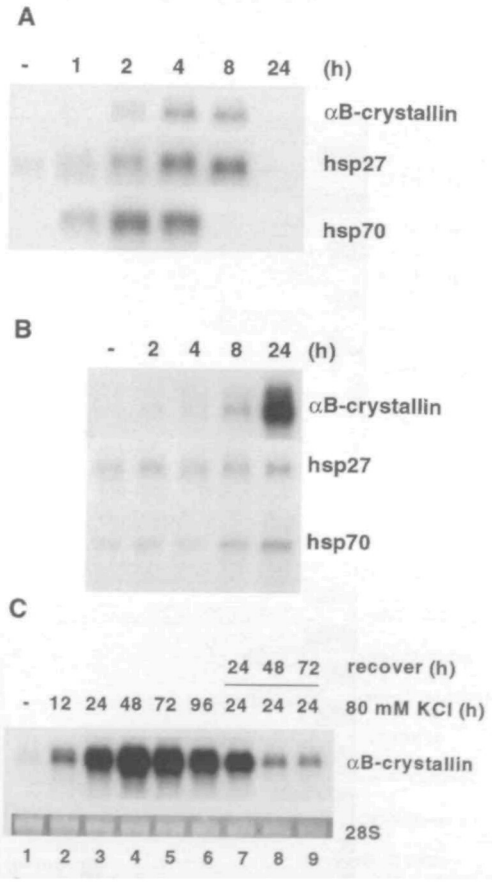

Fig. 1. **Induction of αB-crystallin, hsp27, and hsp70 mRNA expression by heat shock and KCl.** A. After heat shock treatment at 44°C for 15 min, HeLa cells were incubated at 37°C for 1, 2, 4, 8, and 24 h. B. U-251MG cells were exposed to additional 60 mM KCl for 2, 4, 8, and 24 h. C. U-251MG cells were exposed to additional 80 mM KCl for 12, 24, 48, 72, and 96 h (lanes 1–6). After KCl treatment for 24 h, cells were incubated in normal medium at 37°C for 24, 48, and 72 h (lanes 7–9). Untreated cells (–) were left at 37°C with normal medium. Total RNA (10 μg) was loaded onto a 1.5% agarose gel, blotted onto membrane, and hybridized with the ^{32}P-labeled αB-crystallin, hsp27 and hsp70 cDNA probes. Bands of 28S rRNA, stained with ethidium bromide, are shown for reference.

When HeLa cells were transfected with a series of 5′-deletion constructs and subjected to heat shock (Fig. 3A), the αB-crystallin promoter containing the sequence between –534 and +42 (pHXCAT12) showed the maximal induction. Truncation of nucleotides from –431 to –335 led to a decrease in induction, and truncation of nucleotides from –151 to –47 completely abolished gene activation by heat shock. These results indicate that the –151/+42 region, containing HSE-P, is a minimal essential element in conferring heat inducibility upon the αB-crystallin gene, and that the –431/–335 region is required for full induction.

Introduction of mutations at HSE-P in pHXCAT14 and pHXCAT12 dramatically reduced heat-induced activation (see pHXCAT14-Pm and pHXCAT12-Pm, in Fig. 3B). On the other hand, introduction of mutations at HSE-D reduced the activation only slightly (see pHXCAT12-Dm). Moreover, there was no difference in CAT activities between pHXCAT12-DmPm, in which both HSE-P and HSE-D were disrupted, and pHXCAT12-Pm. These data suggest that HSE-P, but not HSE-D, is essential to the heat shock response.

The αB-Crystallin mRNA Level Was Dramatically Increased by KCl Treatment—We have shown previously that elevated extracellular KCl concentration induced an accumulation of αB-crystallin mRNA in C6 rat glioma cells in a dose-dependent manner (*21*). When human glioma U-251MG cells were exposed to continuous KCl treatment for 24 h, αB-crystallin mRNA increased dramatically to 24-fold, whereas only slight increases were observed in hsp27 and hsp70 mRNAs, of 1.5- and 2.7-fold, respectively (Fig. 1B). Moreover, prolonged KCl treatment resulted in further accumulation of αB-crystallin mRNA (Fig. 1C, lanes 1–6). Even after KCl was removed from the culture medium, the high level of αB-crystallin mRNA was maintained for a further 24 h, whereupon it decreased but did not return to the control level (Fig. 1C, lanes 7–9). In HeLa cells, induction of αB-crystallin mRNA by KCl was not observed (data not shown).

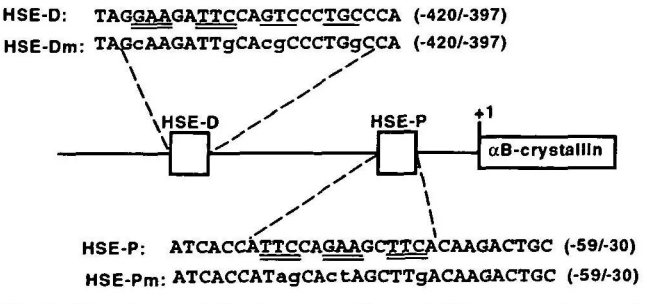

Fig. 2. **Structure of the human αB-crystallin promoter and mutations at HSEs.** Proximal (HSE-P) and distal (HSE-D) HSEs are shown by open boxes. The sequences of the probes used in gel mobility shift assay, HSE-D, HSE-Dm, HSE-P, and HSE-Pm, are also shown. Typical GAA units in consensus HSEs (nGAAn), putatively important for binding to HSF, are doubly underlined; and degenerate GAA units are underlined. Mutated nucleotides in HSE-Pm and HSE-Dm are indicated by lower-case letters.

The Proximal HSE Is Also Required for KCl-Induced Activation of the αB-Crystallin Promoter in U-251MG Cells—Transfection of a series of 5′-deletion constructs into U-251MG cells revealed that the positive regulatory region (–151/+42) in heat-induced activation is also critical for KCl-induced activation of the αB-crystallin gene (Fig. 4A). Inclusion of the sequence between –237 and –151 resulted in a decrease in the level of induction, indicating the presence of some negative regulatory elements. Our data also suggest that the sequence upstream from –237 to –534 contains some positive regulatory elements. We noted that the introduction of a longer promoter sequence (compare pHXCAT07 to pHXCAT01) produced increased CAT activity in control U-251MG cells (Fig. 4A), but not in HeLa cells (Fig. 3A), under non-stress conditions, raising the possibility that the upstream sequence contains glial-specific enhancer elements.

To examine whether the two HSEs are involved in transcriptional activation by KCl, we used the CAT constructs containing mutations at the HSEs (Fig. 4B). Introduction of mutations at HSE-P in pHXCAT14 completely abolished KCl-induced activation (see pHXCAT14-Pm), suggesting that HSE-P is necessary for KCl-induced activation, like heat-induced activation. Mutations at HSE-D had little effect on KCl-induced activation (compare pHXCAT12 to pHXCAT12-Dm, and pHXCAT12-Pm to pHXCAT12-DmPm). As the construct pHXCAT12-DmPm still possessed KCl-inducibility, we could not rule out the presence of some positive regulatory elements other than the HSEs, located between –534 and –152. However, our data clearly demonstrate that HSE-P plays an important role in KCl-induced activation of the αB-crystallin gene.

Binding of HSF2 to the Proximal HSE during KCl Treatment—To characterize the nuclear proteins binding to HSE-P, gel mobility shift assays were performed using the ^{32}P-labeled HSE-P oligonucleotide as a probe (Fig. 5). In nuclear extracts prepared from KCl-treated U-251MG cells, three distinct HSE-binding activities were detected (Fig. 5A, lane 3). Three similar complexes were also observed in heat-shocked cells (lane 4). The appearance of three complexes was consistent with that observed in gel mobility shift assays using extracts from HeLa cells after heat shock (*39*, *40*). The three bands observed in heat-shocked U-

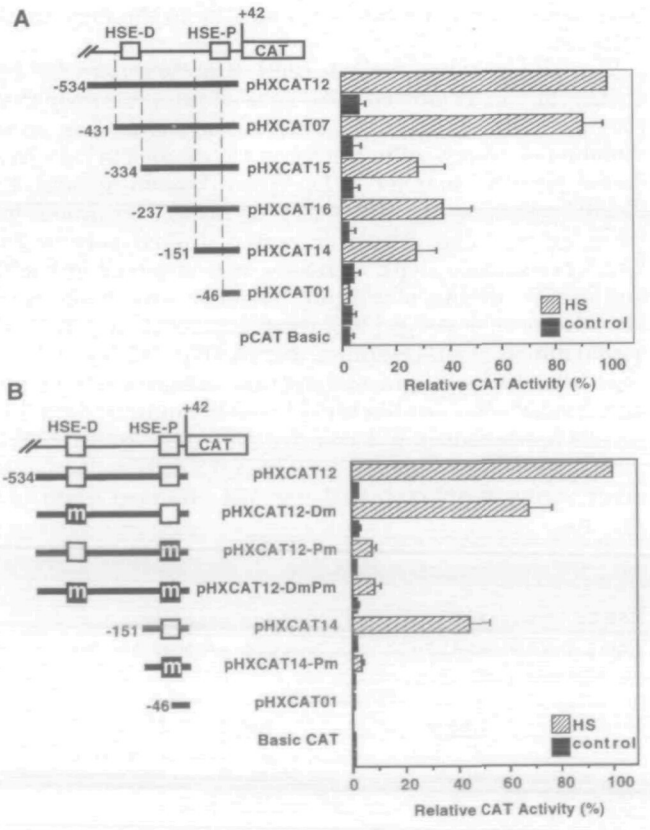

Fig. 3. **Heat-induced activation of the αB-crystallin promoter was mediated by HSE in HeLa cells.** The CAT constructs of deletion (A) and HSE (B) mutants, depicted on the left, were transiently transfected into HeLa cells. After 5 h, cells were exposed to heat shock (44°C, 30 min), subsequently incubated at 37°C for 24 h, then collected (HS). Control cells were incubated at 37°C after transfection and collected at the same time. Schematic representation shows the relative levels of their CAT activities. The CAT activity observed in cells transfected with pHXCAT12, followed by heat shock, was set at 100%. The results are presented as the means ± the standard error (SE) of three independent experiments.

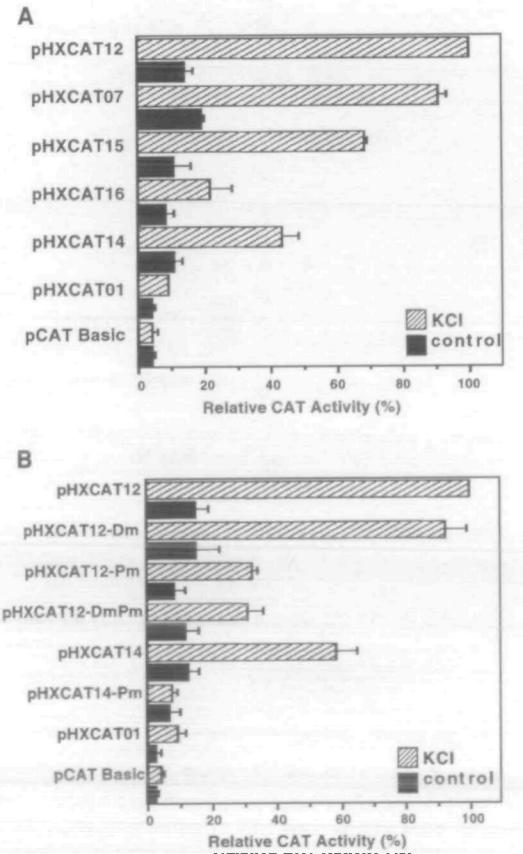

Fig. 4. **KCl-induced activation of the αB-crystallin promoter was mediated by HSE in U-251MG cells.** The CAT constructs of deletion (A) and HSE (B) mutants were transiently transfected into U-251MG cells. After 5 h, cells were exposed to 80 mM KCl for 48 h before harvest. Control cells were cultured with normal medium. Schematic representation shows the relative levels of their CAT activities. The CAT activity observed with pHXCAT12 in the presence of additional KCl was set at 100%. The results are presented as the means ± SE of three independent experiments.

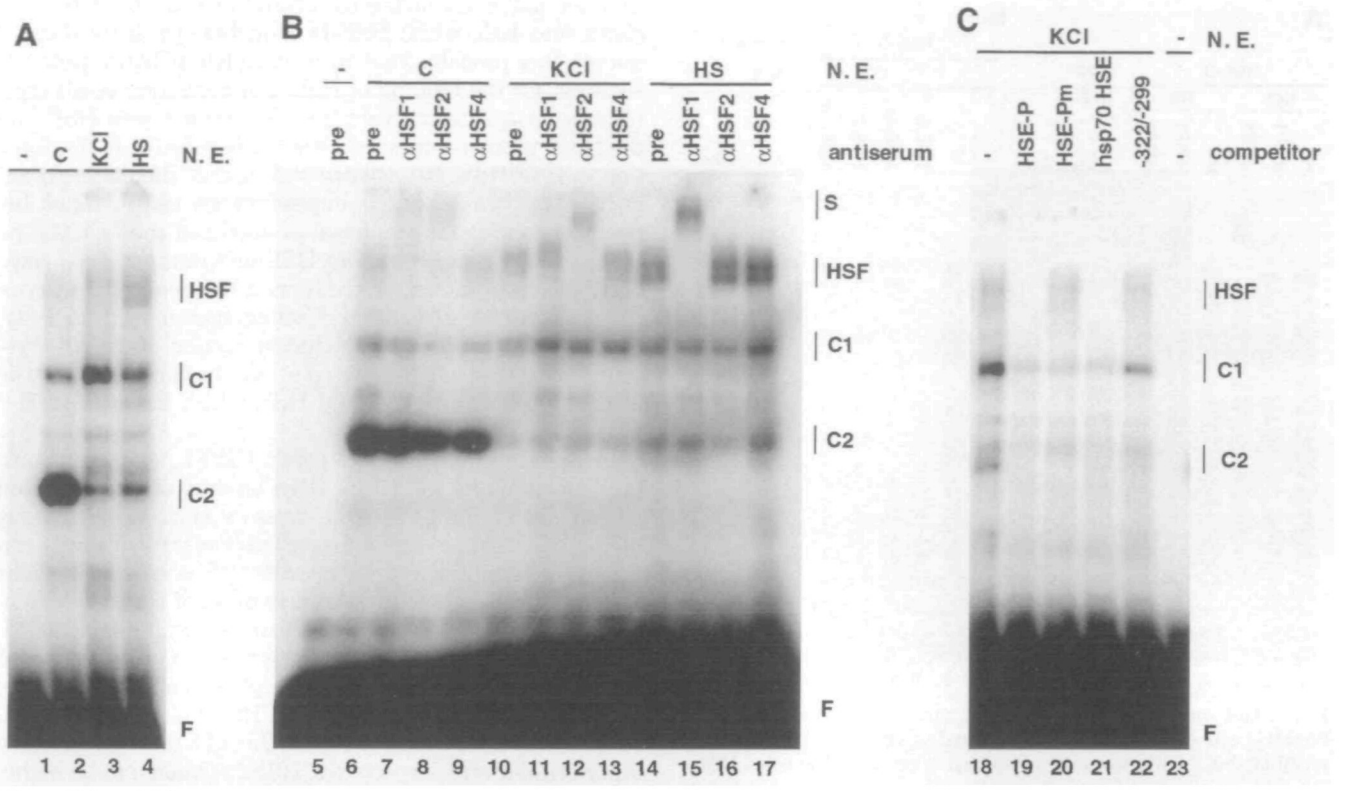

Fig. 5. **Gel mobility shift assays of nuclear extracts from U-251 MG cells exposed to KCl or HS using the HSE-P probe.** Gel mobility shift assays were performed with 2.5 μg of nuclear extracts (NE) prepared from cells treated with 80 mM KCl for 24 h (KCl), or cells subjected to incubation at 44°C for 30 min and subsequently at 37°C for 2 h (HS), and 50 fmol ^{32}P-labeled HSE-P probe. A: Comparison of DNA-binding activities in nuclear extracts exposed to KCl and HS, and control cells (C). B: Antibody supershift experiments were performed using preimmune serum (pre), anti–human HSF1 (αHSF1), anti–human HSF2 (αHSF2), or anti–mouse HSF4 (αHSF4) antisera. C: Competition assays were performed using 100-fold excess of each oligonucleotide: HSE-P, HSE-Pm, a typical HSE from the human hsp70 (hsp70 HSE), and an unrelated 5′-upstream sequence from the αB-crystallin gene (–322/–299). The sequences of HSE-P and HSE-Pm are shown in Fig. 2, and those of the other competitors are described in the experimental procedures. The locations of HSE-HSF complex (HSF), complexes 1 and 2 (C1 and C2), supershift complex (S), and free probe (F) are shown.

251MG cells may correspond to the HSE-HSF complex (HSF), the constitutive HSE-binding activity (C1), and nonspecific protein–DNA interaction (C2).

To examine whether HSFs are involved in the KCl-induced HSE-binding activity, the extracts from cells treated with either KCl or heat were incubated with antisera raised against HSF1, HSF2, or HSF4 (*33*, *34*), then subjected to gel mobility shift assays (Fig. 5B). Interestingly, the HSE-HSF complex in nuclear extracts prepared from KCl-treated cells was completely supershifted with anti-HSF2 antiserum (lane 12) but not with anti-HSF1 or anti-HSF4 antiserum (lanes 11 and 13). In heat-shocked cells, the expected supershift of the HSE-HSF complex with anti-HSF1 was observed (lane 15). Although the HSE-HSF complex was also present in control cells (lanes 2 and 6) and supershifted by either anti-HSF1 (lane 7) or anti-HSF2 (lane 8), its level is relatively low. These data clearly demonstrate that HSF2 binds to the HSE-P after KCl treatment, while HSF1 binds to the same element after heat shock treatment in U-251MG cells.

Although excess unlabeled HSE-P and hsp70 HSE, a consensus HSE from the human hsp70 gene, competed (with labeled HSE-P) for the binding to HSF2 (Fig. 5C, lanes 19 and 21), the mutated HSE-P oligonucleotide HSE-Pm did not compete for the binding to HSF2 (lane 20), indicating that HSF2 has no binding activity to HSE-Pm. This result is consistent with our observation that mutations at HSE-P lead to a loss of transcriptional activity of the reporter gene (Fig. 4B). The intensity of C1 and C2 varied between nuclear extracts prepared in different experiments (data not shown). C1 was competed out partially by HSE-Pm, as well as HSE-P and hsp70 HSE (lanes 19–21), suggesting that C1 can bind to HSE-Pm and may not play a role in HSE-mediated transcriptional activation. C2 is a non-specific DNA-protein complex, because it competed with the all oligonucleotides used as competitors, including an unrelated sequence from the human αB-crystallin gene, coded between positions –322 and –299 (lanes 19–22).

When we used the distal HSE (HSE-D) as a probe in gel mobility shift and antibody supershift assays (Fig. 6), HSE-D formed complexes with HSF1 after heat shock (lane 5) and HSF2 after KCl treatment (lane 3), similar to HSE-P. HSE-D competed with HSE-P (lane 11), but not with HSE-Pm (lane 12) or HSE-Dm (lane 10), for binding to HSF2. These results suggest that HSE-D can bind HSF2 in a similar manner to HSE-P *in vitro*.

DISCUSSION

We showed that KCl treatment induced transcription of the

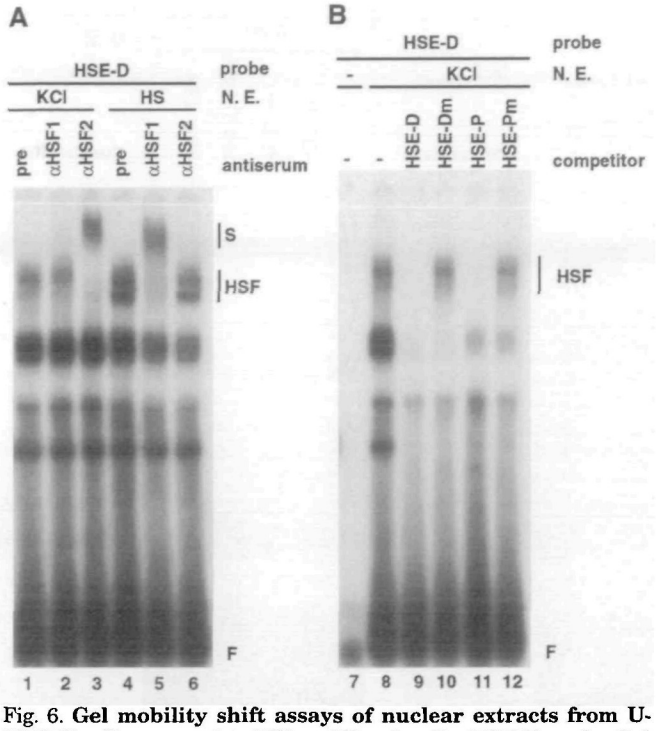

Fig. 6. **Gel mobility shift assays of nuclear extracts from U-251MG cells exposed to KCl or HS using the HSE-D probe.** Gel mobility shift assays were performed using 50 fmol ^{32}P-labeled HSE-D probe. Antibody supershift experiments were performed using preimmune serum (pre), anti–human HSF1 (αHSF1), or anti–human HSF2 (αHSF2) antisera. B: Competition assays were performed using 100-fold excess of each oligonucleotide: HSE-D, HSE-Dm, HSE-P, and HSE-Pm. The sequences of HSE-D, HSE-Dm, HSE-P and HSE-Pm are shown in Fig. 2. The locations of HSF-HSE complex (HSF), supershift complex (S), and free probe (F) are shown.

αB-crystallin gene and enhanced the binding activity of HSF2 to HSE-P of the αB-crystallin promoter. In addition, mutations at HSE-P abolished the KCl-induced activation of αB-crystallin expression and led to a loss of binding activity to HSF2. Therefore, we infer that HSF2 is involved in the transcriptional activation of the αB-crystallin gene by KCl.

The transcriptional activation of HSPs is generally mediated by the binding of an HSF to a specific DNA sequence, HSE, composed of multiple inverted arrays of the pentameric consensus sequence nGAAn. At least three nGAAn units are required for high-affinity binding of HSF (*29–31*). Of the two HSEs in the human αB-crystallin promoter, HSE-P is composed of an array of three perfect inverted repeats of the consensus sequence nGAAn, whereas HSE-D contains two perfect and two imperfect inverted repeats (Fig. 2). There are two reports describing the binding of HSF1 to the HSEs of the αB-crystallin gene. Both HSEs bind to HSF1 following cadmium treatment in rat astrocytes (*32*); the binding activity of HSF1 to HSE-P is present in the ocular lens extracts from post-natal rats and is developmentally regulated (*41*).

We demonstrated that both HSE-P and HSE-D bind to HSF1 upon heat stress and to HSF2 upon KCl induction (Figs. 5 and 6). However, the functional study, using αB-crystallin-CAT reporter constructs with mutations at either HSE-P or HSE-D, revealed that HSE-P is especially important for induction of the αB-crystallin gene by both heat shock and KCl, while HSE-D contributes a little (Figs. 3 and 4). It is probable that three complete nGAAn units are required for the binding of HSF and activation of αB-crystallin *in vivo*. Alternatively, the distance between HSE and the basal promoter may be a critical parameter in the function of HSF/HSE. It has been shown that the degree of induction by heat shock is dependent on the distance between the HSE and the basal promoter of the hsp70 gene (*42*, *43*). Recently, *Drosophila* HSF and human HSF1 have been shown to interact directly with the general transcription factor TBP (TATA-box binding protein) *in vitro* (*44*, *45*). Therefore, it is possible that activation of the αB-crystallin gene transcription *in vivo* may be facilitated by the interaction between TBP and HSF, which binds to HSE-P next to the TATA-box.

In mammals, three HSF genes (HSF1, 2, and 4) have been isolated (*46–48*). HSF1 DNA binding can be activated in many cell types by several stressors, such as heat, heavy metals, and amino acid analogs. Two isoforms of HSF4 can act as an inhibitor and an activator of tissue-specific heat shock expression (*34*, *48*). The activation of HSF1 is accompanied by phosphorylation, nuclear accumulation and trimer formation (*31*). HSF2, however, may not be regulated directly by phosphorylation, since phosphorylated forms of HSF2 have not been detected (*49*). HSF2 is not activated in response to heat shock, nor is it thought to be involved in other cellular stress responses. HSF2 is known to be highly expressed in embryos and testes and considered to play a role in the tissue-specific and developmental stage-specific expression of HSPs (*29–31*). HSE-HSF2 binding activity can be detected in mouse embryos during postimplantation development (*50*), and it may have a role in neural proliferation. HSF2 is activated during hemin-induced erythroid differentiation in K562 cells (*51*). However, recent studies using reporter systems have indicated an apparent lack of correlation between the levels of HSF2 and expression of HSP after hemin treatment (*49*, *52*). In HeLa cells, overexpression of HSF2 stimulates the expression of the CCT, cytosolic chaperonin in the reporter assay (*53*). Recently it has been reported that HSF2 is activated when the ubiquitin-proteasome pathway is inhibited (*54*). The precise role of HSF2 is controversial and not completely understood. Although the mechanism of activation of HSF2 by KCl treatment remains to be resolved, our results point toward a novel regulatory role of HSF2.

We thank Drs. James E. Goldman and Toru Iwaki for helpful discussions, and Dr. Akira Nakai for gifts of antibodies.

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