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An Additional Exon of Stress-Inducible Heat Shock Protein 70 Gene (HSP70-1)

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The HSP70-1 gene, reportedly a single exon, encodes a major stress-inducible 72-kDa heat shock protein (HSP70). We recently demonstrated that patients with major depression had a 162-base pair (bp)-deletion in the 5′-flanking sequence of HSP70-1 mRNA in their peripheral blood mononuclear cells. Present study has been conducted to clarify how this short mRNA is generated, and demonstrated that a novel 385 bp sequence is located 1.1 kb upstream from the HSP70-1 gene of normal subjects. Except for the 162 bp deletion, it is compatible with part of the 5′-flanking sequence of the HSP70-1 gene, and contains another exon of 358 bp (exon 1) that may be connected to the 3′-terminus (exon 3) of the conventional HSP70-1 gene. Alternative transcription of exons 1 and 3 may cause the short mRNA. It is concluded that HSP70-1 gene is constituted of three exons and may cause alternative splicing. © 1999 Academic Press

Key Words: **splicing; exon; intron; heat shock protein; HSP70; molecular cloning.**

Heat shock proteins (HSPs) play an important role as a molecular chaperone to ensure the correct folding, assembly, and transport of newly synthesized polypeptides, and also to restore and clean up damaged polypeptides (1,2). These functions enable cells to maintain homeostasis under normal growth conditions and to survive under stress. Because of their critical roles, their genes are highly conserved during evolution. Their mode of expression is constitutive, stress-inducible, or associated with cell cycle or development (3). A 72-kDa HSP (HSP70) is inducible by stress mediators such as ACTH (4) and catecholamine (5) or by inflammatory cytokines and mitogens (6) and is a consistent biomarker for stress reception (4). We found abnormal HSP70 mRNA expression in patients with major depression, considered a stress-related mood disorder (7,8). We recently found that the mRNA of HSP70-1, a major HSP70 gene, had in-frame deletion in its 5′-flanking sequence in peripheral bood mononuclear cells (PBMCs) in all 18 patients with major depression examined (9). The deleted part corresponded to 29 base pair (bp) in 5′ noncoding and subsequent 133 bp in the coding region of the conventional HSP70-1 gene (10). Because the 5′- and 3′-boundaries of the sequence corresponding to the deleted part are inconsistent with the typical splice consensus sequence that follows the GT-AG rule (11), this deletion may not be caused by alternative splicing within the 5′-flanking region of the conventional HSP70-1 gene. We clarified the gene corresponding to this short HSP70-1 mRNA in patients with stress-related disorders.

EXPERIMENTAL PROCEDURES

Specific primers for HSP70-1 and HSP70-2 genes. In analysis of the HSP70-1 5′-flanking sequence, the sense primer is 5′-TTTCGAGAGTGACTCCCGTT-3′ (primer 3 for HSP70-1 nucleotide 44-63) or 5′-TGCGACAGTCCACTACCTTT-3′ (primer 154 for nucleotide 25-44), and the antisense primer is 5′-AAAGGCCAGTGCTTCATGTC-3′ (primer 4 for nucleotide 473-492). PCR using these primers is conducted as described elsewhere (9).

Genomic DNA: Restriction enzyme digestion, size fractionation on agarose gel, and PCR analysis. Genomic DNA was isolated as described elsewhere (9). Genomic DNAs were digested with *Hind* III, *Bam* HI, and *Bgl* II, and developed in 1% agarose gel electrophoresis. The lane containing DNA was cut uniformly lengthwise at 5 mm intervals using a gelslicer. DNA fragments from each slice (fraction) were isolated using NaI and glass slurry, and suspended in TE, then amplified by PCR using primers 154/4.

Cloning and sequencing of genome between HSP70-1 and HSP70-Hom genes. *Eco* RI and *Hind* III-digested genomic DNAs were cloned using a Gigapack II gold packaging kit (Stratgene, La Jolla, CA) and pWEX cosmid vector. For sequencing, cloned genomic libraries were recloned in a pBS vector. The ligated plasmid DNA was transferred to DH5 α-competent cells, which were then subcloned and deleted using a kilodetection kit (Takara Biochemicals, Osaka, Japan). Sequencing was conducted using a 7-deaza-dGTP kit with T7 DNA polymerase (United States Biochemical, Cleaveland, OH) and [α-^{32}P]-dCTP.

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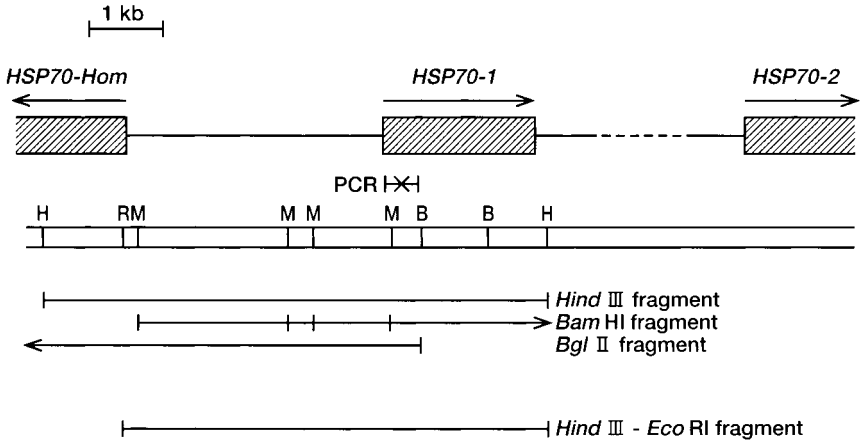

FIG. 1. Restriction map of HSP70 locus. Restriction sites for enzymes *Hind* III (H), *Bam* HI (M), *Bgl* II (B), and *Eco* RI (R). Sequences amplified by PCR primers are shown by PCR. HSP70 locus positions are shown by hatched boxes above in the figure and direction of gene transcription by arrows.

RESULTS AND DISCUSSION

Genomic DNA: Restriction Enzyme Digestion, Size Fractionation on Agarose Gel, and PCR Analysis

We looked at whether a novel HSP70 gene encoding the 5′-flanking sequence with 162-bp deletion existed in addition to the conventional HSP70-1 gene (Fig. 1). Genomic DNA was obtained from the PBMCs of normal subjects and digested with restriction enzymes, followed by size separation on agarose gel (Fig. 2). The DNA fraction in each gel slice was used as a template for PCR amplification of the 5′-flanking sequence of HSP70-1. PCR products were developed on agarose gel (Fig. 3). The *Hind* III fragment containing the whole HSP70-1 gene is reportedly 5.5 kb (10) (Fig. 1), and is presumed to be run on fraction 14 (Fig. 2). A normal-sized (466 bp) PCR product was, in fact, amplified in fraction 14; 162 bp deleted PCR products (304 bp) were also amplified in the same fraction (Fig. 3, above). Because *Bam* HI cleaves the sequence that primers should amplify (Fig. 1), 466 bp-PCR products were not amplified in *Bam* HI fragments, nor were 304 bp products (Fig. 3, center). This suggests that the DNA segment with the deleted sequence may be located at a site affected by *Bam* HI (Fig. 1). *Bgl* II fragments in fractions 12 and 13 were amplified to both 466 and 304 bp products (Fig. 3, below). Both fractions are estimated to contain about 7 kb DNA fragments, and should cover the sequence from HSP70-1 nucleotide 569 to a more telomeric site (Fig. 1). Taken together, the nobel DNA segment encoding the sequence with 162 bp deletion is suggested to be between the HSP70-1 and HSP70-Hom genes (Fig. 1).

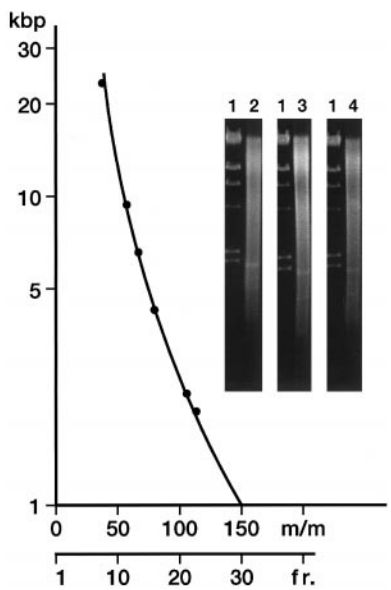

FIG. 2. Size fractionation of genomic DNA on agarose gel after digestion by restriction enzymes. Genomic DNAs were digested with *Hind* III (lane 2), *Bam* HI (lane 3), and *Bgl* II (lane 4), and developed by 1% agarose gel electrophoresis. Lambda DNA *Hind* III markers were eluted for comparison (lane 1). The lane containing DNA fragments was cut uniformly lengthwise at 5 mm intervals using a gelslicer.

Cloning and Sequencing of the 5′-Flanking Region of HSP70-1 and Its Upstream Region, and Its Significance

The *Hind* III fragment including the 5′-flanking region of HSP70-1 and its upstream region was cloned (pBH239), then a 5.8 kb *Hind* III-*Eco* RI fragment subcloned in pBSSV(+) (Fig. 1); 4360 bp were sequenced, with parts shown in Fig. 4. It contained 195 bp of 5′-terminal sequence of HSP70-Hom, identical to that reported by others (10) (data not shown). It also contained 893 bp of the 5′-terminal region of HSP70-1 (nucleotide −273 to 610) which was identical to that

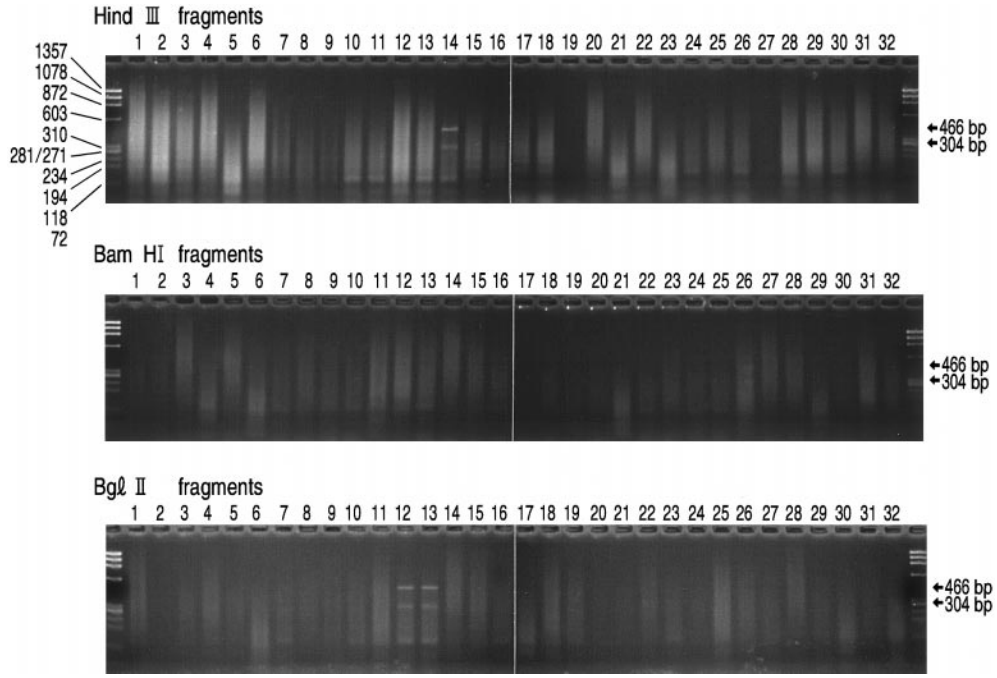

FIG. 3. Size-separated DNA fragments were recovered and amplified by PCR. The lane containing DNA fragments was cut uniformly lengthwise at 5 mm intervals using a gelslicer. DNA fragments from each slice (fraction) were isolated and recovered, then amplified by PCR using primers 154/4 specific to the 5′-region of HSP70-1 and developed on 3% garose gel. Fraction 14 of *Hind* III-digested DNA (B, above), and fractions 12 and 13 of *Bgl* II-digested DNA (B, below) contained PCR products of 466 and 304 bp together. No amplification occurred in *Bam* HI-digested DNA fragments (B, center).

reported elsewhere (10) (Fig. 4B). A novel 385 bp nucleotide sequence (nucleotides −1133 to −749) homologous to the 5′-flanking sequence of HSP70-1 was found to lie about 1.1 kb upstream from the HSP70-1 transcription start site (Fig. 5). A 206 bp sequence from nucleotide −1133 to nucleotide −928 is identical to nucleotide −21 to 186 of 5′-flanking region of HSP70-1 (part "a" in Fig. 5), which starts just after the TATA box. The 179 bp sequence from nucleotide −927 to nucleotide −749 is identical to nucleotide 349 to 527 of HSP70-1 (part "c" in Fig. 5). Thus, a 162 bp sequence corresponding to HSP70-1 nucleotide 187 to 348 is deleted in this sequence (part "b" in Fig. 5). Although we did not determine its transcriptional start site, it is likely to be at a position at nucleotide −1112 similar to that (ATAAC) of conventional HSP70-1 (12). The splice junction donor site (CAAG/GT) is at nucleotide −755. Its acceptor site (AG/G) is in HSP70-1 nucleotide 521. The 358 bp sequence between nucleotide −1112 and −755 is thus recognizable as a novel exon of the HSP70-1 gene, called exon 1. The conventional HSP70-1 gene is now separated into two exons by its splice junction site (nucleotide 521), i. e., exon 2 for the 5′ terminus and exon 3 for the 3′ terminus. When exon 1 is transcribed, the donor site of this exon finds an acceptor site on exon 3. The sequence between nucleotides −756 and 521 is a cryptic intron flanked by a typical splice consensus sequence that follows the GT-AG rule (11). Alternative splicing produces the short mRNA, transcribed from exons 1 and 3 (Fig. 5), compatible to that demonstrated in PBMCs of patients with major depression (9).

Translation start codon ATG is located at the 216 nucleotide in exon 2. Corresponding codons are, however, deleted in exon 1. If exon 1 were translated to the protein, its N-terminal amino acid would start at methionine on ATG at the −903 nucleotide, and a small protein with 60.7 kDa would be synthesized. PBMCs of patients with major depression, however, did not have such small HSP70, even though they had alternatively transcribed mRNA (9), suggesting that alternative mRNA failed to be translated. The 5′ noncoding region was reported to contain the internal ribosomal binding site required for posttranscription (13), suggesting that short HSP70-1 mRNA could not be translated to the protein. Even if it could, the transcription rate would be very weak because HSP70-1 exon 1 does not have a TATA box and because mutations in the TATA box reduce the basal expression of the conventional HSP70 gene (14). Thus, TATA box loss and/or possible loss of the internal ribosome binding site may explain why the 60.7 kDa HSP70 protein was not observed in PBMC lysates of depressive patients with short HSP70-1 mRNA.

Regarding promotor elements, exons 1 and 2 have been found to have distinct elements (Fig. 4). The

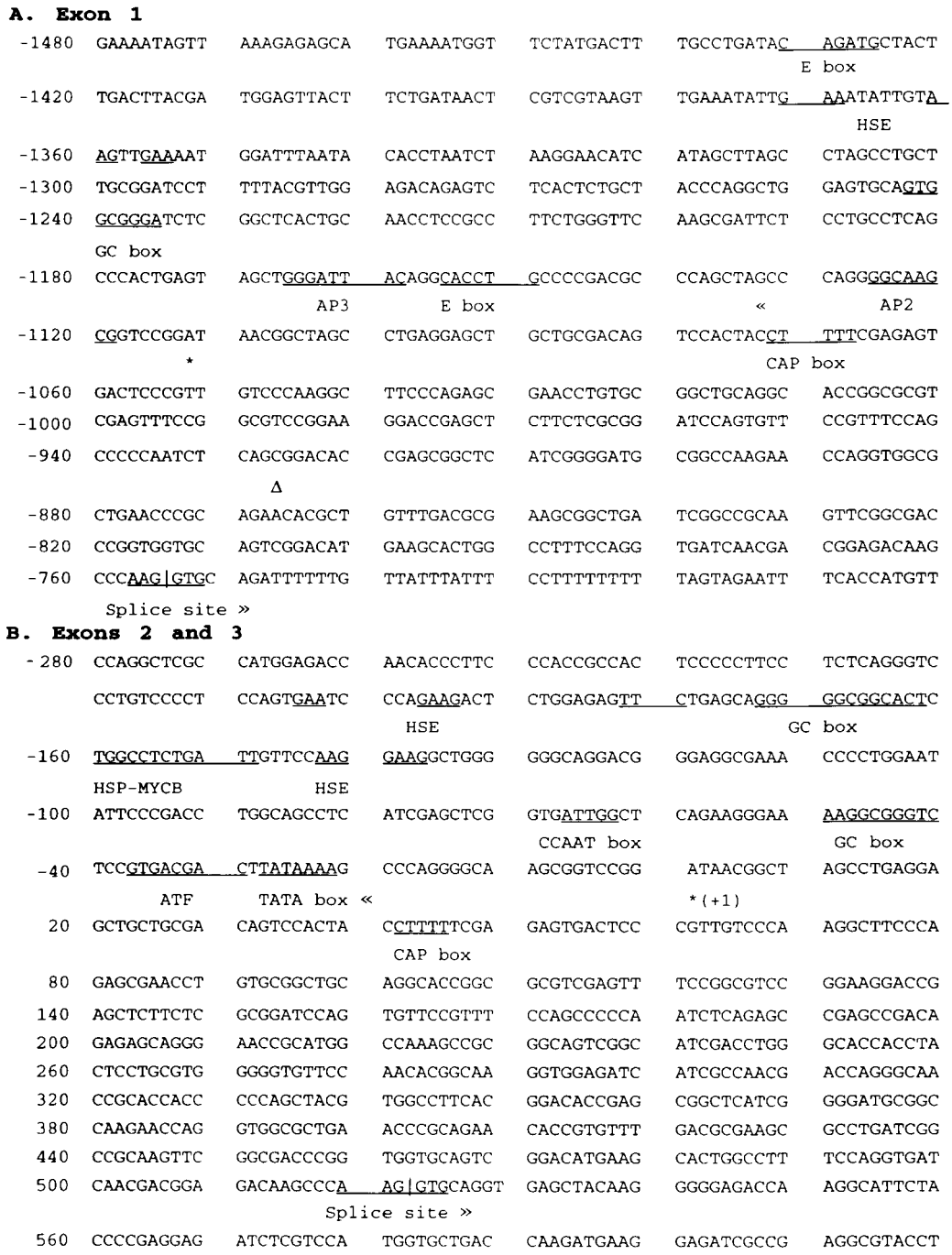

FIG. 4. HSP70-1 gene sequence. A 5.8 kb *Hind* III-*EcoR* I fragment was cloned. Regulatory and coding sequences of exon 1 (A) and of exons 2 and 3 (B) are shown. The nucleotide number starts at the transcription start site located in exon 2 (+1). Transcription start sites in both exons 1 and 2 are shown by asterisks (*). Splice junction sites are shown by the septum at nucleotide −755 and 521. Sequences between « and » in Fig. 3A are identical to that in Fig. 3B except for the 162 bp deletion at the site shown by △. Sequences between * and splice junction site represent exon 1(A) or exon 2 (B). Elements are shown by underlining and abbreviations.

TATA box, ATF binding site, CCAAT box, and HSP-MYCB (15) are present only for exon 2, while the E box and AP3 binding site are present only for exon 1. Exon 1 characteristically has two E box elements (a CANNTG DNA motif) that are the binding site for the family of basic helix-loop-helix (bHLH) transcriptional factors. The E box is present in the regulatory regions of a variety of genes and is critical to tissue-specific expression of these genes. MyoD and myogenin are bHLH transcription factors, and bind to the αB-

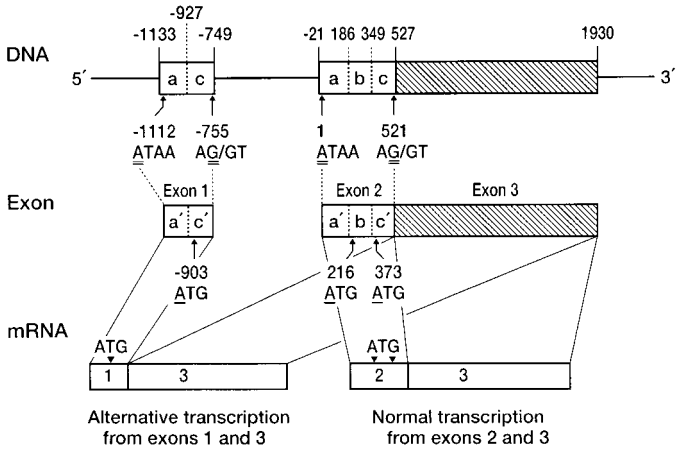

FIG. 5. Normal and alternative transcriptions of HSP70-1 gene. Dot-shaded boxes in DNA show identical sequences between nucleotides −1133 to −749 and nucleotides −21 to 186 plus 349 to 527. Nucleotide 1, ATAA, is reportedly a transcription start site. The identical sequence is found at nucleotide −1112 in exon 1. Splice junction sites are at nucleotides −755 and 521. Note the translation starting coden (ATG). Normal mRNA is transcripted from exons 2 (521 bp) and 3 (1409 bp) and alternative transcription from exons 1 (358 bp) and 3.

crystallin promoter E box and fully transactivate αB-crystallin expression muscle-specifically (16). αB-crystallin is the only HSP known to have an E box in its regulatory region. The E box motif is also involved in B-lymphocytes development (17) and immunoglobulin expression (18). AP3 is the binding site for many transcriptional factors, including NFκB, EBF1, and TEFI. Exon 1 thus has its own regulatory machinery, independent of that for exon 2. When HSP70-1 exon 1 is transactivated by its upstream promotors, the sequence between nucleotides −756 and 421 becomes an intron. This intron contains HSE and other promotors. Does this intron have significant implications? The first intron in HSP86 was reported to make the gene expression constitutive, and its deletion caused a heat-inducible mode of expression (19). This first intron contains HSE the same as the intron in HSP70-1. When this first intron is connected to the HSP70 5′ flanking sequence, a constitutive expression of transcriptional activity is observed (19). These findings indicate that HSP70-1 nucleotide sequence between −756 and 521 may enable gene expression to be constitutive when the sequence becomes an intron. Taken together, we propose the following hypothesis: Sustained psychological stress induces alternative transcription of the HSP70-1 gene and switches the expression mode from inducible to constitutive. Transcription and translation of this short and alternative HSP70-1 gene would be weak, if any, and prevent excessive and sustained HSP70 protein expression. A sustained increase in HSP70 gene expression reportedly caused lymphoma in transgenic mice expressing the human HSP70 gene (20), and confered tumorigenecity to mouse fibrosarcoma cells (21). Epstein-Barr virus (EBV) binds to CD21 virus receptors on human B lymphocytes, and the virus infection triggers a cascade of transformation-requisite cellular events and induces HSP70 expression. HSP70 expression blockage prevents transformation to lymphoma (22), so the HSP70 protein expression escape phenomenon during sustained stress helps prevent malignant lymphocyte transformation. Even if HSP70-1-derived protein were not produced, it can be substituted by constitutively expressed HSC70 or HSP70-2 or -Hom-derived proteins. It remains unclear, however, whether this alternative HSP70 transcription is primarily linked to the pathophysiology of major depression or is secondary to specific pleiotropic effects.

The present study has demonstrated that the human HSP70-1 gene is composed of two distinct 5′-terminal sequences (exons 1 and 2) and a common 3′-terminal sequence (exon 3). Alternative transcription may be induced from exons 1 and 3, and is associated with short mRNA. Studying this alternative HSP70 gene transcription may open up new approaches to clarifying the stress response and stress-related disease.

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