The Kruppel-like Factor Zf9 and Proteins in the Sp1 Family Regulate the Expression of HSP47, a Collagen-specific Molecular Chaperone*

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In several cells and tissues the synthesis of HSP47, a collagen-specific molecular chaperone in the endoplasmic reticulum, is closely correlated with the synthesis of collagen. We previously reported that the Sp1 binding site at −210 bp in the promoter region and the first and second introns are required for the tissue-specific expression of HSP47 in transgenic mice (Hirayoshi, H., Yamamura, I., Yasuda, K., Kobayashi, A., Tada, N., Suzuki, M., Hirayoshi, K., Hosokawa, N., and Nagata, K. (1999) J. Biol. Chem. 274, 35706–35710). Here, we analyze how these introns influence the transcriptional regulation of the hsp47 gene in BALB/c 3T3 cells, which produce high levels of HSP47. In vitro promoter analysis using a luciferase reporter and gel mobility shift analysis revealed that two cis-acting elements in the first and second introns, BS5-B and EP7-D, respectively, are required for the activation of hsp47 in BALB/c 3T3 cells. Several members of the Kruppel-like factor (KLF) family of proteins were identified as BS5-B-binding proteins by yeast one-hybrid analysis using these elements as baits. One of these proteins, KLF-6/Zf9, binds to the BS5-B element and activates expression of the reporter construct when transfected into cells. Chromatin immunoprecipitation assay analysis revealed that the endogenous KLF-6/Zf9 binds the BS5-B elements that contain the CACCC motif, which is a consensus recognition sequence for other proteins in the KLF family. We also showed that BS5-B and EP7-D are bound by two members of the Sp1 family, Sp2 and Sp3. These results suggest that at least three sequences are required for the constitutive expression of hsp47 in BALB/c 3T3 cells: the −210 bp Sp1 binding site, the BS5-B element in the first intron, and the EP7-D element in the second intron. We suggest that KLF proteins regulate the transcription of hsp47 by binding the BS5-B element in cooperation with Sp2 and/or Sp3.

HSP47 is an endoplasmic reticulum (ER) resident stress-protein that transiently binds to newly synthesized procollagen. It belongs to the serpin (serine protease inhibitor) superfamily but is not secreted because it contains the ER retention signal (RDEL) at the C terminus (1). HSP47 associates with procollagen during its assembly, folding, and/or post-translational modification in the ER and dissociates from it in the ERGIC (ER-Golgi intermediate compartment) or in the cis-Golgi (2). Biochemical studies using synthetic model peptides of collagen have revealed that HSP47 preferentially binds to Gly-Xaa-Arg triplets in the procollagen triple helical region (3). Recent studies have shown that HSP47 plays a crucial role in collagen biosynthesis as a collagen-specific molecular chaperone; hsp47 null mice cannot synthesize collagen normally and cannot survive beyond the E11.5 stage of embryogenesis (4). These mice exhibit severe impairments in the processing of the collagen N- and C-propeptides and in type I procollagen triple helix formation, resulting in the absence of collagen fibrils in mesenchymal tissues and basement membranes that lie between the epithelial cell layer and the mesenchyme.

All known ER-localized stress proteins in mammalian cells are induced by various ER stresses through the unfolded protein response pathway (5). HSP47 is the only heat shock protein that resides in the ER of mammalian cells that is induced by cytosolic stresses, including heat shock, but it is not induced by ER stress (6). This induction depends on the presence of the heat shock element in the hsp47 promoter (7). On the other hand, the constitutive expression of hsp47 appears to be coordinated with that of several types of collagens. For example, during the differentiation of mouse F9 teratocarcinoma cells the rates of synthesis of HSP47 and type IV collagen coordinately increase (8), and following the malignant transformation of fibroblasts the rates of synthesis of the two proteins, HSP47 and type I collagen, decrease (9). Collagen nonproducing cells such as mouse myeloid leukemia M1 cells and rat pheochromocytoma PC12 cells do not synthesize HSP47. The spatiotemporallyconcerted expression of HSP47 with collagens is also observed during mouse and chick embryonic development (10). In addition, a marked induction of HSP47 has been reported in the progression of fibrosis in various experimental fibrosis models, including liver cirrhosis (11), kidney fibrosis (12), and atherosclerosis (13). The down-regulation of hsp47 caused by introducing hsp47 antisense oligoribonucleotides into rat renal cells after the initiation of fibrosis markedly reduces the rate of the progression of fibrosis and reduces the levels of types I and III collagens in the kidney (14).

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1 The abbreviations used are: ER, endoplasmic reticulum; KLF, Kruppel-like factor; GST, glutathione S-transferase; CHIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; TGF, transforming growth factor.
In a previous paper (15), we demonstrated that both the 280-bp promoter region and the first and second introns are required for the tissue-specific expression of HSP47 in transgenic mice harboring a β-galactosidase reporter gene under the control of these elements. In vitro promoter analysis using a luciferase reporter gene revealed that the Sp1 binding site at −210 bp is necessary for the basal expression of hsp47, and the addition of a downstream intron region caused a marked up-regulation of reporter activity in HSP47- and collagen-producing cells but not in nonproducing cells. In this paper, we have further analyzed the cis-acting elements in the intron regions that are responsible for the activation of hsp47 expression, and we identify two such elements in the first and second introns. We also used a yeast one-hybrid assay, gel mobility shift analysis, and CHIP analysis to identify the transcription factors that bind these elements. In addition to the Kruppel-like factor (KLF) proteins, which were identified in the yeast one-hybrid screen, we have now used the DNA affinity precipitation (ChIP) assay to identify the regulation of hsp47 expression. Among the KLF proteins, we further focused on the KLF-6/Zf9 protein in the activation of hsp47, as shown by gel mobility shift analysis and reporter analysis of cells transfected with Zf9 cDNA.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection Assays—Murine BAL/c 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (with low glucose) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO2 atmosphere.

DNA was transfected into BAL/c 3T3 cells using FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. BAL/c 3T3 cells were plated at a density of 5 × 104 cells in 35-mm culture dishes 16 h before transfection. Cells were harvested 48 h after transfection, and luciferase activities were measured by using a luciferase reporter assay system (Promega, Madison, WI). The plasmid pact-Zf9, which has deletions within the first intron of the hsp47 promoter upstream of lacZ, was used as an internal control to normalize control of these elements.

To make the BS-5 and BS5-B mutant constructs, which replaced a downstream intron region caused a marked up-regulation of reporter activity in HSP47- and collagen-producing cells but not in nonproducing cells. To construct the BST-5/Zf9 expression plasmid pBST/Zf9, the XhoI and BamHI site was added with mixing by rotation for 30 min at 4 °C. The BST-5/Zf9 plasmids were then transfected into BAL/c 3T3 cells to confirm the expression levels of Zf9 in mammalian cells, and additional plasmids were subcloned into the SphI/EcoRI fragment of pT7/Zf9 into the expression vector pCAGGS. pCAGGS was used to transfect the plasmid into a human fibroblastic cell line, was constructed by subcloning the SphI/EcoRI fragment of pT7/Zf9 into the expression vector pCAGGS. pCAGGS was constructed by the insertion of a multicloning site containing SnaBI, KpnI, SmalI, BclI, EcoRV, NotI, BstBI, NsiI, and SmalI into pCAGGS at the EcoRI site.

Electrophoresic Mobility Shift Assay (EMSA)—Nuclear extracts from BALB/c 3T3 cells were prepared by the method as described in Ref. 16. Briefly, BALB/c 3T3 cells were washed twice with TBS and then harvested in TBS with a cell scraper. The cell pellets were resuspended in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 min. After centrifugation, the pellets were resuspended in buffer C (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and rocked on ice for 15 min. The nuclear extracts were recovered by centrifugation.

GST-tagged Zf9 was expressed in Escherichia coli BL21 and purified with glutathione-sepharose beads as described previously (17). The concentration of the GST fusion proteins eluted from the glutathione beads was determined by SDS-PAGE followed by Coomassie Blue staining with known amounts of bovine serum albumin as a standard.

The DNA probes BS-1 to 9 and EP-1 to 7 were prepared by PCR with specific primers (Table II). The sequences of the oligonucleotide probes BS5-A to E and EP7-A to G are indicated in Table III. All probes were labeled at the 5′ end with 32P-ATP by T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA).

EMSAs were performed in a 10-μl final volume containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 0.5 mM dithiothreitol, 50 mM NaCl, 12% glycerol, and 1 μg of poly(dI-dC). The binding reaction was carried out with 10 μg of nuclear extract and ~1 pmol of 3P-labeled probe at room temperature for 30 min. Unlabeled probes or antibodies were added to the reaction mixture 30 min before the addition of 3P-labeled probes. Probes bound to proteins were separated on 4% nondenaturing polyacrylamide gels in 0.25× TBE at 250 V for 2 h.

One-hybrid Screening—One-hybrid screening was carried out essentially as described (18). Four repeats of the BS5-B sequence from the hsp47 first intron or the EP7-D sequence from the second intron were cloned into the BglII site of pHEHEL2 (19); these plasmids were designated as pHEHEL/BS5-B and pHEHEL/EP7-D, respectively. Plasmids were linearized by digestion with NotI and integrated into the his3 yeast strain KMY1015 (18). The resulting yeast strains were transformed with a multicopy plasmid DNA library containing mouse 11-day-old embryo cDNAs fused to the transcription activation domain of Gal4 (Clontech, Palo Alto, CA). Transformants were plated on medium lacking histidine and containing 1 mM 3-amino-triazole. After 4 days culture at 30 °C, His+ colonies were inoculated into liquid medium, and plasmid DNA was extracted. Following re-amplification in E. coli, the nucleotide sequence of each clone was determined.

DNA Affinity Precipitation Assay—Nuclear extracts from BALB/c 3T3 cells were prepared by the same procedure as for EMSA. Exact bound DNA probes BS5-B and EP7-D were made by PCR with a 5′-biotinylated primer. Probe (1 μg) was added to nuclear extracts (100 μg) containing poly(dI-dC) (15 μg) and the mixture was incubated on ice for 30 min. Streptavidin-Dynabeads (Dynal, Great Neck, NY) were added with mixing by rotation for 30 min at 4 °C. The Dynabeads were washed twice with 3T3 cell nuclei lysis buffer and resuspended in 5 μl of washing buffer, and added to the electrophoresis gel.
collected with a magnet and washed twice with buffer. The trapped pellets were resuspended in 50 μl of sample DNA. PCR products were separated on 10% nondenaturing polyacrylamide gels in 1X TBE at 4°C for 2 h. The beads were heated at 65°C for 5 min each with 1 ml of low and high salt buffer, then with 1 ml of SDS lysis buffer containing protease inhibitors and sonicated for 10 s. Cell lysates were collected by centrifugation. An aliquot of the lysate (10 μl) was reserved as a control for amplification by PCR. The remainder was incubated overnight at 4°C in CHIP dilution buffer containing protease inhibitors with 5 μl of anti-Sp2, -Sp3, and -Zf9 antibodies (Santa Cruz Biotechnology). Immune complexes were recovered by the addition of 60 μl of salmon sperm DNA and a protein A-agarose bead suspension, followed by incubation at 4°C for 2 h. The beads were washed for 5 min each with 1 ml of low and high salt buffer, then with 1 ml EDTA, Tris-HCl (pH 8.1) containing LiCl and finally with TE. Quantitative PCR was carried out for 1.0 h.

RESULTS

Identification of cis-Acting Elements in HSP47 Introns That Activate Expression—In a previous study, Hirata et al. (15) reported that a 280-bp region of the promoter upstream of the transcriptional start site is necessary for basal level expression of hsp47 in murine cells. However, the cell type-specific expression of hsp47 could not be attributed to this promoter region alone. Hirata et al. (15) also showed that the first two introns, located upstream of the translation initiation site, are necessary in addition to the 280-bp promoter region for the tissue-specific expression of hsp47. Considering these previous results, we made systematic deletion constructs fused to the luciferase gene to identify the cis-acting element(s) in the first and the second introns that are responsible for the constitutive expression of hsp47. Luciferase reporter analysis was performed after transfecting these deletion constructs into BALB/c 3T3 cells; the results of these experiments are shown in Fig. 1.

As reported previously, full activity was observed for the pLuc280(III) construct, which contains the first and second introns in addition to the 280-bp promoter region, upon transfection into BALB/c 3T3 cells, whereas modest luciferase activity was observed for a pLuc280 construct without introns (Fig. 1). pLuc280(III) derivatives with 500- and 480-bp deletions of the first and second introns (pLucABS and pLucEPm, respectively) had levels of luciferase activity that were half that of pLuc280(III), if the activity of pLuc280 is considered as the basal level of activity. When both regions were simultaneously deleted, the activity of the resulting construct (pLucEPm) was the same as that of pLuc280(III). Error bars represent the mean ± S.D. of at least three experiments in duplicate.

**FIG. 1. The effect of deletions in the intron region of the hsp47 gene on the expression of luciferase activity in BALB/c 3T3 cells.** Constructs with deletions of the intron region are shown at the left. These constructs were transfected into BALB/c 3T3 cells with β-actin-galactosidase as an internal control for transfection efficiency, and luciferase activity was normalized to β-galactosidase activity. The relative luciferase activity was determined by comparing the activity of each construct with that of pLuc280(III). Error bars represent the mean ± S.D. of at least three experiments in duplicate.
lapping PCR-amplified fragments that collectively cover the two introns (Fig. 2A). Incubation of [32P]phosphate-labeled probes with BALB/c 3T3 nuclear extracts produced bands of reduced mobility as seen by EMSA (Fig. 2, B and C). The specificity of the alterations in band mobility was tested by the addition of an excess of unlabeled probe. Arrows indicate two bands commonly detected for extracts containing the BS-5 or EP-7 probe. D, nuclear extracts were incubated with [32P]labeled BS-5 (lanes 1–3) or EP-7 (lanes 4–6) in the presence of an excess of unlabeled BS-5 (lanes 2 and 5) and EP-7 (lanes 3 and 6). The two arrows indicate protein-DNA complexes commonly observed for extracts incubated with the BS-5 or EP-7 probe.

To further define the cis-acting elements in the region covered by the BS-5 (100 bp) and EP-7 (120 bp) probes that are responsible for hsp47 expression in BALB/c 3T3 cells, a series of 30-bp probes that collectively covers the BS-5 and EP-7 regions was synthesized as shown in Fig. 3A for use in EMSA. Fig. 3B clearly shows that incubation of nuclear extracts with both the BS5-B and EP7-D probes results in the formation of similar DNA-protein complexes (lanes 2 and 9, arrows). Interestingly, these bands could be eliminated by the presence of either of the unlabeled oligonucleotides; the band shift observed for the [32P]labeled BS5-B probe could be suppressed by the presence of an excess of the unlabeled BS5-B as well as of the EP7-D probes (Fig. 3C, lanes 2 and 4). The bands that were produced by incubation of extracts with EP7-D also did not appear in the presence of unlabeled BS5-B (lane 7). Other competitors, including BS5-C and EP7-E, did not interfere with band mobility (Fig. 3C, lanes 3, 5, 8, and 10). These results
indicate that BS5-B and EP7-D share an element(s) that is recognized by the same transcription factor(s).

To identify the core cis-acting elements that activate hsp47 expression in BALB/c 3T3 cells, we systematically introduced 4-bp mutations into the BS5-B and EP7-D DNA fragments as shown in Fig. 4A and performed EMSA using $^{32}$P-labeled BS5-B and EP7-D probes in the presence of an excess of each mutated construct as a competitor (Fig. 4B). For the $^{32}$P-labeled BS5-B probe, the two mobility shifted bands depicted by arrows disappeared when the unlabeled competitors BS5-B/mt-4, mt-5, and mt-6 were present, indicating that the mutated sites are not necessary for transcription factor binding. In contrast, these shifts in mobility were not affected by the presence of the competitors BS5-B/mt-1, mt-2, and mt-3. These results indicate that a 12-bp sequence (GAGGCCACACCC, Fig. 4A) in the BS5-B element is necessary for transcription factor binding. Similar experiments were performed with mutated EP7-D constructs (Fig. 4B). For EP7-D, the presence of the competitors EP7-D/mt-2 and mt-6 caused the mobility shifted bands indicated by arrows to disappear (Fig. 4B, lanes 10 and 14). However, the mutant probes EP7-D/mt-1, mt-3, mt-4, and mt-5 had no effect on the mobility of the shifted bands. Thus, a 10-bp sequence (GCCCTCCCA) in the EP7-D element was identified as necessary for transcription factor binding. Interestingly, the 12-bp sequence in the BS5-B element and the 10-bp sequence in the EP7-D element are similar and can be characterized as GC-rich, as shown in Fig. 4C. It is noteworthy that the band indicated by an asterisk in Fig. 4B disappeared when the competitor BS5-B/mt-1 was present (lane 2), suggesting that this DNA-protein complex contains a protein different from that in the complexes indicated by the arrows and that this protein binds to the CCACACCC motif of the 12-bp sequence in the BS5-B element (see below).

To verify that these two elements, which we assume are core sequences for transcription factor(s) binding, are also necessary for the transcriptional activation of hsp47 in BALB/c 3T3 cells, we performed luciferase reporter assays by transfecting a series of reporter gene constructs with 2-bp mutations in the BS5-B element (Fig. 5). The pLuc$^{\Delta}$BS, pLuc$^{\Delta}$BS-5, and pLuc$^{\Delta}$BS5-B deletion constructs showed reduced luciferase activities as compared with pLuc280(III). This result suggested that the BS5-B 30-bp region is necessary for the full activation of the reporter gene. The mutant constructs pLucBS5-B/mt-3 to mt-8 exhibited reductions in luciferase activities similar to those of pLuc$^{\Delta}$BS, pLuc$^{\Delta}$BS-5, and pLuc$^{\Delta}$BS5-B. Other reporter constructs, including pLucBS5-B/mt-1, mt-2, mt-9, and mt-10 showed luciferase activities comparable with that of pLuc280(III). Thus, the 12-bp sequence (GAGGCCACACCC) is a core element necessary for the activation of the reporter gene in BALB/c 3T3 cells in combination with the 280-bp promoter region. This core sequence is the same as that identified by EMSA (Fig. 4). By a similar analysis of luciferase reporter constructs with systematic mutations in the EP7-D region, we also confirmed that the 10-bp sequence identified by EMSA is necessary for transcriptional activation of the reporter gene in BALB/c 3T3 cells (data not shown).

**Identification of Transcription Factors in a Yeast One-hybrid Screen**—To identify the transcription factors that bind to the core sequences (the 12-bp sequence in the BS5-B fragment and

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**Fig. 3.** EMSA using various constructs covering BS-5 and EP-7 regions as probes. A, DNA probes used for EMSA. The sequences of all probes, the lengths of which are between 30 and 35 bp, are indicated in Table III. B, nuclear extracts from BALB/c 3T3 cells (10 µg) were incubated with $^{32}$P-labeled DNA probes as shown in A. Arrows indicate the two major bands detected for incubation of extracts with the BS5-B or EP7-D probe. C, a competition experiment in which nuclear extracts were incubated with $^{32}$P-labeled BS-5 (lanes 1–5) or EP-7 (lanes 6–10) in the presence of unlabeled BS5-B (lanes 2 and 7), BS5-C (lanes 3 and 8), EP7-D (lanes 4 and 9), or EP7-E (lanes 5 and 10).
the 10-bp sequence in the EP7-D fragment) and activate hsp47 expression, we performed a yeast one-hybrid screen using constructs containing a quadruplication of the BS5-B or EP7-D core sequences as baits. We screened a total of 1.2\times10^7 independent clones from an 11-day embryonic mouse cDNA library and obtained 23 positive clones when the BS5-B repeat was used as the bait (Table IV). No positive clones were obtained from 1.0\times10^7 independent clones screened from the same cDNA library when the EP7-D repeat was used as the bait. Sequencing of all the positive clones identified as interacting with the BS5-B repeat revealed that they encoded several members of the KLF family, KLF-6 (Zf9/CPBP), KLF-1 (EKLF), KLF-3 (BKLF), and KLF-4 (GKLF) in the order of the numbers of positive clones obtained (Table IV).

KLFs are DNA-binding transcriptional regulators that play diverse roles during differentiation and development (20, 21). They form a subset of the broad class of Cys2/His2 zinc finger-containing proteins that bind to DNA via their C-terminal ends. KLFs reportedly bind to the consensus sequence CACCC, which is found in the 12-bp core sequence of the BS5-B element (Table IV). The EP7-D element also contains similar sequences (GCCCC and CTCCC) within the 10-bp core sequence (Fig. 4C).

However, EP7-D does not contain the KLF consensus binding sequence, which may explain why no KLF family genes were identified in the yeast one-hybrid screen when the EP7-D repeat was used as the bait.

KLF-1 has been reported to regulate globin and other erythroid cell-specific gene expression (22), and KLF-4 is specifically expressed in lung, testis, skin, and thymus (23). KLF-3 is reported to be a negative regulator (24). Interestingly, KLF-6, also called Zf9/CPBP, is reported to positively regulate the expression of collagen α1(I) (25) and the TGF-β and TGF-β receptors in the fibrosis (26), and it appears to be up-regulated.

**TABLE IV**

| cis-Element | KLF proteins obtained by one-hybrid screening |
|-------------|---------------------------------------------|
| BS5-B × 4 repeats | KLF-6 (CPBP/zf-9) 8 |
| BS5-B × 4 repeats | KLF-1 (EKLF) 6 |
| BS5-B × 4 repeats | KLF-3 (BKLF) 6 |
| BS5-B × 4 repeats | KLF-4 (GKLF) 3 |
| EP7-D × 4 repeats | None None |

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**TABLE IV**

| KLF family/consensus sequence : CACCC |
| BS5-B : TAAAGCCCTCCCCCTCTCTCGAT |

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Experimental Procedures.

Recombinant proteins were prepared as described previously (1, 14, 15). Because Zf9 was cloned in a yeast one-hybrid screen using the BS5-B repeat as a bait, the direct binding of Zf9 to the BS5-B element was examined in vitro by EMSA. Although supershift analysis using an anti-Zf9 antibody followed by EMSA would be the most efficient way to examine the binding of endogenous Zf9 to this sequence, the Zf9 antibody currently available was ineffective for this purpose. To overcome this problem, we performed a DNA affinity precipitation assay. In this method, a protein-DNA complex is isolated using a specific biotinylated oligonucleotide probe, and bound proteins are detected by immunoblot analysis with a specific antibody. The biotinylated BS5-B element was incubated with BALB/c 3T3 nuclear extracts, and DNA-protein complexes were isolated with streptavidin-conjugated magnet beads. These complexes were then examined by immunoblotting using an anti-Zf9 antibody. Zf9 in BALB/c 3T3 nuclear extracts (Fig. 6A, lane 2) was recovered in the precipitate fraction with biotinylated BS5-B (Fig. 6A, lane 4). This result suggests that endogenous Zf9 bind to the BS5-B element.

GAGGCCACACC was identified as a core sequence by mutational analysis combined with EMSA (Fig. 4B) and by luciferase reporter analysis (Fig. 5). EMSA was performed using the BS5-B probe in the presence of varying amounts of GST-tagged recombinant Zf9 to confirm the direct binding of Zf9 to the BS5-B core sequence. As shown in Fig. 6A, the incubation of GST-tagged Zf9 with the 32P-labeled BS5-B probe resulted in a dose-dependent manner the appearance of two bands of reduced mobility. Both of these bands were supershifted by the addition of anti-GST antibody to the incubation mixture (Fig. 6B, lane 8). GST alone did not bind to the BS5-B probe. The more slowly migrating of the two shifted bands was likely produced by the dimerization of GST. To determine the binding sequence of Zf9 within the BS5-B element, we performed EMSA in the presence of mutant versions of BS5-B as competitors. The binding of recombinant GST-Zf9 to the BS5-B probe was not inhibited by the presence of the BS5-B/mt-2 (GAG-GAACAACCC) (Fig. 6C, lane 4) and mt-3 (GAGGCCACAAAA) mutant probes (Fig. 6C, lane 5), whereas the mutants BS5-B/mt-1 (TCCTTCCACCCC) did interfere with binding (Fig. 6C, lane 3). This suggests that Zf9 does not bind to the 5' upper portion of this core sequence. In addition, the lower band indicated by the asterisk in Fig. 4B disappeared when BALB/c 3T3 nuclear extracts were incubated with 32P-labeled BS5-B in the presence of BS5-B/mt-1 (Fig. 4, lane 2), suggesting that this band might represent Zf9 bound to the core sequence of the BS5-B element. In considering these results, we speculated that factors other than Zf9 might bind to this 5'-upper portion in the 12-bp sequence (GAGGCCACACC) of the BS5-B element. This upper portion contains a GC-rich sequence, which is known to be a possible binding site for proteins in the Sp1 family (27). Furthermore, these proteins are reported to cooperate with members of the KLF family in activating the transcription of target genes (see “Discussion”).

To address whether an Sp1 protein binds to the upper sequence of this core sequence, we next performed EMSA using 32P-labeled BS5-B as the probe and antibodies against specific Sp1 proteins. Each of the two major bands depicted by arrows was supershifted in the presence of the anti-Sp2 antibody (Fig. 7, lane 3), and the mobility of the lower band was also affected by the presence of anti-Sp3 antibody (lane 4). Antibodies against Sp1 and Sp4 had no effect on probe mobility (lanes 2 and 5). These results, combined with those shown in Fig. 4B, suggest that Sp2 and/or Sp3 binds to the 5'-upper portion (GAGGCCA) of the 12-bp core sequence in the BS5-B element. Supershift analysis with the EP7-D probe revealed that Sp2 and Sp3, but not Sp1 and Sp4, also bind to the EP7-D element, producing the same shifted band as seen by EMSA (Fig. 7). The band depicted by an asterisk was not supershifted by the presence of antibodies to Sp1-Sp4 (Fig. 7). This result is consistent with the proposal that this band represents a complex of Zf9 protein.
and the BS5-B core sequence. Thus, we suggest that KLF-6/Zf9 binds to sites distinct from those bound by Sp2 and/or Sp3 within the core sequence of the BS5-B element.

To examine whether these transcription factors bind to the BS5-B core sequence in vivo, we next performed a CHIP assay using anti-Sp2, anti-Sp3, and anti-Zf9 antibodies. BALB/c 3T3 cells were fixed with formaldehyde and sonicated in SDS lysis buffer to fragment the DNA to an average size of 200 bp. Protein-DNA fragments were precipitated with control IgG and amplified using the BS5-B and EP7-D primer sets. Fig. 8 shows that DNA fragments precipitated with the anti-Sp2 and anti-Sp3 antibodies could be amplified with both the BS5-B and EP7-D primer sets, whereas those precipitated with control IgG could not be amplified with either set. DNA fragments precipitated with the anti-Zf9 antibody also could be amplified with the BS5-B primer set, but not with the EP7-D primer set (Fig. 8). This result clearly indicates that in vivo, Sp2 and Sp3 bind to both BS5-B and EP7-D but Zf9 binds only to BS5-B.

Finally, we examined with the luciferase reporter assay whether exogenously expressed Zf9 activates the transcription of hsp47. An expression vector containing Zf9 driven by the CAG promoter was transiently transfected into BALB/c 3T3 cells together with the luciferase reporter constructs shown in Fig. 1. Exogenously transfected Zf9 enhanced the lucerase activity of the pLuc280(III) construct by more than 3-fold (Fig. 9). However, the levels of activation of the reporter constructs that lack the BS5-B sequence in the first intron, such as pLucΔBS-5, pLucBS5-B, and pLucΔPsPm, were only about half the level exhibited by pLuc280(III) in the presence of exogenous Zf9. In contrast, the transfection of Zf9 activated the pLucΔEPpm construct, which contains the BS5-B domain in the first intron, to an extent similar to pLuc280(III) (Fig. 9). These results clearly indicate that Zf9 activates reporter genes that contain the BS5-B element. Thus, we conclude that Zf9 is the transcription factor that specifically binds to the core sequence of the BS5-B element in the first intron and in collaboration with Sp2 and/or Sp3 activates the expression of hsp47.

**DISCUSSION**

HSP47 is induced by various stresses including heat shock (28), and it is the only heat-inducible protein among the stress proteins residing in the ER of mammalian cells. Other stress proteins in the ER are induced by the accumulation of unfolded proteins in the ER, a phenomenon referred to as the unfolded protein response (5). A well conserved heat shock element in the promoter region at −100 bp is responsible for the heat inducibility of the hsp47 gene (7).

On the other hand, the constitutive expression of hsp47 is always correlated with the constitutive expression of various types of collagen in several cell lines and tissues; collagen-producing cells also synthesize hsp47, but the cells that do not produce collagens do not synthesize hsp47 (15). Transgenic mice harboring a construct consisting of the promoter and the first and second introns upstream of a lacZ reporter gene express β-galactosidase in those tissues that express hsp47. We also reported that this constitutive expression of hsp47 is regulated not only by the promoter region but also by the first intron of the hsp47 gene in vitro. In the present study, we showed that in addition to cis-acting elements in the promoter and the first intron, the second intron is also involved in regulating hsp47 constitutive expression. Here, we identified two elements, BS5-B and EP7-D, in the first and second introns, respectively, which are necessary for the expression of hsp47 in BALB/c 3T3 cells. We also identified in a yeast one-hybrid screen four Kruppel-like factors (KLF family proteins) as proteins that bind to the BS5-B element in the first intron, but not to the EP7-D element in the second intron. Among KLF family proteins, Zf9 was reported to activate the expression of various genes, including type I collagen, TGF-β, and the TGF-β receptor, which are associated with the progression of hepatic fibrosis (25, 26). We confirmed in this study that transfected Zf9 activates the expression of a simultaneously transfected reporter gene under the control of the BS5-B element and the 280 bp promoter region.

KLF family proteins are characterized by a highly conserved C-terminal DNA binding domain containing three zinc finger motifs that recognize the sequence CACCC (20, 21). We showed by EMSA that Zf9 binds to the CACCC motif in the core sequence of the BS5-B element. However, the EP7-D element in the second intron does not have the CACCC motif, and we showed by CHIP analysis that Zf9 does not bind this element (Fig. 8). This result is consistent with our failure to identify any members of the KLF family in a yeast one-hybrid screen using EP7-D as a bait. This was also confirmed by an analysis of luciferase reporter activity after the transfection of Zf9 cDNA into BALB/c 3T3 cells (Fig. 9). Transfected Zf9 up-regulated
expression in hsp47 examine whether KLF-3 is involved in the repression of this study, we did not examine the influence of KLF-3 on the repressor activity that depends on the recruiting of CtBP2, a Zf9 does not involve the Sp1 binding site.

Struct with that of pLuc280(III) in the absence of Zf9. Error bars represent the mean ± S.D. of at least three experiments in duplicate.

Fig. 9. Zf9 transactivates the luciferase reporter gene through the CACCC sequence in the first intron. The luciferase reporter constructs shown in Fig. 1 were transfected into BALB/c 3T3 cells together with the recombinant Zf9 expression plasmid. Relative luciferase activity was determined by comparing the activity of each construct with that of pLuc280(III) in the absence of Zf9. Error bars represent the mean ± S.D. of at least three experiments in duplicate.

luciferase activity to levels 3-fold greater than observed for the pLuc280(III) construct. The pLucΔEPm construct, which lacks the EP7-D element, was up-regulated to the same extent as the pLuc280(III) construct by transfected Zf9, which suggests that the EP7-D element is not involved in Zf9-mediated transcriptional activation. We have first demonstrated here that Zf9 trans-activates gene expression by interacting with the cis-acting elements in the intron region.

In contrast, an ∼2-fold increase in the level of expression was observed in cells co-transfected with Zf9 and the pLucΔBS-5, pLucΔBS-5-B, and pLucΔPsPm constructs (Fig. 9), which do not contain the CACCC motif. This up-regulation might be because of the binding of overexpressed Zf9 to the promoter region of the hsp47 gene. In fact, we confirmed by EMSA that a GST/Zf9 fusion protein binds to the GC-rich sequence, a putative Sp1 binding site, in the hsp47 promoter (data not shown). However, CHIP analysis using anti-Zf9 antibody indicated that endogenous Zf9 does not bind to the Sp1 site in the hsp47 promoter in BALB/c 3T3 cells, suggesting that activation by endogenous Zf9 does not involve the Sp1 binding site in vivo.

Among the KLF family proteins that were isolated in this study by yeast one-hybrid screening, KLF-3 is reported to have a repressor activity that depends on the recruiting of CtBP2, a general co-repressor protein, to the transcription factor complex (24). Because we aimed to identify activator proteins in this study, we did not examine the influence of KLF-3 on the transcription of hsp47. However, it should be necessary to examine whether KLF-3 is involved in the repression of hsp47 expression in hsp47 nonproducing cells in the future. KLF-4 is reported to activate the β-globin gene and other erythroid cell lineage-specific genes in human and mouse by binding to the CACCC motif in the promoters of these genes (22). KLF-4 is expressed mainly in the gastrointestinal tract, but transcript is also detected in the lung, testis, skin, and thymus (23). This protein activates the expression of CYP1A1 (29), a cytochrome P-450 drug-metabolizing enzyme, keratin 4 (30), keratin 19 (31), cyclin D1 (32), and p21WAF1/CIP1 (33), a cyclin-dependent kinase inhibitor. KLF-4 was shown to co-activate the human keratin 4 gene through an interaction with Zf9/KLF-6 (30). Although Zf9 was clearly shown in this study to be involved in the activation of hsp47 expression in BALB/c 3T3 cells, a possible involvement of KLF-4 in cooperation with Zf9/KLF-6 might be an interesting issue to be examined in relation to the regulation of the hsp47 gene in the future.

The C-terminal zinc finger motif of KLF family proteins is also found at the C termini of Sp1 family proteins, which can bind the GC box. Thus, Sp1 and KLF family proteins, both of which belong to the same superfamily, recognize similar GC-rich DNA elements (34, 35). The Sp3 protein was previously reported to bind to a GC-rich site at −210 bp in the promoter of hsp47 and to be required for basal levels of reporter activity in a luciferase assay in both hsp47-producing and nonproducing cells (15). CHIP analysis using an anti-Sp3 antibody showed that Sp3 binds to this GC-rich sequence in vivo.2

The involvement of Sp1 family proteins in the transcriptional regulation of collagen has been reported. Both the Sp1 binding site in the promoter region and the first intron are necessary for the up-regulation of COL2A1 transcription in cartilage (36). Sox9 has been shown to trans-activate the COL2A1 gene by binding to sequences in the intron (37). Sp1 also binds to one of the two GC boxes in the type II collagen promoter, and it also interacts with a zinc finger protein, CIIZF, that is bound to the first intron, resulting in the activation in chondrocytes of the type II collagen gene (38). Sox9 is also reported to activate the COL11A2 gene by binding to the CTCAAGAG motif in the first intron and interacting with the promoter region (39).

In this study, we revealed that two Sp1 family proteins, Sp2 and Sp3, bind to the BSS-B and EP7-D elements in the first and second introns, respectively. Vergeer et al. (40) reported that the type I collagen gene has several cis-acting regulatory elements, including AP2 binding sites in the promoter and Sp1 binding site clusters in the first intron. Electron microscopic analysis showed that heterologous and homologous protein-protein interactions involving Sp1 and AP2 bring the promoter and the intron into close contact, facilitating transcription initiation. Recently, the importance of the Sp1 binding sites in the first intron of the type I collagen gene was revealed by the observation that the polymorphic substitution of G by T in the Sp1 binding site in the first intron of the COL1A1 gene causes a reduction in bone density in the osteoporosis by reducing the expression of its mRNA (41).

Sp1 family proteins are ubiquitously expressed whereas KLF family proteins are not. Cooperative but distinctive functions between members of the two families have been shown in various cases in addition to the activation of the COL1A1 gene and TGF-β and TGF-β receptor genes (25, 26). The core promoter of leukotriene C4 synthase, which catalyzes the conjugation of glutathione with leukotriene A4 to form leukotriene C4, is composed of the CACCC motif and a downstream GC box (42). The basal expression of leukotriene C4 synthase is regulated by Sp1 bound to the GC box, and cell type-specific expression in THP-1 cells is regulated by both Sp1 and Zf9 bound to the CACCC motif. The tissue-specific expression of the laminin γ1 chain and keratin 19 genes is also regulated by cooperation between KLF-4 and Sp1 (31, 43). Whereas it is not clear at present whether Zf9/KLF-6 is involved in the tissue-specific expression of hsp47, the observations that Sp2 and/or Sp3 bind to three sites in the promoter, the first and second introns and that Zf9 binds to the cis-acting element in the first intron, resulting in an activation of hsp47 gene expression in HSP47-producing cells, lead us to propose Zf9 as the co-activator for the tissue-specific expression of hsp47.

The expression of HSP47 is dramatically up-regulated in fibrogenic pathophysiological conditions including liver, lung and kidney fibrosis (12), keroid (44), systemic fibrosis (45), and atherosclerosis (13), all of which are characterized by the abnormal accumulation of several types of collagens, including.

2 K. Yasuda, K. Hirayoshi, H. Hirata, H. Kubota, N. Hosokawa, and K. Nagata, unpublished observation.
types I and III, in various tissues. Interestingly, in the rat model of kidney fibrosis, the transient down-regulation of hsp47 expression by the administration of antisense oligonucleotides causes a reduction in the progression of fibrosis as well as a reduction in the accumulation of HSP47 and types I and IV collagens in mesangial cells (14). Thus, the regulation of hsp47 is interesting and important from the therapeutic point of view and is highly relevant for future studies of fibrogenic diseases.

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The Kruppel-like Factor Zf9 and Proteins in the Sp1 Family Regulate the Expression of HSP47, a Collagen-specific Molecular Chaperone
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