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Tissue-Dependent Expression of Heat Shock Factor 2 Isoforms with Distinct Transcriptional Activities

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Heat shock factor 2 (HSF2) functions as a transcriptional regulator of heat shock protein gene expression in mammalian cells undergoing processes of differentiation and development. Our previous studies demonstrated high regulated expression and unusual constitutive DNA-binding activity of the HSF2 protein in mouse testes, suggesting that HSF2 functions to regulate heat shock protein gene expression in spermatogenic cells. The purpose of this study was to test whether HSF2 regulation in testes is associated with alterations in the HSF2 polypeptide expressed in testes relative to other mouse tissues. Our results show that mouse cells express not one but two distinct HSF2 proteins and that the levels of these HSF2 isoforms are regulated in a tissue-dependent manner. The testes express predominantly the 71-kDa HSF2-α isoform, while the heart and brain express primarily the 69-kDa HSF2-β isoform. These isoforms are generated by alternative splicing of HSF2 pre-mRNA, which results in the inclusion of an 18-amino-acid coding sequence in the HSF2-α mRNA that is skipped in the HSF2-β mRNA. HSF2 alternative splicing is also developmentally regulated, as our results reveal a switch in expression from the HSF2-β mRNA isoform to the HSF2-α isoform during testis postnatal development. Transfection analysis shows that the HSF2-α protein, the predominant isoform expressed in testis cells, is a more potent transcriptional activator than the HSF2-β isoform. These results reveal a new mechanism for the control of HSF2 function in mammalian cells, in which regulated alternative splicing is used to modulate HSF2 transcriptional activity in a tissue-dependent manner.

Heat shock factors (HSFs) function as transcriptional regulators of heat shock protein (hsp) gene expression in eukaryotic cells (7, 8, 10, 22). Cells of a number of eukaryotic species contain several related HSF polypeptides, which are expressed from different members of an HSF gene family (12, 13, 17–19). Mouse cells express two different HSF polypeptides, HSF1 and HSF2, which have distinct functional roles in the regulation of hsp gene expression. HSF1 is responsible for mediating the cellular stress response, which is the induction of hsp gene expression that occurs in cells following exposure to elevated temperature and other environmental stress conditions (1, 15). In response to stress conditions, HSF1 undergoes oligomerization from a non-DNA-binding monomeric form to a trimeric, DNA-binding form which interacts with hsp gene promoter sequences to increase transcription of these genes (7, 8, 10, 22).

HSF2, on the other hand, appears to function as a regulator of hsp gene expression under nonstress conditions, particularly in cells involved in processes of differentiation and development (11, 16, 20, 21). We have previously shown that both the expression and functional properties of HSF2 are regulated in mouse testis cells (16). HSF2 mRNA expression in testes is subject to developmental, spermatogenesis stage-dependent, and cell-type-dependent regulation, with the highest levels of HSF2 mRNA and protein being found in pachytene spermatocytes and round spermatids. The DNA-binding activity of the HSF2 protein is also regulated in cells of the testis. While this factor exists in most cell types in a non-DNA-binding state, the HSF2 protein expressed in testis cells exhibits constitutive DNA-binding activity (15, 16). This constitutive HSF2 DNA-binding activity interacts in vitro with promoter sequences of the *hsp70.2* gene, a testis-specific member of the mammalian *hsp70* gene family, suggesting a functional role for HSF2 in regulating hsp gene expression in spermatogenic cells (16).

The purpose of this study was to gain insight into the mechanisms which regulate HSF2 function in testes by characterizing differences between the HSF2 polypeptide expressed in testes and that expressed in other mouse tissues. Our results show that mouse cells express two distinct HSF2 polypeptide species, which are generated by alternative splicing of the HSF2 precursor mRNA. We also show that the expression of these HSF2 protein isoforms is regulated in a tissue-dependent manner, with the testis expressing primarily the larger HSF2-α protein isoform and the heart and brain expressing predominantly the smaller HSF2-β protein isoform. The biological significance of the regulated expression of HSF2-α and HSF2-β isoforms in mouse tissues is that these two proteins are functionally distinct. Transfection analysis demonstrates that the HSF2-α isoform is a more potent transcriptional activator than the HSF2-β isoform. These results reveal a new mechanism for HSF2 regulation in mammalian cells, involving tissue-dependent modulation of the transcriptional activity of this factor via alternative splicing of HSF2 mRNA.

MATERIALS AND METHODS

Experimental animals. CBA/J mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and maintained under a controlled light cycle (14 h light/10 h dark). Testes were removed from mice at the ages of 7, 14, and 21 days and 6 to 8 weeks (adult), rapidly frozen on dry ice, and then kept at −80°C until use. Other mouse tissues were taken at 8 weeks of age.

RT-PCR analysis. Total RNA was prepared from mouse tissues and isolated spermatogenic cells by homogenization in guanidine isothiocyanate and centrifugation through cesium chloride as described previously (16). Isolated pachytene spermatocytes and round spermatids were obtained by centrifugal elutriation of enzymatically dissociated testes of adult mice (9). Reverse transcription-PCR (RT-PCR) of RNA samples was performed as described previously (16). Total RNA (2 to 5 μg) was reverse transcribed at 42°C, using random hexamer primers and avian myeloblastosis virus reverse transcriptase in a 20-μl reaction mixture.

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Two oligonucleotide primers 22 nucleotides in length (55% GC content) which hybridize to nucleotides 1171 to 1192 and 1623 to 1644 of the mouse HSF2 cDNA (17) were used to amplify 473- and 527-bp isoform products from the HSF2 cDNA, while a 104-bp fragment of the mouse ribosomal protein S16 mRNA was amplified as an internal control (23). A mix containing oligonucleotide primers (200 ng of each), [α-^{32}P]dCTP (2 μCi at 3,000 Ci/mmol), deoxynucleoside triphosphates (2 μl of a 10 mM solution), and *Taq* DNA polymerase (2.5 U) was added to 2 μl of each reverse transcription reaction, the total volume was brought to 100 μl with 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), and the sample was overlaid with mineral oil. Amplification was carried out for 20 cycles, using an annealing temperature of 65°C, in a Perkin-Elmer Cetus thermal cycler. The quantitative nature of the RT-PCR assay was determined by quantitation of PCR products resulting from reactions utilizing bracketed cycle numbers (15, 20, 25, etc.). The RT-PCR assay is linear with respect to input template cDNA over at least 25 cycles of amplification. The amplified products were separated by electrophoresis on 5% polyacrylamide gels and visualized by film autoradiography. The intensities of the bands in the RT-PCR analysis were quantitated with a Molecular Dynamics PhosphorImager, using the ImageQuant (version 3.3) program, and the level of each HSF2 mRNA isoform was calculated after normalization to the S16 mRNA internal control level.

Western blot (immunoblot) and gel shift analysis. Mouse tissues and isolated spermatogenic cells (pachytene spermatocytes and round spermatids) were boiled in 2× Laemmli buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% sodium dodecyl sulfate [SDS], 200 mM dithiothreitol), electrophoresed on an SDS–8% polyacrylamide gel, and then blotted to nitrocellulose by using a Bio-Rad Semidry transfer apparatus. The blot was then probed with the HSF2 polyclonal antibodies, using methods described previously (15). The native gel mobility shift assay was performed as described previously (15) with a self-complementary consensus heat shock element-containing oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) which contains four perfect inverted 5′-NGAAN-3′ repeats.

Isolation and cloning of HSF1 and HSF2 cDNAs and genomic DNA sequences. HSF2 cDNA fragments spanning the alternative splice site were obtained by RT-PCR of total mouse testis RNA. Following reverse transcription of total RNA, cDNA fragments were amplified by PCR as described above except that radioactivity was not incorporated and bands were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. After digestion with restriction enzymes using sites on opposite sides of the splice variant junctions, HSF2 cDNA fragments were subcloned into vector plasmids and then sequenced by the Sanger dideoxy method.

The DNA sequences of the HSF2 gene that comprise the alternative splice junctions were isolated from phage DNA of HSF2 genomic clones obtained from a mouse genomic DNA library (Clontech). Because of large intronic interruptions in the corresponding regions of the HSF2 gene, each side of the HSF2 alternative splice junction as well as the 54-nucleotide alternative exon was isolated separately after identification of appropriate restriction fragments by Southern blot hybridization using oligonucleotide probes specific for each sequence. After subcloning into plasmid vectors, the sequences of the HSF2 genomic fragments were determined by the Sanger dideoxy method.

Transfection of NIH 3T3 cells with HSF2 expression vectors. The plasmids used were β-actin-mHSF2-β and β-actin-mHSF2-α expression plasmids and the hsp promoter-luciferase reporter plasmid (15). The β-actin-mHSF2-α plasmid was constructed by replacing a *Bgl*II-*Hin*dIII fragment of the HSF2-β coding sequence with a corresponding fragment of the HSF2-α cDNA. The transfections were performed by using calcium phosphate precipitation as described by Espeseth et al. (5). Mouse NIH 3T3 cells were seeded at a density of 500,000 per plate and transfected with 10 μg of plasmid DNA per plate the following day. The cells were incubated overnight at 37°C, and fresh medium was added after removal of the DNA precipitate by one wash with phosphate-buffered saline. After 24 h, the cells were harvested, and whole cell extracts were prepared as described previously (15). Luciferase assays were performed as previously described (4), and the results were normalized for transfection efficiency as previously described (15). Western blot and gel shift analysis of transfected cell extracts was performed as described above.

RESULTS

Regulated expression of HSF2 protein isoforms via tissue-dependent alternative splicing. To characterize the properties of the HSF2 protein in cells of different mouse tissues, we performed Western blot analysis on extracts of heart, brain, testis, and two isolated spermatogenic cell types (pachytene spermatocytes and round spermatids), using polyclonal antibodies specific to the HSF2 protein (15). The results (Fig. 1) show that these mouse tissues and isolated germ cell types express two distinct HSF2 protein isoforms, with sizes of 69 and 71 kDa. We will refer to the smaller, 69-kDa polypeptide as the HSF2-β isoform and the larger, 71-kDa polypeptide as the HSF2-α isoform. This analysis also shows that the relative levels of the two HSF2 protein isoforms vary significantly between different tissues. The heart and brain contain predominantly the HSF2-β protein, while testes, isolated pachytene spermatocytes, and round spermatids express almost exclusively the HSF2-α protein.

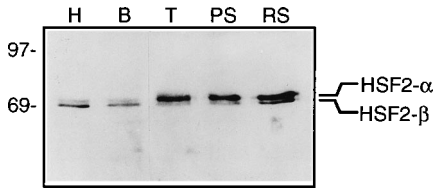

FIG. 1. Western blot analysis of HSF2 protein isoforms in mouse tissues and isolated spermatogenic cells. Extracts of mouse heart (H), brain (B), testis (T), and isolated spermatogenic cell types, pachytene spermatocytes (PS) and round spermatids (RS), were subjected to Western blot analysis using HSF2 polyclonal antibodies (15). The positions of the HSF2-α and HSF2-β protein isoforms are indicated on the right; positions of migration of molecular mass standards are indicated in kilodaltons on the left.

Alternative mRNA splicing is one mechanism by which distinct polypeptide products can be expressed from a single gene. To explore whether this is the mechanism by which the HSF2-α and HSF2-β protein isoforms are generated, we probed for the existence of HSF2 mRNA isoforms in the mouse tissues and isolated spermatogenic cells examined in Fig. 1, using RT-PCR analysis. For this analysis, we used an oligonucleotide primer pair that spans a region in the C-terminal portion of the HSF2 mRNA (nucleotides 1171 to 1644 of the mouse HSF2 cDNA [17]), which was suggested by previous sequence analysis to be a potential site for alternative mRNA processing events. Comparison of the deduced amino acid sequences of cloned mouse and human HSF2 cDNAs reveals a single sequence of 18 amino acids that is present in the human HSF2 sequence (amino acids 393 to 411) but missing from the mouse HSF2 sequence (14a, 17, 19).

The results of the RT-PCR analysis (Fig. 2A) reveal two amplification products of 473 and 527 bp in all of the tissues and isolated spermatogenic cell types tested, demonstrating the existence of HSF2 mRNA variants in these tissues and cell types. On the basis of evidence presented below, we will refer to the shorter HSF2 mRNA variant as the HSF2-β mRNA isoform and the larger HSF2 mRNA variant as the HSF2-α mRNA isoform. These results also show a significant variation in levels of the two HSF2 mRNA isoforms between the different tissues and cell types. The heart and brain exhibit primarily the HSF2-β mRNA isoform, while testes and the isolated spermatogenic cell types exhibit predominantly the HSF2-α mRNA isoform. The tissue-dependent variation in levels of the HSF2-α and HSF2-β mRNA isoforms is remarkably similar to that observed for the HSF2-α and HSF2-β protein isoforms in the Western blot analysis shown in Fig. 1. In Fig. 2B, the RT-PCR bands shown in Fig. 2A were quantitated and normalized to internal control S16 mRNA levels. The heart and brain contain 2.8- and 5.3-fold-higher levels, respectively, of the HSF2-β mRNA isoform than the HSF2-α isoform, while the testes, pachytene spermatocytes, and round spermatids contained 1.9-, 2.6-, and 2.1-fold-higher levels, respectively, of the HSF2-α mRNA isoform.

To determine the identities of the HSF2-α and HSF2-β mRNA isoforms, the two HSF2 RT-PCR products amplified from testis RNA (shown in Fig. 2A) were isolated and subcloned, and the sequences were determined. This sequence analysis revealed that the two HSF2 mRNA isoforms are iden-

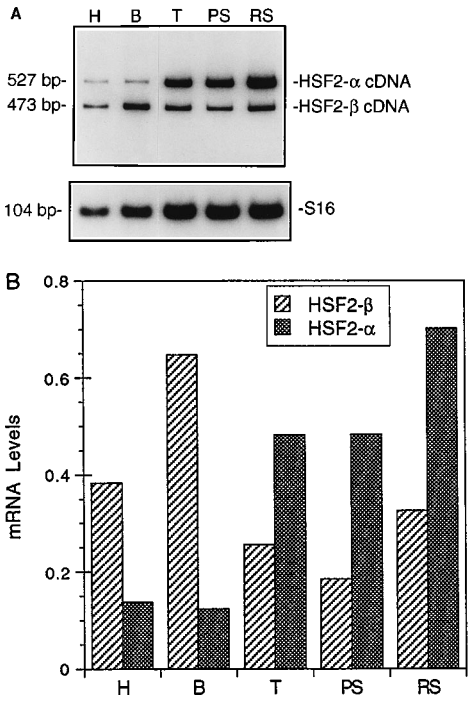

FIG. 2. (A) RT-PCR analysis of HSF2 mRNA isoforms in mouse tissues and isolated spermatogenic cells. Total RNA from mouse heart (H), brain (B), testis (T), and isolated spermatogenic cell types, pachytene spermatocytes (PS) and round spermatids (RS), was subjected to RT-PCR analysis using an oligonucleotide primer pair that amplifies the region corresponding to nucleotides 1171 to 1644 of the full-length mouse HSF2 cDNA (clone C9) (17). (B) Quantitation of HSF2-α and HSF2-β mRNA isoform levels in mouse tissues and isolated cell types. The HSF2-α and HSF2-β RT-PCR bands in panel A were quantitated and normalized to values of S16 mRNA internal control bands.

tical except for a single short nucleotide sequence found in the HSF2-α isoform but missing from the HSF2-β isoform. The nucleotide sequences of the HSF2-α and HSF2-β mRNA isoforms within this region of variation, along with the deduced amino acid sequences, are shown in Fig. 3. The sequence of the HSF2-β mRNA isoform is identical to that of a previously isolated mouse HSF2 cDNA (clone C9) (17). The HSF2-α mRNA isoform differs from the HSF2-β isoform by a single 54-nucleotide insertion, which encodes an 18-amino-acid sequence. This extra amino acid sequence in the HSF2-α isoform is identical to the 18-amino-acid sequence noted above that is encoded in a human HSF2 cDNA but missing from the mouse HSF2 cDNA (mouse HSF2-β mRNA) (17, 19).

Although we cannot formally rule out a potential role for posttranslational modification in generating the HSF2 protein isoforms, several pieces of evidence strongly suggest that the HSF2-α and HSF2-β protein isoforms arise from translation of HSF2-α and HSF2-β mRNAs, respectively. First, as demonstrated above, there is a tight correlation between the levels of the HSF2-α and HSF2-β proteins and mRNA isoforms in different tissues and cell types. Second, RT-PCR analysis of testis RNA, using a primer pair spanning the entire HSF2 mRNA coding region, yields only two products, with sizes matching those expected for the HSF2-α and HSF2-β mRNA species, suggesting that these are the only major HSF2 mRNA species present in these cells from which the HSF2-α and HSF2-β protein isoforms could be translated (data not shown). Third, previous studies showed that exogenous expression of a cloned mouse HSF2 cDNA, which corresponds to the HSF2-β mRNA isoform, yields a polypeptide product whose migration on SDS-polyacrylamide gels is identical to that of the natural HSF2-β protein isoform (15, 17). Finally, the theoretical molecular mass of the 18-amino-acid insertion in the HSF2-α isoform is 2,022 Da, which accounts perfectly for the observed size difference between the HSF2-β and HSF2-α protein isoforms on SDS-polyacrylamide gels (69 and 71 kDa; Fig. 1).

Sequence analysis of HSF2 gene alternative splice junctions. To verify that the HSF2-α and HSF2-β mRNA isoforms arise by alternative splicing of the HSF2 pre-mRNA, the regions of the HSF2 gene corresponding to the exon-intron boundaries of the putative alternative splice junctions were isolated and sequenced. The results of this sequence analysis (Fig. 4A) reveal that the additional 54-nucleotide sequence found in the HSF2-α mRNA isoform exists as a distinct exon in the HSF2 gene, bounded by mammalian splice site consensus sequences, thus providing strong evidence that the HSF2-α and HSF2-β mRNAs are generated by alternative splicing events from a single HSF2 precursor mRNA. Shown in Fig. 4B is a schematic representation of the functional domain organization of the HSF2 protein and the alternative splicing events by which the HSF2-α and HSF2-β protein isoforms are generated (numbers refer to HSF2 amino acid sequences as encoded by the two mRNA isoforms). The extra 18 amino acids found in the HSF2-α protein (designated SV) are inserted in the C-terminal region of the protein, immediately adjacent to a conserved leucine zipper domain implicated in maintaining HSFs in the inactive, non-DNA-binding form (14, 24).

Developmental switch in HSF2 mRNA alternative splicing. Prompted by the high regulated expression of the HSF2-α mRNA isoform in adult testes, we wanted to determine whether levels of the HSF2 mRNA isoforms are regulated during postnatal development of the testis. Therefore, we performed RT-PCR analysis on RNA from mouse testes at different time points of postnatal development (Fig. 5A). Testes from mice at day 7 after birth, which contain somatic testis cell types but only immature spermatogonial germ cells, express significantly higher levels of the HSF2-β mRNA isoform than the HSF2-α isoform. However, as development proceeds through days 14 and 21 to 6 weeks, the relative levels of the HSF2-α mRNA isoform gradually increase, leading to the predominance of this larger HSF2 mRNA isoform in mature testes. These results are consistent with the observation of high levels of the HSF2-α mRNA isoform in pachytene spermatocytes and round spermatids, as the proportion of these developing germ cells relative to somatic cell types increases during postnatal development of the testis (2). Quantitation of these results after normalization to internal control S16 mRNA levels (Fig. 5B) reveals that the change in ratio of the HSF2-α and

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      asp leu leu val asp leu phe thr ser ser val gln met asn pro thr asp asn ile asn asn thr lys ser glu asn lys gly
[--//--GAT CTT CTG GTT GAT CTT TTC ACT AGT TCT GTG CAG ATG AAT CCC ACA GAT AAC ATC AAT AAT ACA AAA TCT GAG AAT AAG GGA--//--] mHSF2-α cDNA
[--//--GAT CTT CTG GTT GAT - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - TCT GAG AAT AAG GGA--//--] mHSF2-β cDNA
      1317                                                                                                  1347
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FIG. 3. Nucleotide and deduced amino acid sequences of HSF2 mRNA isoform cDNAs. RT-PCR products corresponding to HSF2-α and HSF2-β mRNA isoforms (as shown in Fig. 2A) were isolated, subcloned, and sequenced. The nucleotide and deduced amino acid sequences shown correspond to the region of variation between the HSF2-α and HSF2-β mRNA isoforms. Numbers refer to nucleotide position relative to the previously cloned full-length mouse HSF2 cDNA (clone C9) (17).

A

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1317 GATCT TCTGGTTGAT gtaggtattt ttgatactct ttactatact ggtggtttgt gtacttaca
     CTAGA AGACCAACTA catccataaa aactatgaga aatgatatga ccaccaaaca catgaatgt

                                                    1.8 kb
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     acacggtaaat tttagttatg agaacataag atgcgaaag  //  aatttgaata gtagaaaaaa

     gtttggtctt ttcagCTTTTCACTAGTTCT GTGCAGATGA ATCCCACAGA TAACATCAAT ATACA
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B

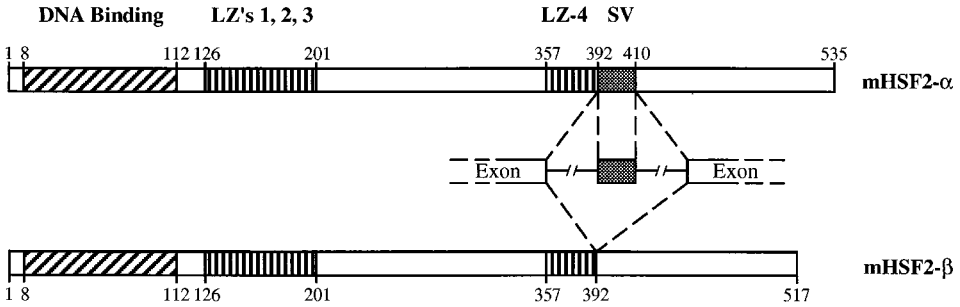

FIG. 4. (A) Sequence of HSF2 gene regions corresponding to alternative splice junctions. HSF2 genomic fragments containing the exon-intron boundaries of interest were isolated from a mouse genomic library, subcloned, and sequenced. Exons are represented by boldface capital letters, while introns are represented by normal lowercase letters. Numbers at the beginning and end of the genomic sequences are nucleotide positions relative to the full-length mouse HSF2 cDNA (clone C9), which are the same as those indicated in Fig. 3 in order to allow easy comparison of the corresponding HSF2 mRNA isoform and gene sequences (17). (B) Schematic representation of HSF2 mRNA alternative splicing pathways and functional domains of HSF2-α and HSF2-β protein isoforms. The HSF2 exon encoding the 18-amino-acid sequence which differs in the HSF2 isoforms is indicated (SV), along with the conserved DNA-binding domain, oligomerization domain (leucine zippers 1 to 3 [LZ's 1, 2, 3]), and carboxy-terminal leucine zipper motif (LZ-4).

HSF2-β mRNA isoforms during testis postnatal development is due primarily to a large increase in expression of the HSF2-α mRNA isoform, as levels of the HSF2-β mRNA isoform are relatively unchanged during postnatal development. Levels of the HSF2-α mRNA isoform in the testis increase 7.6-fold between day 7 and 6 weeks of postnatal development.

HSF2 protein isoforms are functionally distinct. Finally, we investigated the biological significance of the regulated expression of HSF2-α and HSF2-β protein isoforms in mouse tissues by determining whether there is any difference in the transcriptional activation functions of these two isoforms. HSF2-α and HSF2-β expression plasmids were transfected into NIH 3T3 cells along with an hsp promoter-luciferase reporter gene plasmid (hsp70-luciferase) and assayed for luciferase activity (Fig. 6A). Cells transfected with the HSF2-β expression plasmid exhibited a mean 3.6 (±0.5)-fold-higher luciferase activity relative to cells transfected with the expression vector (β-actin-1-neo) and hsp70-luciferase, while cells transfected with the HSF2-α expression plasmid exhibited a mean 9.6 (±1.1)-fold-higher luciferase activity.

These results indicate that the addition of the 18-amino-acid sequence to the HSF2-α protein confers an increase in transcriptional activity on this HSF2 isoform. Western blot analysis reveals very similar levels of the HSF2-β and HSF2-α protein isoforms in extracts of the transfected cells (Fig. 6B; compare lanes 2 and 3), indicating that the observed difference in transcriptional activities cannot be ascribed to differences in the expression and/or stability between the two HSF2 protein isoforms in the transfected cells. In addition, native gel mobility shift analysis demonstrates that the transfected cell extracts exhibit very similar levels of HSF2 DNA-binding activity (Fig. 6C; compare lanes 2 and 3). This result suggests that the insertion of the additional 18-amino-acid sequence does not positively regulate the transcriptional activity of the HSF2-α protein isoform by causing an increase in the DNA-binding ability of this HSF2 isoform.

DISCUSSION

Our results show that mouse cells express two distinct isoforms of the HSF2 protein and that the levels of these HSF2 isoforms are regulated in a tissue-dependent manner. The HSF2 protein isoforms arise via alternative splicing of the HSF2 pre-mRNA, which results in the insertion of an 18-amino-acid sequence in the HSF2-α isoform that is lacking in the HSF2-β isoform. The biological significance of the regulated expression of HSF2-α and HSF2-β isoforms in mouse tissues is that these two proteins are functionally distinct. We have shown that the HSF2-α isoform, the predominant isoform expressed in testes, is a more potent transcriptional activator than the HSF2-β isoform, which is the predominant isoform expressed in the heart and brain. These results reveal a new mechanism for the control of HSF2 function in mammalian cells, in which regulated alternative splicing of HSF2 pre-mRNA is used to modulate the transcriptional activation ability of the HSF2 protein in a tissue-dependent manner.

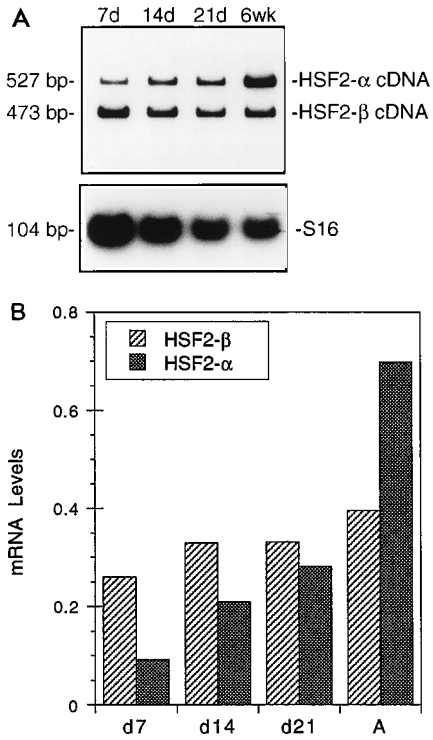

FIG. 5. (A) RT-PCR analysis of HSF2 isoforms during postnatal development of mouse testes. Total RNA isolated from mouse testes at days 7, 14, and 21 (7d, 14d, and 21d) and 6 weeks (6 wk) after birth was subjected to RT-PCR analysis using an oligonucleotide primer pair that amplifies the region corresponding to nucleotides 1171 to 1644 of the full-length mouse HSF2 cDNA (clone C9) (17). (B) Quantitation of HSF2-α and HSF2-β mRNA isoform levels in testes at different times of postnatal development. The HSF2-α and HSF2-β RT-PCR bands in panel A were quantitated and normalized to values of S16 mRNA internal control bands. A, adult.

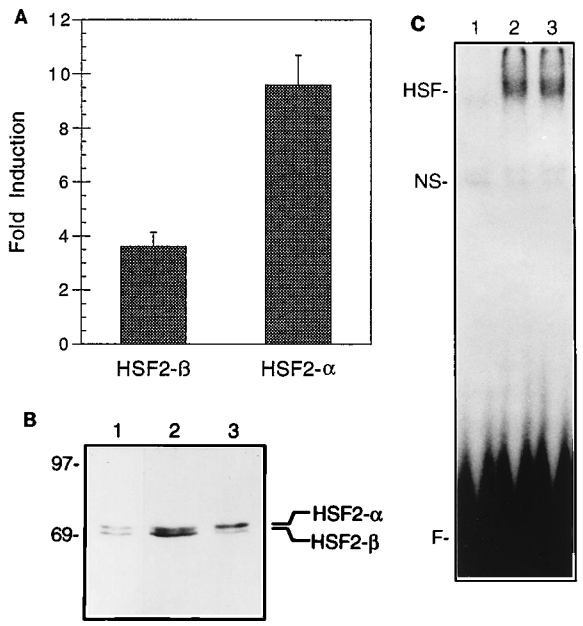

FIG. 6. Transfection analysis of HSF2-β and HSF2-α protein isoforms. (A) Transcriptional activity of HSF2-β and HSF2-α protein isoforms in transfected cells. HSF2-β and HSF2-α expression plasmids were transfected into NIH 3T3 cells along with an hsp promoter-luciferase reporter gene plasmid (hsp70-luciferase) and assayed for luciferase activity. The results are expressed as the mean ± standard deviation of the fold increase in luciferase activity over cells transfected with the reporter construct and parental expression vector (β-actin-1-neo). Cells transfected with the HSF2-β or HSF2-α expression plasmid exhibited mean 3.6 (±0.5)-fold and 9.6 (±1.1)-fold increases in luciferase activity, respectively. (B) Western blot analysis of HSF2-β and HSF2-α protein levels in transfected cells. Extracts of NIH 3T3 cells transfected with the reporter plasmid alone (lane 1), HSF2-β expression plasmid (lane 2), or HSF2-α expression plasmid (lane 3) were subjected to Western blot analysis using HSF2 polyclonal antibodies (15). Positions of the HSF2-α and HSF2-β protein isoforms are indicated on the right; positions of size markers are indicated in kilodaltons on the left. (C) DNA-binding activity of HSF2-β and HSF2-α isoforms in transfected cells. Extracts of NIH 3T3 cells transfected with the reporter plasmid alone (lane 1), HSF2-β expression plasmid (lane 2), or HSF2-α expression plasmid (lane 3) were subjected to a native gel mobility shift assay using a radiolabeled heat shock element-containing probe (15). NS, nonspecific complex; F, free probe.

Our results show that the insertion of a single 18-amino-acid sequence in the HSF2-α protein isoform is sufficient to cause a significant increase in the transactivation function of this HSF2 isoform relative to the HSF2-β isoform. Since this effect does not appear to be mediated through alteration of DNA-binding activity, the most likely explanation is that the extra 18-amino-acid sequence in the HSF2-α isoform increases the transactivation function of this protein by enhancing its ability to interact with other components of the transcription apparatus. This sequence may itself comprise a transcriptional activation domain, or alternatively, its addition to the HSF2 polypeptide sequence may alter the positioning of the actual transcriptional activation domains within the structure of the HSF2 protein so as to create a more favorable orientation for interactions with the other proteins in the transcription complex. Previous studies have shown that the function of another transcriptional regulator, the cyclic AMP-responsive element modulator (CREM), is subject to this type of regulation during mammalian spermatogenesis. During male germ cell development, a switch occurs in the pattern of alternative splicing of CREM mRNA, which results in a change in expression from CREM isoforms with repressor properties (CREMα, -β, and -γ) to a CREM isoform that functions as a transcriptional activator (CREMτ) (3, 6). CREMτ differs from the other CREM isoforms by the addition of two glutamine-rich sequences which comprise transcriptional activation domains (6).

HSF2 is subject to a complex pattern of control in testis cells, mediated by regulatory events occurring at multiple levels. Our previous studies have demonstrated developmental, cell-type-dependent, and stage-dependent up-regulation of HSF2 expression, as well as testis cell-specific activation of HSF2 DNA binding (16). To this already complex regulation we can now add a new level: potentiation of HSF2 transcriptional activity in testis cells via tissue-dependent alternative splicing of HSF2 mRNA. In addition to revealing a new mechanism of HSF2 regulation in mammalian cells, our results strengthen support for a biological role for HSF2 as a regulator of gene expression in cells of the testis. We have previously shown that HSF2 present in extracts of testis cells can bind to consensus heat shock element sequences as well as to promoter sequences of the *hsp70.2* gene, a testis-specific member of the mammalian *hsp70* gene family (16). We hypothesize that HSF2 expression and function are up-regulated in spermatogenic cells in order to increase expression of one or more hsps, whose functions as molecular chaperones may be required to handle the unique patterns of protein expression in male germ cells. Future studies will be required to characterize the *cis*-acting sequence elements and *trans*-acting factors that mediate each of the levels of HSF2 regulation in spermatogenic cells. Given the complexity of this regulation, these studies are likely to reveal

many interesting insights into the diverse mechanisms by which gene expression and function are controlled in male germ cells.

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ADDENDUM IN PROOF

We have recently published results showing the existence and regulated expression of two HSF1 mRNA splicing isoforms in cells of mouse tissues (M. L. Goodson and K. D. Sarge, Biochem, Biophys. Res. Commun. **211:**943–949, 1995). We also note that another laboratory has recently published results which demonstrate the existence of the HSF1 and HSF2 α and β isoforms in mouse cells (M. T. Fiorenza, T. Farkas, M. Dissing, D. Kolding, and V. Zimarino, Nucleic Acids Res. **23:**467–474, 1995).

REFERENCES

1. **Baler, R., G. Dahl, and R. Voellmy.** 1993. Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. Mol. Cell. Biol. **13:**2486–2496.
2. **Bellve, A. R., J. C. Cavicchia, C. F. Millette, D. A. O'Brien, Y. M. Bhatnagar, and M. Dym.** 1977. Spermatogenic cells of the prepuberal mouse: isolation and morphological characterization. J. Cell Biol. **74:**68–85.
3. **Delmas, V., F. van der Hoorn, B. Mellstrom, B. Jegou, and P. Sassone-Corsi.** 1993. Induction of CREM activator proteins in spermatids: down-stream targets and implications for haploid germ cell differentiation. Mol. Endocrinol. **7:**1502–1514.
4. **deWet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
5. **Espeseth, A., S. P. Murphy, and E. Linney.** 1989. Retinoic acid receptor expression vector inhibits differentiation of F9 embryonal carcinoma cells. Genes Dev. **3:**1647–1656.
6. **Foulkes, N. S., B. Mellstrom, E. Benusiglio, and P. Sassone-Corsi.** 1992. Developmental switch of CREM function during spermatogenesis from antagonist to activator. Nature (London) **355:**80–84.
7. **Kingston, R. E.** 1991. Transcriptional regulation of heat shock genes, p. 377–398. *In* P. Cohen and J. G. Foulkes (ed.), The hormonal control of gene transcription. Elsevier Science Publishers, New York.
8. **Lis, J., and C. Wu.** 1993. Protein traffic on the heat shock promoter: parking, stalling, and trucking along. Cell **74:**1–4.
9. **Meistrich, M. L., J. Longtin, W. A. Brock, S. R. Grimes, and M. L. Mace.** 1981. Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. Biol. Reprod. **25:**1065–1077.
10. **Morimoto, R. I., K. D. Sarge, and K. Abravaya.** 1992. Transcriptional regulation of heat shock genes: a paradigm for inducible genomic responses. J. Biol. Chem. **267:**21987–21990.
11. **Murphy, S. P., J. J. Gorzowski, K. D. Sarge, and B. Phillips.** 1994. Characterization of constitutive HSF2 DNA-binding activity in mouse embryonal carcinoma cells. Mol. Cell. Biol. **14:**5309–5317.
12. **Nakai, A., and R. I. Morimoto.** 1993. Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. Mol. Cell. Biol. **13:**1983–1997.
13. **Rabindran, S. K., G. Giorgi, J. Clos, and C. Wu.** 1991. Molecular cloning and expression of a human heat shock factor, HSF1. Proc. Natl. Acad. Sci. USA **88:**6906–6910.
14. **Rabindran, S. K., R. I. Haroun, J. Clos, J. Wisniewski, and C. Wu.** 1993. Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. Science **259:**230–234.

14a. **Sarge, K. D.** Unpublished data.

15. **Sarge, K. D., S. P. Murphy, and R. I. Morimoto.** 1993. Activation of heat shock gene transcription by HSF1 involves oligomerization, acquisition of DNA binding activity, and nuclear localization and can occur in the absence of stress. Mol. Cell. Biol. **13:**1392–1407.
16. **Sarge, K. D., O.-K. Park-Sarge, J. D. Kirby, K. E. Mayo, and R. I. Morimoto.** 1994. Expression of heat shock factor 2 in mouse testis: potential role as a regulator of heat-shock protein gene expression during spermatogenesis. Biol. Reprod. **50:**1334–1343.
17. **Sarge, K. D., V. Zimarino, K. Holm, C. Wu, and R. I. Morimoto.** 1991. Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. Genes Dev. **5:**1902–1911.
18. **Scharf, K.-D., S. Rose, W. Zott, W. Schoff, and L. Nover.** 1990. Three tomato genes code for heat stress transcription factors with a remarkable degree of homology to the DNA-binding domain of the yeast HSF. EMBO J. **9:**4495–4501.
19. **Schuetz, T. J., G. Gallo, L. Sheldon, P. Tempst, and R. E. Kingston.** 1991. Isolation of a cDNA for HSF2: evidence for two heat shock factor genes in humans. Proc. Natl. Acad. Sci. USA **88:**6911–6915.
20. **Sistonen, L., K. D. Sarge, and R. I. Morimoto.** 1994. Human heat shock factors 1 and 2 are differentially activated and can synergistically induce *hsp70* gene transcription. Mol. Cell. Biol. **14:**2087–2099.
21. **Sistonen, L., K. D. Sarge, B. Phillips, K. Abravaya, and R. I. Morimoto.** 1992. Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. Mol. Cell. Biol. **12:**4104–4111.
22. **Sorger, P. K.** 1991. Heat shock factor and the heat shock response. Cell **65:**363–366.
23. **Wagner, M., and R. P. Perry.** 1985. Characterization of the multigene family encoding the mouse S16 ribosomal protein: strategy for distinguishing an expressed gene from its pseudogene counterparts by an analysis of total genomic DNA. Mol. Cell. Biol. **5:**3560–3576.
24. **Zuo, J., R. Baler, G. Dahl, and R. Voellmy.** 1994. Activation of the DNA-binding ability of human heat shock transcription factor 1 may involve the transition from an intramolecular to an intermolecular triple-stranded coiled-coil structure. Mol. Cell. Biol. **14:**7557–7568.