Heat shock factor-1 (HSF-1) activates transcription of heat shock proteins in eukaryotes. Several overlapping genomic clones containing the murine HSF-1 gene were isolated from a phage genomic library. Results indicate that the HSF-1 gene contains 13 exons that span at least 30 kilobase pairs. Sequence analysis of the 5′-untranslated region of HSF-1 suggests that it contains sequences of a recently described Bop1 gene in reverse orientation within its first 331 base pairs (bp) upstream of the translation initiation site. The minimal promoter sequence required for HSF-1 basal expression was identified by deletion analysis from −4 kilobase pairs to −331 bp of the promoter fused to a luciferase reporter gene using transient transfection assays. Results indicate that 331 bp upstream of the HSF-1 translation start site is required for maximal basal expression in NIH3T3 and F9 cells. This fragment also results in high levels of luciferase activity in the reverse orientation, that is, 5′ to the Bop1 gene, suggesting that this segment is bidirectional and could be utilized for basal expression of both HSF-1 and Bop1 genes. This segment of the promoter contains recognition elements for Sp1 and CCAAT-box binding transcription factors, which when mutated in either sense or antisense orientations to the HSF-1 gene results in a reduction of basal expression by 50–75% relative to wild type, suggesting that these sites are critical for basal expression of both HSF-1 and Bop1 genes.

The heat shock transcription factors (HSFs)1 have been cloned from a variety of organisms. Studies suggest that yeast (Saccharomyces cerevisiae and Schizosaccharomyces pombe) and Drosophila each contain one gene (1–3), whereas two separate genes have been cloned in mouse cells and three genes each in human and chicken cell (4–7). Comparisons of HSF protein structure in a variety of organisms indicate the presence of a conserved DNA binding domain and three hydrophobic heptad repeats that constitute the trimerization domain. These domains are located within the amino-terminal region of the protein. The stress-responsive transcriptional activation domain is located in the carboxyl-terminal end of the molecule. Intramolecular interactions between the amino- and carboxy-terminal coiled coil domains of HSF keep the protein in an inactive state under nonstress growth conditions (8).

In eukaryotes, HSF-1 binds to conserved regulatory sequences known as heat shock elements, where it controls the expression of heat shock proteins in response to stress (9–14). The heat shock elements consists of multiple inverted repeats of AGAAN located upstream of heat shock genes (15–17). In the yeast S. cerevisiae, HSF is constitutively bound to heat shock elements, whereas in most organisms a stress is required for HSF to trimerize and bind to DNA (11, 18–20). In Drosophila and mammals, stress causes an increase in trimerization, DNA binding, and phosphorylation of HSF-1 (11, 21, 22). In mammalian cells, HSF-1 is phosphorylated under normal physiological growth conditions, and this phosphorylation has been shown to repress the activity of HSF-1 (22–26). HSF in yeast is an essential gene, whereas in Drosophila it is not required for general cell growth and viability (1, 27), but it is required during oogenesis and early larval development (27). These data suggest that the function of HSF may not be solely to control transcription of heat shock proteins under stress conditions, but it may also control the expression of non-heat shock genes under normal physiological growth conditions (27). The presence of multiple HSF genes in higher eukaryotes, in contrast to one HSF gene present in yeast and Drosophila, could indicate that individual proteins may be responsible for different biological functions. In human and mouse, HSF-1 has been shown to activate transcription of various heat shock proteins in response to heat shock as well as other environmental stresses (5, 7, 14). HSF-2, on the other hand, does not respond to heat stress but has been shown to activate transcription of heat shock proteins in response to heme in erythroleukemia cells (28, 29). HSF-2 activity is also detected during mouse spermatogenesis (30). The third HSF isoform, HSF-3, is found in chicken and has recently been shown to be activated by c-Myb in the absence of cellular stress (6, 31). Another isoform, HSF-4, has been found in human cells but seems to lack the property of a transcriptional activator (32). Other levels of regulation described for HSF-1 and HSF-2 in mammalian cells is the presence of α and β isoforms (33, 34). These are alternate splice variants of HSF-1 and HSF-2 that recently were shown to be expressed differentially during development (35).

In these studies, we have isolated and analyzed several overlapping genomic clones of murine HSF-1 to examine its gene structure. The HSF-1 promoter was also analyzed to determine the minimal promoter sequences required for basal expression and the binding sites for transcription factors responsible for HSF-1 basal expression. Our results show that HSF-1 contains
13 exons, which span an area of more than 30 kb. The promoter analysis of HSF-1 suggests the presence of binding sites for several transcription factors, some of which appear to be involved in tissue-specific expression for HSF-1. Further, the first exon of the recently described Bop1 gene is found within the first 331 bp upstream of the HSF-1 translation initiation site. The Bop1 gene is in an opposite orientation to the HSF-1 gene. Furthermore, HSF-1 and Bop1 control elements are at least partly shared, as the fragment between them drives transcription of both genes.

**Materials and Methods**

**Cell Culture**—The murine NIH3T3 and F9 embryonal carcinoma cells were obtained from American Type Culture Collection. The 3D0-548 and 5KC T cells were the gift of Dr. L. Ignatowicz (Medical College of Georgia). Cells were maintained in a 37 °C humidified incubator in an atmosphere of 5% CO2 in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and antibiotics.

**Isolation of Genomic Clones Encoding Murine HSF-1**—Approximately 10\(^6\) plaques of the \(\alpha\)FFII vector containing a 4–8-week-old female 129 SJV mouse liver genomic phage library (Strategene, La Jolla, CA) were screened with the EcoRI fragment of murine HSF-1 cDNA. Positive clones were picked in plasmid pBluescript II (pBluescript II SK\(^+\)). The phage lysate of the library was mixed with host cells, plated at 5 \(\times\) 10\(^5\) phage/plate, and lifted onto nylon filters for subsequent denaturation and neutralization.

The filters were prehybridized with 6 \(\times\) SSPE (0.9 mM NaCl, 60 mM Na\(_2\)PO\(_4\), 6 mM EDTA), 5 \(\times\) Denhart's solution, 0.5% SDS, denatured salmon sperm DNA (100 \(\mu\)g/ml), and 50% formamide for 2 h at 42 °C and then hybridized with an [\(\alpha\)-\(32\)P]dCTP-labeled probe using random prime kit purchased from New England BioLabs (Beverly, MA). The filters were washed twice with 0.3 \(\times\) SSC (45 mM NaCl and 4.5 mM sodium citrate) + 0.1% SDS for 30 min at 65 °C. After washing, filters were exposed to x-ray film and aligned to plates for the identification of positive clones. Phage DNA from three partially overlapping independent positive clones (3–1, 3–2, and 19–1; see Fig. 1) were isolated and digested with NotI restriction enzyme and subcloned into pBluescript II SK\(^+\) for further characterization. All three clones were partially sequenced by fluorescence-based cycle sequencing with a model 377 ABI Prism DNA Sequencer (Perkin-Elmer) at the Medical College of Georgia sequencing facility. Most of the genomic sequences were obtained by using oligonucleotide primers whose sequences were based on available murine cDNA sequence (9) and primer walking.

The three clones described above do not contain a translation start site. To clone the exon containing this site and the 5'-untranslated region of HSF-1, the above library was rescreened with an [\(\alpha\)-\(32\)P]dCTP-labeled, 200-bp PCR fragment spanning a portion of the translation initiation site and the 5'-untranslated region of HSF-1. Two overlapping clones (21–1 and 8–1; see Fig. 1) were isolated and partially sequenced as above. The GenBank accession numbers are A561503 and A561504.

5'-Rapid Amplification of cDNA Ends (RACE)—To determine the HSF-1 transcription initiation site, total RNA was isolated from NIH3T3 cells by Trizol reagent according to the manufacturer's instructions (Life Technologies, Inc.). Appropriate primers (2 μg of expression plasmid and 0.1 μg of Renilla luciferase plasmid) were added to 100 μl of serum-free Dulbecco's minimal essential medium mixed with 100 μl of the same medium plus 6 μl of LipofectAMINE and incubated at 25 °C for 30 min. 0.8 μl of serum-free Dulbecco's minimal essential medium was added and poured over the cultures. Cultures were incubated at 37 °C for 5 h. The medium was then replaced with 1 ml of Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, and cells were incubated at 37 °C for an additional 48 h. Cells were then lysed in lysis buffer (Promega), and the amount of protein in cell lysates was determined by biocinchonic acid (Pierce). Luciferase activity present in 20 μg of protein was measured using luciferase assay system or the dual luciferase assay system (Promega). Renilla luciferase was used as an indicator of transfection efficiency (23).

**Transient Transfection Assays**—3–10\(^6\) NIH3T3 or F9 embryonal carcinoma cells were grown in 35-mm culture dishes. The cells were transfected with the indicated DNA constructs by LipofectAMINE as described by the manufacturer (Life Technologies, Inc.). Appropriate plasmids (2 μg of expression plasmid and 0.1 μg of Renilla luciferase plasmid) were added to 100 μl of serum-free Dulbecco's minimal essential medium mixed with 100 μl of the same medium plus 6 μl of LipofectAMINE and incubated at 25 °C for 30 min. 0.8 μl of serum-free Dulbecco's minimal essential medium was added and poured over the cells. Cultures were incubated at 37 °C for 5 h. The medium was then replaced with 1 ml of Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, and cells were incubated at 37 °C for an additional 48 h. Cells were then lysed in lysis buffer (Promega), and the amount of protein in cell lysates was determined by biocinchonic acid (Pierce). Luciferase activity present in 20 μg of protein was measured using luciferase assay system or the dual luciferase assay system (Promega). Renilla luciferase was used as an indicator of transfection efficiency (23).

The murine D30-548 and 5KC T cells were transfected with 15 μg of plasmid constructs as well as 0.2 μg of Renilla luciferase using electroporation (Gene Pulser; Bio-Rad) (336 V, 975 microfarads).

**Gel Mobility Shift Assays**—Gel mobility shift assays using whole cell extracts have been described in detail previously (10, 14). Briefly, NIH3T3 cells were grown in 100-mm tissue culture dishes to confluence, rinsed with cold PBS, and lysed by homogenization in 100 μl of extraction buffer (10 mM HEPES, pH 7.9, 0.4 mM NaCl, 0.1 mM EDTA, 1 mM 1,4-bis[2-hydroxyethyl]ethylenediamine tetraacetic acid, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min on ice. After ultracentrifugation at 100,000 g for 10 min, the protein concentration in the supernatant of each sample was estimated by biocinchonic acid (Pierce). Equal amounts of protein (15 μg) in extraction buffer

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\(^{2}\) W. Woessmann, W., Y.-H. Meng, and N. F. Mivechi, unpublished data.
Structural Organization and Promoter Analysis of HSF-1 Gene

RESULTS

Structure of the Mouse HSF-1 Gene—Three positive clones containing portions of the HSF-1 gene were isolated following primary screening of $10^6$ independent clones and secondary screening to isolate individual plaques of the AFix II mouse genomic library. All clones were partially sequenced using oligonucleotide primers containing sequences from the known mouse HSF-1 cDNA sequence and primer walking. Sequence analysis indicated that clones 3–1 and 3–2 contained the entire HSF-1 cDNA with the exception of the initiation of translation (Fig. 1A). To isolate clones containing the translation start site, the genomic library was rescreened, and two other overlapping clones (3–1 and 3–2) were isolated (Fig. 1A). An additional 7.5-kb of clone 8–1 was sequenced.

The structure of the HSF-1 gene was deduced from a comparison of the known murine HSF-1 cDNA sequences and our genomic sequences. Several salient features can be identified (Fig. 1B and Tables I and II). The HSF-1 gene contains 13 exons. Exons 2 through 13 cover an area of approximately 5 kb. The distance between exons 1 and 2 exceeds 18 kb (Table II). The smallest exon is exon 11, which codes for 22 amino acids, and the largest is exon 9, which codes for 91 amino acids (Table I). The sizes of the introns vary. The smallest introns, 70 bp each, are between exons 11 and 12 and exons 12 and 13. The largest intron, as mentioned above, separates exons 1 and 2 and spans over 18 kb. The exon/intron boundaries contain the 5’-splice sequence gt, and 3’-splice sequence ag, as commonly observed in the mammalian genome (Table II) (37).

Various domains of the HSF-1 protein that have been defined previously (22, 24, 25, 38) are located as follows. The DNA binding domain of HSF-1 spans exons 1, 2, and 3. Leucine zippers 1, 2, and 3 are located in exons 4, 5, 6, and 7. The negative transcriptional regulatory domain is located within exons 5, 6, and 7. The potential mitogen-activated protein kinase (extracellular signal-regulated kinase) and glycogen synthase kinase (GSK-3) phosphorylation motifs (serines 303 and 307) that have been shown to negatively regulate HSF-1 function under normal physiological growth conditions are located in exon 9 (23, 26). Leucine zipper 4 is located in exon 10. The transcriptional activation domain is located within exons 9, 10, 11 (alternative splice isoform), 12, and 13.

Another interesting feature of the HSF-1 gene is the presence of the Bop1 gene in the amino-terminal 5’-untranslated region of HSF-1. The Bop1 translation initiation site is located in the reverse orientation, within the −379 bp of the HSF-1 translation initiation site (Fig. 1). The Bop1 cDNA has recently been sequenced, although its function in mammalian cells is unknown at the present time (GenBank accession number U77415).

Determination of Promoter Architecture and Transcription Start Site—Clones 8–1 and 21–1 were isolated from the genomic library using as a probe the 200-bp sequence surrounding the translation start site and were partially sequenced (approximately 7.5 kb). The sequence of −472 bp of the HSF-1 promoter is shown in Fig. 2. A distinguishing feature is that it contains Bop1 exon 1 as indicated in bold face. Exon 2 of the Bop1 gene is located further upstream within −3 kb of the HSF-1 promoter (data not shown). In addition, the HSF-1 promoter lacks a classical TATA box. To determine the transcription initiation sites, 5’-RACE analysis was performed on total RNA isolated from NIH3T3 cells. The results indicate that the HSF-1 gene has one major transcription start site, an adenosine at −190 bp from the initiation of translation (Fig. 2).

Computer analysis was employed to identify potential transcription factor binding sites in 1 kb of the HSF-1 promoter. The search revealed potential binding sites for various transcription factors such as Sp1, AP1, AP2, CCAAT-box binding protein (CBP), C/EBP, early growth response-1 gene (EGR-1), GATA-1, CTF, and TCF-1.

Promoter Activity of the 5’-Flanking Region of the HSF-1 Gene in Transient Transfection Assays—The 5’-flanking region (−4 kb) of the HSF-1 gene contains potential binding sites for several transcription factors. This region also contains the se-
quences of exons 1 and 2 and a portion of the first and second introns of the Bop1 gene (Fig. 2). To test if this region functions as a promoter, we transiently transfected NIH3T3 or F9 cells with constructs containing various fragments fused upstream of a luciferase reporter gene, and luciferase activity was measured (Fig. 3A). The results indicate that most of the basal transcriptional activity is located in the first 331 base pairs (pHSF-1P331; Fig. 3A). Relative to the 331-bp fragment, the transcription activity of the 379-bp fragment in the pHSF-1P379 construct that contains the entire sequence between the translation start site of Bop1 and HSF-1 genes is decreased by 50% in NIH3T3 and by 25% in F9 cells. The mutant construct containing the 1-kb fragment of the HSF-1 promoter (pHSF-1P1kb) shows further reduction in basal transcription of the luciferase reporter gene, but the activity remains significantly higher (100-fold) than that of pGL2-Basic. The activities of the 2-, 3-, and 4-kb fragments (pHSF-1P2, -3, or -4 kb) fused to the luciferase reporter gene are also reduced (Fig. 3B). The same pattern of expression was observed for all constructs transfected into NIH3T3 or F9 cells (Fig. 3, B and C).

Because the Bop1 gene is in the reverse orientation of the HSF-1 gene, we performed experiments designed to investigate whether the 379-bp, 1-kb, or 2-kb fragments have transcriptional activity when fused to the luciferase gene in the reverse orientation (Fig. 4). The constructs are shown in Fig. 4A. NIH3T3 or F9 cells were transfected with constructs pBop1P379, pBop1P1kb, or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene. Furthermore, NIH3T3 or F9 cells transfected with constructs pBop1P1kb or pBop1P2kb luciferase, and luciferase activity was measured (Fig. 4, B and C). The results show that the luciferase activity is 3-fold higher when the 379-bp fragment is in the sense orientation with respect to the Bop1 gene than when it is in the sense orientation relative to the HSF-1 gene.
cleotides representing some of the potential transcription factor binding sites were tested in gel mobility shift assays. Two potential Sp1 binding sites (Sp1–1 and Sp1–2) and three potential TCF-1 binding sites (TCF-1a–1, -2, and -3) are present in the first 331 bp of the HSF-1 5′-untranslated region (Fig. 2).

**Fig. 3.** Determination of the minimal promoter required for basal transcription of the murine HSF-1 gene. A, map of promoter deletion mutants fused to luciferase reporter gene. The numbers indicate the nucleotide positions of the 5′-untranslated region with the first nucleotide of the codon for initiation of translation as +1. B, NIH3T3 cells were transfected with constructs shown in A, and luciferase activity was determined in cell extracts after 48 h. C, F9 cells were transfected with constructs shown in A, and luciferase activity was determined in cell extracts after 48 h. pGL2-Basic is the plasmid containing the luciferase gene without any promoter. Data is shown as percent of the luciferase activity observed with plasmid constructs containing the 379-bp fragment. Error bars show standard deviations calculated from the results of more than three experiments.

**Fig. 4.** Determination of the minimal promoter required for basal transcription of the murine Bop1 gene. A, map of promoter deletion mutants fused to luciferase reporter gene. The numbers indicate nucleotide positions of the 5′-untranslated region of HSF-1, with the first nucleotide of the codon for initiation of translation as +1. B, NIH3T3 cells were transfected with constructs shown in A, and luciferase activity was determined in cell extracts after 48 h. C, F9 cells were transfected with constructs shown in A, and luciferase activity was determined in cell extracts after 48 h. pGL2-Basic is the plasmid containing the luciferase gene without any promoter. Data is shown as percent of luciferase activity observed with plasmid construct containing the 379-bp fragment. Error bars show standard deviations calculated from the results of more than three experiments.

Fig. 5A shows a gel mobility shift assay for the Sp1 sites. The results indicate that Sp1–2 is an active binding site for Sp1 transcription factor, whereas no binding was observed in the case of Sp1–1 (lanes 4 and 7). The binding activity of consensus Sp1 is shown for comparison (lane 1). Furthermore, antibody to
Fig. 5. Identification of the nuclear factors that bind to the Sp1 and TCF-1 binding motifs in the promoter region of HSF-1. A, gel mobility shift assay using a synthetic double-stranded oligonucleotide from the consensus Sp1 (lanes 1, 2, and 3), Sp1–2 (lanes 3, 4, and 5), and Sp1–1 sites (lanes 5, 6, and 7) in the HSF-1 promoter. Lanes 1, 4, and 7 show DNA binding activity observed in 15 μg of NIH3T3 whole cell extracts. Lanes 2, 5, and 8 show reactions containing 200-fold excess unlabeled double-stranded oligonucleotides as competitors. Lanes 3, 6, and 9 show reactions that were incubated for 30 min at 25 °C with 0.3 μg of anti-Sp-1 antibody. B, gel mobility shift assay using 1 μl of monoS fractions containing purified TCF-1α (LEF-1) and a synthetic double-stranded oligonucleotide from the sequences of the consensus TCF-1α (lane 1) (32), TCF-1α-1 (lane 2), TCF-1α-2 (lane 3), and TCF-1α-3 (lane 4) in the 5′-untranslated region of HSF-1.

Sp1 is capable of interacting with this factor, causing a retardation in mobility of the complex (lanes 3 and 6). Fig. 5B shows that purified TCF-1α (LEF-1) can also interact with a control consensus TCF-1α site as well as the TCF-1α-1 site that is located in the first exon of HSF-1 downstream of the transcription start site. The DNA binding activity observed with TCF-1α-1, however, was 10 times lower than that observed for the consensus TCF-1α.

To investigate the significance of the Sp1–2 and TCF-1α-1 binding sites located in the 379-bp segment that were found to contain DNA binding activity as well as testing the authenticity of the CTF and AP2 binding sites (Fig. 2), these sites were mutated, and the fragments were fused to the luciferase gene in sense or antisense orientations relative to the HSF-1 gene (Fig. 6). For Sp1–2 and CTF, which could potentially be bidirectional, they were mutated in both sense and antisense orientations. The TCF-1α-1 site, which is located in the first exon of HSF-1 downstream of transcription start site and could only be utilized for the expression of the Bop1 gene, was mutated in the sense orientation to the Bop1 gene. The AP2 binding site that is located proximal to the Bop1 exon 1 was mutated in the sense orientation to the HSF-1 gene. These constructs were transiently transfected into NIH3T3 or T cells (in the case of the plasmid constructs containing the TCF-1α mutation), and luciferase activity was determined. The results show that for the promoter fragment oriented toward the HSF-1 gene, mutation of the AP2 transcription factor had no effect, and luciferase activity was the same as that obtained for the wild-type 379-bp segment (Fig. 6B). Mutations of the CTF and Sp1 sites resulted in a 60 and 75% reduction of luciferase activity, respectively, when compared with the wild-type 379-bp fragment. For the promoter constructs oriented toward the Bop1 gene (Figs. 6, C and D), the mutation of CTF or Sp1–2 resulted in a 70 and 75% reduction of luciferase activity, respectively, when compared with the wild-type 379-bp fragment. The plasmid construct with mutation of the TCF-1α-1 site was transiently transfected into 3DO-548 T lymphoma cells (Fig. 6D) or 5KC T cells (data not shown), and luciferase activity was determined 48 h after transfection with or without treatment of cells with PMA (50 ng/ml for 15 h) to stimulate growth. Results showed that T cells transfected with constructs containing the mutation of the TCF-1α-1 fragment showed a 50% reduction of luciferase activity compared with cells transfected with the wild-type 379-bp fragment. Moreover, the promoter activity was not affected when plasmid constructs containing the mutation in the TCF-1α-1 was transfected into NIH3T3 cells, in which TCF-1α is not expressed (data not shown). These results suggest that the TCF-1α-1 site can drive the expression of Bop1 gene in a tissue-specific manner. The data was the same whether cells were pretreated or untreated with PMA.

**DISCUSSION**

We have reported the cloning and sequencing of a large segment of the murine HSF-1 gene as well as its 5′-flanking region. The gene spans over 30 kb of DNA and contains 13 exons. Exon 1 and 2 are separated by an intron larger than 18 kb. The murine HSF-1 gene expresses two alternative splice variants (33, 34). Exon 11 is present in the α isoform but deleted in the β isoform. The significance of the α and β isoforms is not clear at the present time. However, in the case of HSF-2, it has been suggested that the shorter version, HSF-2β, is a negative regulator of HSF-2α during development (35). Thus, the differential expression of α and β isoforms could regulate the activity of HSF-2 by possibly competing for trimer formation. A similar function has not been proposed for murine HSF-1β. Although the cDNA sequence originally cloned for HSF-1 represented the shorter HSF-1β isoform, it appears to be transcriptionally active in the context of an HSF-1 chimeric protein in which the heterologous GAL-4 DNA binding domain was fused to HSF-1 (39).

An interesting finding is the observation that the murine HSF-1 promoter belongs to the family of TATA-less promoters. Other genes whose expression is regulated in a tissue-specific manner and often are housekeeping genes also lack TATA consensus sequences. These include synapsin I (40), brain specific aldolase C (41), nerve growth factor (42), and lymphocyte CD4 (43). There is also no CAAT box at the appropriate position, usually 25 bp upstream of the transcriptional start-site (44), although there is one located 95 bp upstream of the transcription start site, and it appears to be an active site (Fig. 6).

Another feature of the murine HSF-1 gene is the presence of the Bop1 gene within 331 bp of the HSF-1 translation initiation site. This region of the HSF-1 promoter appears to be bidirectional and drives the expression of both HSF-1 and Bop1 genes in a manner similar to the human monamine oxidase A or mouse thymidylate synthase genes (45, 46). However, the 331-bp fragment of the HSF-1 promoter yields higher levels of basal expression for Bop1 than for HSF-1 by as much as 3-fold. The activity of the larger promoter fragments, that is, the 379-bp or 1-kb fragments fused to the luciferase reporter gene in the direction of HSF-1 gene, were progressively reduced. The activity of the 2-kb promoter fragment was consistently higher than the 1-kb fragment. The activities of 3- and 4-kb fragments were again reduced from that observed for the 2-kb fragment fused to the luciferase gene.

Basal promoter function for HSF-1 was demonstrated by comparison of relative luciferase activities of specific constructs in NIH3T3 and F9 cells. Studies designed to show inducibility by serum following serum starvation or cotransfection with c-Ha-Ras expression plasmids failed to show significant response to these inducers in NIH3T3 cells (data not shown). As our results indicate, the 331-bp upstream of the HSF-1 translation start site drives the highest levels of basal expression. This segment contains two Sp1 sites, which were tested for DNA binding activity. Binding was observed for the Sp1–2 site.
but not the Sp1–1 site. This perhaps is not surprising because the Sp1–1 site is located downstream of the HSF-1 cDNA start site, although upstream of the initiator codon. Supershift experiments using anti-Sp1 antibody confirmed the identity of the factor binding at the Sp1–2 site. The Sp1 transcription factor has previously been shown to be bidirectional (47) and, according to the data shown in Fig. 6, the Sp1–2 site contributes to the expression of both the HSF-1 and Bop1 genes. Sp1 sites represent functional elements in different cell types and promoter contexts and contribute to basal transcription from minimal promoters by promoting interactions between Sp1 and general factors of the preinitiation complex (48). Furthermore, it is known that Sp1 elements can confer either negative or positive gene expression (49, 50).

There are several other potential binding sites within the 331-bp fragment of the HSF-1 5'-untranslated region that were also tested for their ability to drive basal expression of the HSF-1 and Bop1 genes. These included CTF, that is known to also drive basal expression and is found in promoters of heat shock genes (51). The mutation of this CTF site in sense or antisense orientations to the HSF-1 gene also reduced basal transcriptional activity, suggesting CTF is also contributing to the basal expression of both HSF-1 and Bop1 genes. The binding sites for transcription factors TCF-1α (T cell factor 1) and AP2, which may regulate tissue-specific expression of HSF-1, were also tested by gel mobility shift assay or mutational analysis. The TCF-1 binding activity using purified TCF-1α (LEF-1) protein also showed binding activity at the TCF-1α site. The TCF-1α binding site is located in the first exon of HSF-1 (Fig. 2) and therefore, most likely does not drive HSF-1 expression. However, it could drive the expression of the Bop1 gene. TCF-1 and the closely related LEF-1 are transcription factors expressed during murine T cell differentiation, which regulates the T cell receptor α enhancer (52, 53), although LEF-1 is 10 times more efficient in stimulating the T cell receptor α enhancer than TCF1. TCF-1α and LEF-1 have identical DNA binding properties, and binding sites have been identified in transcriptional control regions of several T lymphocyte-specific genes. Mutation of the TCF-1α site in the sense orientation to Bop1 gene followed by its transfection into T cells suggested that this site could stimulate the expression of the Bop1 gene. The transcription factor AP2 was originally purified from HeLa cells. There are three different isoforms, namely, α, β and γ coded by three different genes. All three genes are coexpressed strongly in early promigratory and migrating neural crest cells (54, 55). Mutation of the AP2 site, however, suggested it was not an active binding site for the AP2 transcription factor in NIH3T3 cells that have previously been shown to express this factor (36).

In terms of expression patterns of the HSF-1 and Bop1 genes, it appears that both genes are expressed constitutively via the activity of two transcription factors involved in basal expression, namely Sp1 and CTF. This is consistent with the previous reports that HSF-1 is present constitutively in all
adult tissues (5, 32), although less is known about its expression pattern during embryonic development. In the case of the Bop1 gene, there is no published report of its expression pattern or whether this gene is also a transcriptional regulatory factor. However, in the studies reported here, the promoter fragment of the HSF-1 gene can drive high levels of basal expression of a luciferase reporter gene by as much as 500- to 1000-fold when fused to the luciferase gene in sense or antisense orientations.

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