Oligomerization of the heat shock factor (HSF) and its interaction with the heat shock element (HSE) are the hallmark of active transcriptional response to tangible physical or chemical stress. It is unknown if these interactions are subject to control and modulation by developmental cues and thus have tissue or stage specificity. By using promoter sequences containing a canonical HSE from the αB-crystallin gene, we demonstrate a tissue-specific transition from monomeric (in fetal and early neonatal stages that lack oligomeric HSF-HSE complexes) to oligomeric HSF-HSE interactions by postnatal day 10–21 in the ocular lens. Developmental control of these interactions is further demonstrated by induction of oligomeric HSF-HSE complexes in neonatal extracts by in vitro manipulations, interestingly, only in the lens and not in the brain, heart, or liver extracts. The exclusive presence of oligomeric HSF-HSE complexes in the postnatal/adult lens corresponds to known highly increased number of αB-crystallin transcripts in this tissue.

The heat shock response entails activation of transcription from genes containing the heat shock promoter. Transcriptional activation is brought about by the interaction of the heat shock element (HSE) and the pre-existing transactivating protein, the heat shock factor (HSF). With the exception in yeast, in which the HSF is constitutively bound to DNA, interaction of HSF with HSE is initiated by trimerization of the monomeric HSF. Most of our knowledge about activation of HSF and its binding to HSE comes from cells cultured in vitro that have been subjected to a physical or chemical stress (1, 2). In these investigations, the appearance of a multimeric HSF concomitant with its binding to HSE upon heat shock has been well elaborated (1–5).

It is well known that small heat shock proteins including αB-crystallin are developmentally expressed without any apparent stress intervention (2, 6–10). αB-crystallin (αB) is a polymeric protein (~20-kDa monomeric) with a chaperone-like activity (11, 12). Although the highest amount of αB protein is found in the ocular lens, appreciable expression of the αB-crystallin gene is detected very early in the developing heart (13–14). Elevated expression of αB has been associated with a myriad of pathologies such as desmin-related cardiomyopathy and cataractogenesis (15), and most notably with the neuronal diseases including Alzheimer’s disease, Alexander’s disease (16), scrapie (17), and multiple sclerosis (18). Increased levels of αB have been reported in NIH3T3 cells transformed with plasmids expressing v-mos and Ha-ras oncogenes (reviewed in Refs. 19 and 20). Given its wide range of expression and the presence of a canonical HSE in its promoter, the αB-crystallin gene presents an attractive paradigm for investigating the potential HSF-HSE interactions in the context of developmental control and tissue-specific expression.

The rodent (21–23) and the human αB-crystallin gene (24) have been well characterized. The rat αB-crystallin gene employs one predominant transcription initiation site, 44 base pairs upstream from the ATG. The sequences between −164/+44 of the mouse αB-crystallin gene have been suggested to regulate lens-specific expression in transgenic mice (25). The HSE is one of the significant landmarks of these sequences. Activation of the αB heat shock promoter-reporter gene constructs upon exposure to heat stress in cultured cells (26) and HSF1 binding to HSE in the αB promoter in rat astrocytes, exposed to osmotic and chemical stress, have been demonstrated (27). There are two HSEs in the rat αB-crystallin gene promoter (22) as follows: a dimeric HSE (5′-GGAGATTCC-3′) with the 5′-NGAA-3′ motifs arranged in a head:head fashion at position −391 and a trimeric element that contains the pentamer motif arranged in a tail:tail:head fashion at position −54. In this report we have used this HSE (HSEαB) (Fig. 1) to explore the status of HSF-HSE interactions in four rat tissues (lens, liver, heart, and brain) that express αB-crystallin mRNA in a tissue-specific manner.

EXPERIMENTAL PROCEDURES

Animals and Oligonucleotide Probes—Harlan Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Newborn rat pups, 3–4 days after birth, and rats of different ages were sacrificed under ether, and different organs were harvested for the preparation of whole cell extracts (WCEs). Oligonucleotide probes were synthesized on an ABI DNA synthesizer (Perkin-Elmer) or purchased from commercial sources (Life Technologies, Inc.). Protein standard markers were purchased from Life Technologies, Inc. The oligonucleotide probes were end-labeled with polynucleotide kinase (New England Biolabs Inc., Beverly, MA) with [γ-32P]ATP (NEN Life Science Products) (28).

Preparation of WCEs—Harvested tissues (400–500 mg) were rinsed in cold tissue culture medium (Dulbecco’s modified Eagle’s medium without serum) before extraction. WCEs were prepared according to the procedure described previously (29), with minor modifications. After the final ammonium sulfate precipitation the 16,000 × g pellet was dis-
solved in 0.1 volume of the supernatant in dialysis buffer (200 mM HEPES, pH 7.9, 100 mM KC1, 12.5 mM MgCl2, 0.1 mM EDTA, pH 7.9, 2 mM dithiothreitol, and 17% glycerol) and dialyzed (Spectropor membranes, 3500-kDa cut-off, Pierce). After 10–12 h of initial dialysis, the buffer was changed and dialysis continued for an additional 2-h period. After a brief centrifugation in a microcentrifuge to remove any insolubles, aliquots (20–30 μl per tube) were quick-frozen and stored in liquid nitrogen. The aliquots were used only once. Protein estimation was done using the BCA assay kit (Pierce).

**Gel Mobility-shift Assay**—Gel mobility-shift assays were performed as described (30). Twenty to thirty femtomoles of 32P-labeled oligonucleotides and whole cell extracts (~30 μg of protein) were used in a typical assay that contained 20 mM HEPES, pH 7.9, 40 mM KC1, 0.1 mM EDTA, 10% glycerol, 1.0 μg of poly(dI-dC) (Sigma), and 250 μg/ml of bovine serum albumin (New England Biolabs). The reaction mix was assembled on ice and incubated at 30 °C for 15 min. Whole reaction volumes were electrophoresed on an 8% acrylamide gel in a buffer containing 50 mM Tris, pH 7.9, 40 mM glycine, and 1 mM EDTA. The gels were run until the tracking dye (bromphenol blue), loaded a few minutes ahead of the reaction mix, reached the bottom. The gels were transferred onto Whatman DE81 paper, dried, and exposed to Hyperfilm MP (Amersham Pharmacia Biotech) at ~70 °C with or without an intensifier screen. For super-shift experiments, we used a commercially available anti-human HSF1 antibody (ABR Inc., Golden, CO). For these reactions we first determined the minimum amount of WCE needed for obtaining the bound complexes. This minimum amount was then used in the super-shift reactions. This was done to optimize the interaction of the antibody with the gel-shift complexes.

**UV Cross-linking of Protein-DNA Complexes and Their Characterization by Gel Electrophoresis**—The DNA-protein complexes resolved in the gel-shift assays were exposed to UV (in-gel), (Stratalinker, Stratagene Inc., La Jolla, CA). The gels were kept at a distance of 4–5 cm from the UV source. The top 3–4 cm of the gel (containing all the three complexes, see Fig. 3 A) were cut away and equilibrated with the SDS-solubles, aliquots (20–30 μl per tube) were quick-frozen and stored in liquid nitrogen at −70 °C with or without an intensifier screen. For super-shift experiments, we used a commercially available anti-human HSF1 antibody (ABR Inc., Golden, CO). For these experiments we first determined the minimum amount of WCE needed for obtaining the bound complexes. This minimum amount was then used in the super-shift reactions. This was done to optimize the interaction of the antibody with the gel-shift complexes.

**RESULTS**

A gel mobility-shift assay using HSEαB with WCEs, made from the neonatal rat lens (3–4-day-old), produces three complexes (I, II, and III) (Fig. 1 and Figs. 2–6). The formation of these three complexes is dependent on the presence of 5'-NGAAN-3' motifs in the probe as ascertained by using HSEαB probes, M1 and M2, mutated at different positions in the HSE. Using HSEαB-M1 oligonucleotide, wherein all the 5'-NGAAN-3' motifs were mutated, abolished almost all the complexes (Fig. 1 A, M1). However, HSEαB-M2 oligonucleotide, which retained one 5’-NGAAN-3’ motif, showed reduced intensity of binding in all complexes (Fig. 1 A, M2) in comparison to the wild-type HSEαB. Competition experiments demonstrated the specific nature of the three complexes. An oligonucleotide competitor containing consensus HSE sequences (HSEcon) competes efficiently with HSEαB, whereas HSE probe derived from the promoter of the hsp82 gene is a less efficient competitor (Fig. 1 B).

Although of reproducible mobility, the complexes I–III varied in intensity from experiment to experiment. This was particularly the case with complex III. Most of the fresh extracts made from neonatal 3–4-day-old rat lenses either produced a very weak intensity complex III or none at all. We discovered that this variability was created by the length of time that a particular lens extract spent in storage before the assay was conducted. The longer the period of storage the higher the intensity of complex III (data not shown). We used these stored extracts to study the effect of other parameters such as pH on the generation of HSP-HSE complexes. At pH 6.0 only a weak complex I was obtained. At pH 7.9, the formation of the complex III is most favored (data not shown). Fig. 2 shows the temperature dependence of the appearance of complex III. There seems to be an inverse relationship between the intensities of complexes I and II and the complex III as the temperature of incubation is increased. Incubation of liver extracts at higher temperatures did not produce complex III, and the pH optima for the only complex obtained (complex I) in liver (see below) was 6.5 (data not shown).

In order to understand the relationship of the three complexes obtained with lens extracts to data published previously (4, 27, 31–33), we ran a gel mobility-shift assay using lens and liver WCEs. Interestingly, with the liver WCEs only one complex is obtained in comparison to three complexes obtained with the lens WCE (Fig. 3 A). Complex I obtained with the liver

![Fig. 1. A. gel-shift assays with 32P-HSEαB and 32P-HSEαB mutant M1 and 32P-HSEαB mutant M2. Asterisks in the sequences shown below the autoradiograph indicate mutant bases in M1 and M2. a, free probe; b, experimental. B, gel-shift with 32P-HSEαB in the presence of unlabelled competitors HSE(con) (a consensus HSE sequence) and HSE82 (HSE from the hsp82 gene). HSE(con), 5'-GTCGACGGATCGAGCGCCTCGGATTCCCTTACTGAAATAGG-3'; HSE82, 5'-TCCCCTGGCCATCCGAA-AGGCCTCTAGAAGTTTCTAGAGACTTCCAGTTCGGG-3' (Both these sequences were taken from Ref. 43; the underline indicates the 5'-NGAAN-3' monomeric unit of the HSE). C, indicates control without WCE. EXP, experimental. a, 100-fold molar excess of the competitor; b, 200-fold molar excess of the competitor. F, indicates position of the unbound probe at the bottom of the gel. WCEs from 3- to 4-day-old rat lenses were used in both A and B. The gel-shift complexes obtained in A and B are indicated by I, II, and III.](http://www.jbc.org/)
and lens extracts has similar mobility. Complexes I and II have molecular masses between 70 and 87 kDa (Fig. 3A), suggesting that these two complexes possibly represent monomeric complexes of HSF with HSEαβ. The molecular mass of complex III is more than 200 kDa. Thus the complex III seems to be an oligomeric complex (at least trimeric) of HSF with HSEαβ (Fig. 3A). The following observation further strengthen this assessment. The commercially available recombinant HSF1 (which is known to exist as a trimer), when used in the gel mobility-shift assay with HSEαβ also produces a complex of mobility similar to the complex III (Fig. 3B). The generation of complex III with recombinant HSF1 is, however, very inefficient, possibly due to lack of other molecular inputs (in this in vitro assay) that may be normally present in vivo. To explore further the relationship between them, complexes I/II and complex III were analyzed by two-dimensional SDS-PAGE (two-dimensional SDS-polyacrylamide gel electrophoresis) after photo-cross-linking. Fig. 3C shows that complexes I/II and the complex III contain DNA-protein complexes that have similar molecular masses. The data presented in Fig. 2 and Fig. 3 suggest that the complex III is related to complexes I/II, at least in the composition of the monomeric units that make all or part of the complex III. The photo-cross-linking of the complex III was far more efficient than the photo-cross-linking of either the complex I or complex II (see open arrows in Fig. 3C), confirming known instability of the constitutive monomeric HSF-HSE interactions (33). Fig. 4 shows super-shift of the complexes obtained with a commercially available anti-HSF1 antiserum further supporting the above data that the complex III does indeed contain HSF (8). Examination of the super-shifted complex obtained (Fig. 4) shows that the addition of the antiserum enhances the intensity of binding, corroborating previously published data on the enhanced activation of HSF binding to the DNA by anti-HSF antibodies (34).

By having established conditions for the generation of trimomeric HSF-HSEαβ complex in neonatal lens extracts, which do not possess this capability when freshly assayed, we decided to...
The HSF assays were conducted with frozen-stored extracts at pH 7.9 prepared from 3- to 4-day-old neonatal rat tissues. Gel-shift mobility-shift assays were conducted within 3–4 days of the preparation of WCEs. The WCE used in these reactions contained 5 μg of protein and about 100 fmol of 32P-HSEaB. 6, no antiserum added, S indicates the position of the super-shifted complex. Lane containing the free probe is not shown. Experiments with a control antibody (anti-actin, ICN, Costa Mesa, CA) did not produce the same results.

In light of the data presented in Fig. 5, we asked the question if complex III can be found naturally without the intervention of “long storage”? WCEs were prepared from temporally defined different stages of the lens and the liver extracts. The gel mobility-shift assays were conducted within 3–4 days of the preparation of the extracts. The oligomeric HSF-HSEaB complex appears gradually with a maximum at day 11 to day 21 in the postnatal rat lens (Fig. 6). The intensity of the complex III decreases with age. Interestingly, over the same period complex III does not appear in the liver extracts (Fig. 6).

DISCUSSION

The presence of such a constitutive state has been demonstrated and seems to be present differentially in a number of tissues including the rat liver (32, 33). Based on the data presented in this investigation, complex I may represent this constitutive monomeric HSF-HSE interaction. Complex I is seen in all the extracts made from lens, liver, heart, and brain (Fig. 5). With lens extracts, HSEaB produces three complexes as follows: complex I (similar to that obtained in other tissues), and complexes II and III, which are lens-specific. The molecular masses of the complexes I and II range from 70 to >70 kDa (Fig. 3). This is very similar to the published data (Ref. 33 and references cited in the Introduction). The data presented in Fig. 3, A–C, strongly suggest that complex III represents an oligomeric (trimeric) aggregate of one or both of the lower molecular mass monomeric complexes.

The actual identity of the HSF in the rat lens is not known. The sequence of the rat HSF2 is very similar (87% sequence similarity) to the sequence of the human HSF1. The data presented in Fig. 1 establish that the appearance of complexes I–III is dependent on the 5′-NGAAN-3′ unit in HSEaB. Considering the complexity of the expression of HSFs (2, 35, 36), it is possible that the complexes II and even complex I (although with mobility similar to that obtained with other tissues) represent less specific-isosforms of HSF. This is also suggested by competition experiments, which show that HSF derived from hsp82 gene is a less efficient competitor as opposed to a consensus HSE (Fig. 1B). HSEaB is very similar to consensus HSE than to HSE82 (see sequences in Fig. 1A and legend to Fig. 1B). The organization of the monomeric 5′-NGAAN-3′ units and possibly the surrounding sequences also contribute to the specificity and modulation of the HSF-HSE interactions. Special sequence preferences may also be inherent in the HSFs. HSF2 has been previously suggested to be involved in hemin-induced activation of HSP70 in K562 cells (2). However, recently it was reported that it is HSF1 that mediates this activation (37). Also, it is known that heat shock has no effect on the DNA-binding ability and oligomeric state of HSF2 (31). We compared the generation of complex III in extracts made from lenses preincubated at 37 and 4 °C before preparation of WCEs. The enhanced presence of complex III was detected in lenses preincubated at 37 °C indicating the stress (heat) sensitivity of its appearance. The heat sensitivity of the appearance of the complex III is further established by the data presented in Fig. 2. The data presented in Fig. 4, in which a human HSF1 antibody was used for super-shift analyses, establish that these complexes contain heat shock factors.

The presence of the monomeric HSF-HSEaB constitutive complexes (complex I in all tissues examined and complex II in the lens) does not correspond to the known expression patterns of aB. Both in the heart (which is known to express aB) and in the liver (which is known not to express aB), there is a robust presence of the constitutive (monomeric) HSF-HSEaB complexes. The neonatal brain extracts show a general lack of binding to HSEaB (Fig. 5) corresponding well with absence of significant aB expression (13, 14).

The appearance of the trimeric complex (complex III), on the other hand, corresponds very well with the known expression patterns of the aB-cry optic gene. The fact that the complex III (produced in vitro by long storage and heat) is the same complex that increases in a temporally defined fashion (Fig. 6) in the lens extracts without any in vitro manipulation clearly establishes that the forma-
tion of these complexes is related to the expression of the αB-crystallin gene. This conclusion is evident because this very heat shock element has been previously shown (a) to respond to heat stress by activation of transcription of the downstream gene in cells transfected with αB heat shock promoter-reporter constructs (26), and (b) to result in the increase of the transcription of the αB-crystallin in cells exposed to heat or osmotic stress (27). A quantitative analysis shows that the adult lens contains about 10-fold more αB transcripts than either the fetal or early postnatal lens. These data suggest that although the expression of αB starts early in the fetal life (e.g., in the heart and the lens), the lens may depend on the activation of the heat shock promoter for high expression during late postnatal/adult stages.

It is noteworthy that among different extracts made from 3–4-day-old rat tissues, only the lens extract can produce the trimeric complex when facilitated in vitro by long storage (Fig. 5). Interestingly, the extracts from day 11 to 21 lens do not need any in vitro manipulation for the production of the complex III (Fig. 6). These data suggest that “competence” for the production of complex III exists in the day 3–4 lenses; however, it is not functional until activated in vitro (by long storage or heat) or in vivo by the developmental cues (or temporally defined stages). It will not be out of place to speculate that “long
storage" of extracts or exposure to high temperatures may facilitate conformational changes within the HSF. It has been suggested that in HSF, transitions from intramolecular interactions to intermolecular interactions may lead to trimerization and DNA binding (38). The tissue specificity of such transitions may suggest that special HSF isoforms are involved as suggested above or that tissue-specific activator or facilitator molecules are present in the lens. The in vivo mechanism that leads to trimerization of the HSF and its binding to DNA at day 11–21 remains to be investigated. It is tempting to speculate that the presence of a developmentally controlled activator-switch that leads to HSF oligomerization and DNA binding. HSF3 has been shown to be activated by direct interaction with other transcription factors (39).

The exclusive presence of the multimeric HSF-HSEoB complex only in the lens and its absence in the liver clearly establish involvement of the heat shock promoter in tissue- and developmental stage-specific control of the αB-crystallin gene expression. We believe that other genes containing heat shock elements in their promoters may utilize HSF-HSE interactions to modulate their expression under normal non-stress conditions (40, 41). Furthermore, although it has been suggested by in vitro studies that HSF may have an “intrinsic” ability to respond to temperature by oligomerization and DNA binding (42), these data point to a tissue-specific and developmental control of this ability.

Acknowledgments—We thank Dr. Michael Bova and Sylvia Rayner for reading the manuscript and Ric Grambo for assistance with the preparation of the figures.

REFERENCES
