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HSF1 Down-regulates XAF1 through Transcriptional Regulation*

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Studies have indicated the role of HSF1 (heat-shock transcription factor 1) in repressing the transcription of some nonheat shock genes. XAF1 (XIAP-associated factor 1) was an inhibitor of apoptosis-interacting protein with the effect of antagonizing the cytoprotective role of XIAP. XAF1 expression was lower in gastrointestinal cancers than in normal tissues with the mechanism unclear. Here we showed that gastrointestinal cancer tissues expressed higher levels of HSF1 than matched normal tissues. The expression of XAF1 and HSF1 was negatively correlated in gastrointestinal cancer cell lines. Stress stimuli, including heat, hypo-osmolarity, and H_2O_2, significantly suppressed the expression of XAF1, whereas the alteration of HSF1 expression negatively correlated with XAF1 expression. We cloned varying lengths of the 5′-flanking region of the *XAF1* gene into luciferase reporter vectors, and we evaluated their promoter activities. A transcription silencer was found between the −592- and −1414-nucleotide region that was rich in nGAAn/nTTCn elements (where n indicates G, A, T, or C). A high affinity and functional HSF1-binding element within the −862/−821-nucleotide region was determined by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. Inactivation of this "heat-shock element" by either site-directed mutation or an HSF1 inhibitor, pifithrin-α, restored the promoter activity of the silencer structure. Moreover, pretreatment with antioxidants suppressed HSF1 binding activity and increased the transcriptional activity and expression of XAF1. These findings suggested that endogenous stress pressure in cancer cells sustained the high level expression of HSF1 and subsequently suppressed XAF1 expression, implicating the synergized effect of two anti-apoptotic protein families, HSP and inhibitors of apoptosis, in cytoprotection under stress circumstances.

Heat-shock proteins (HSPs)[2] are conserved molecules present in all prokaryotes and eukaryotes (1, 2). The expression of these proteins is very low under normal physiological conditions and can be induced by stress factors, including physiological (growth factors, oxidative stress, and hormonal stimulation), environmental (heat shock, heavy metals, and ultraviolet radiation), or pathological stimuli (inflammation and autoimmune reactions and viral, bacteriological, or parasitic infections) (3, 4). Some stress factors, such as oxidative stress, have been considered as tumorigenic agents at low concentrations (5, 6). The main function of HSPs is to operate as an intracellular chaperone for aberrantly folded or mutated proteins and to provide cytoprotection against the stress conditions (31). For this reason, the presence of a cellular stress response in cancer cells reduces their sensitivity to chemical stress caused by insufficient tumor perfusion of chemotherapeutic agents (2).

Heat-shock transcription factors (HSFs or HSTFs) were originally characterized as regulators of the expression of the heat-shock protein, through binding to specific sequences ("heat-shock element" (HSE)), typically a pentanucleotide nGAAn structure (where n indicates G, A, T, or C) oriented in inverted dyad repeats (7, 8). The HSF family consists of three members in human, namely HSF1, HSF2, and HSF4. HSF1 is specifically responsible for the stress-mediated HSP induction. In unstressed cells, HSF1 is present in the cytoplasm either as a monomer or forming heteromeric complexes. Upon treatment with stress inducers, HSF1 homotrimerizes, translocates to the nucleus, and binds the HSE for its transactivation capacity (9, 10). Recent studies have shown that HSF1 can also act as a negative regulator of certain nonheat-shock genes, including *IL-1β*, c-*fos*, and *TNF-α* (11–13).

Inhibitors of apoptosis (IAPs) constitute another family of anti-apoptotic proteins. They were identified in baculoviruses where they function to prevent the death of infected host cells (14). XIAP is a potent member of IAPs that is expressed in all adult and fetal tissues with the exception of peripheral blood leukocytes. XIAP binds directly to caspases and functions as a competitive inhibitor of caspase catalytic function (15).

Yeast two-hybrid studies identified a XIAP-interacting N-terminal zinc finger protein designated XAF1 (XIAP-associated factor-1) (16). The incubation of recombinant XIAP with caspase-3 in the absence or presence of XAF1 demonstrated that XAF1 blocked the inhibitory activity of XIAP for caspase-3, and co-expression of XAF1 and XIAP inhibited XIAP-dependent caspase-3 suppression (17). XAF1 has been implicated as a tumor suppressor because its expression was lower in tumor cells than in normal tissues, and transient expression of XAF1 sensitized tumor cells to the pro-apoptotic effects of etoposide as well as tumor necrosis factor-related apoptosis-inducing ligand (17, 18). In gastrointestinal (GI) cancers, Byun *et al.* (23) reported gastric cancer tissues expressed lower levels of XAF1 than normal tissues. However, few studies have focused on the regulation of XAF1. In this study, we described the presence of a high affinity HSF1-binding sequence within the 5′-flanking region of the *XAF1* gene. GI cancer cells expressed high levels of HSF1, which enhanced cell survival under stress stimulation, by negatively regulating XAF1 expression.

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[2] The abbreviations used are: HSP, heat-shock protein; IAP, inhibitor of apoptosis; XIAP, X-linked IAP protein; HSF, heat-shock factor; HSE, heat-shock element; HS, heat shock; HO, hypo-osmosis; NAc, *N*-acetyl-L-cysteine; PFA, pifithrin-α; RACE, rapid amplification of cDNA ends; EMSA, electrophoretic mobility shift assay, TSS, transcription starting site; GSP, gene specific primer; AP, adapter primer; RLU, relative luciferase unit; ChIP, chromatin immunoprecipitation; GI, gastrointestinal; DTT, dithiothreitol; siRNA, small interfering RNA; ROS, reactive oxygen species; TNF, tumor necrosis factor; nt, nucleotide.

EXPERIMENTAL PROCEDURES

Primers, Oligonucleotides, and Probes—All oligonucleotides were synthesized by Proligo, Singapore. Table 1 shows the sequences of each oligonucleotide used for reverse transcription-PCR, 5′-rapid amplification of 5′-cDNA ends (5′-RACE), electrophoretic mobility shift assays (EMSA), luciferase construction, site-directed mutagenesis, and chromatin immunoprecipitation (ChIP).

Reagents—Catalase, *N*-acetyl-L-cysteine (NAc), and pifithrin-α (PFA) were purchased from Sigma. Goat anti-human XAF1 (C-16), goat anti-human actin (I-19), normal goat IgG, goat anti-human HSTF-1 (C-19), and horseradish peroxidase-conjugated anti-goat IgG were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Tissue Specimens and Human Cell Lines—Three gastric cancer and nine colon cancer specimens and their adjacent normal tissues were obtained from patients by surgical resection in the Nanfang Hospital (Guangzhou, China). All colon tissues were from sporadic colon cancer patients. Tissue specimens were snap-frozen in liquid N_2 and stored at −70 °C until used. Tissue slices were subjected to histopathological review, and tumor specimens consisting of at least 80% carcinoma cells were chosen for molecular analysis. Gastric cancer cell lines AGS and Kato-III and colon cancer cell lines SW1116, HT-29, Lovo, and Colo205 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Gastric cancer cell lines MKN45 and BCG823 were maintained by our laboratory and were described in a previous study (21). They were all maintained in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified incubator at 37 °C with an atmosphere of 5% CO_2. For stress treatment, the cells were incubated in complete medium at 42 °C (heat stress (HS)) or in hypo-osmotic (HO) medium for 30 min, followed by culture in normal medium at 37 °C for 24 h. The hypo-osmotic medium contained 67% complete medium and 33% sterile double distilled water with the osmolarity of about 209 mosM/kg. For oxidative stress, the cells were exposed to 200 μM of H_2O_2 for various time points.

Transient Transfection—For transient transfection, 4 μg of the pcDNA3.1 construct encoding HSF1 (pcDNA3.1/HSF1, kindly provided by Dr. R. E. Kingston) was mixed with 250 μl of serum and antibiotics-free medium containing 10 μl of LipofectAMINE2000 reagent for 20 min at room temperature. The mixtures were overlaid onto monolayers of cells seeded in a 6-well tissue culture plate preincubated under serum-free conditions. After 4 h of incubation at 37 °C, the DNA-liposome complex was replaced with complete medium without antibiotics and cultivated at 37 °C. Whole cell lysates were prepared 48 h later to evaluate the protein expression.

Immunoblotting—The whole cell lysates were prepared with lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). To prepare protein sample for tissue specimens, homogenization was performed in protein lysis buffer. The protein concentration was determined by the bicinchoninic acid assay (BCA protein assay kit, Pierce) with bovine serum albumin (Sigma) as the standard. Equal aliquots of total cell lysates (30 μg) were solubilized in sample buffer and electrophoresed on denaturing SDS-polyacrylamide gel (5% stacking gel and 12% separating gel). The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked with 10 mM Tris-HCl buffer saline, pH 7.6, plus 0.05% Tween 20 containing 2% skimmed milk. The blots were probed with primary anti-human XAF1 antibody for 1 h at room temperature followed by the horseradish peroxidase-conjugated anti-goat secondary antibody. Goat anti-human actin antibody (1:1000) was used as an internal control. Antigen-antibody complexes were visualized by the ECL system (Amersham Biosciences).

5′-RACE—To extend the cDNA transcript, 5′-extension PCRs were performed by using SMART RACE cDNA amplification kit (Clontech) with Human Colon 5′-STRETCH PLUS cDNA Library (Clontech) as the template, as described previously (19). Briefly, the first round of touchdown PCR was performed using HotStart Taq polymerase (Qiagen, Hilden, Germany) with AP1 (adapter primer 1) provided by the kit and the *XAF1* GSP1 (gene-specific reverse primer 1). The PCR product was separated in a 1% gel. Because no intensified PCR product was found under UV light, a pair of nested primers was used to re-amplify the PCR product by using AP2 (adapter primer 2) provided by the kit and XAF1 GSP2. Both GSP1 and GSP2 were located at exon 2 of *XAF1* gene. The sequences of primers are listed in Table 1. The conditions of touchdown PCR were as follows: 94 °C for 30 min; 5 cycles at 94 °C for 30 s and 72 °C for 4 min; 5 cycles at 94 °C for 30 s and 70 °C for 4 min; and 25 cycles at 94 °C for 30 s and 68 °C for 4 min. The PCR product was separated in a 1% gel. DNA was isolated using a GFX PCR DNA and gel band purification kit (Amersham Biosciences) and cloned into a pGEMT-T cloning vector (Promega, Madison, WI). Plasmid DNAs were purified using a commercial kit (Promega) and sequenced using the ABI PRISM 377 DNA Sequencer (Applied Biosystems), according to the manufacturer's instructions.

Generation of XAF1-Promoter Luciferase Constructs—Genomic DNA was isolated from cancer cells by proteinase K digestion and sequential phenol extraction. To locate the regulatory promoter of XAF1, five DNA segments that shared the same proximal site and different distal sites were obtained by PCR amplification. The distal sites were located at −1414, −920, −592, −254, and −107 nt, respectively, and the proximal primers were located at −42 to −20 bp of the *XAF1* gene. The upstream nucleotide adjacent to the translation starting ATG codon is defined here as −1 (20). KpnI site was added into the 5′ terminus of all of the forward primers, and the XhoI site was added into the reverse primer. The primers used were listed in the Table 1. Genomic DNA of AGS cell was used as the template for PCR amplification with HotStart Taq polymerase. PCR products were visualized on 1% agarose gels by ethidium bromide staining and were purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences). After digestion of both the pGL3 basic vector (Promega) and the PCR products with KpnI and XhoI, the purified products were inserted in the forward orientation upstream of a luciferase reporter gene of pGL3 basic vector to generate pLuc-1414, pLuc-920, pLuc-592, pLuc-254, and pLuc-107 constructs.

XAF1 Promoter-Luciferase Reporter Expression—For luciferase assay, the cells were seeded into 24-well plates to 70–80% confluence and transfected with the various pLuc constructs by Lipofectamine 2000 as described previously (21). pRL-CMV (Promega) was used to normalize the reporter gene activity. 0.8 μg of pLuc plasmids and 0.008 μg of pRL-CMV vector were mixed with 50 μl of serum and antibiotics-free medium containing 4 μl of LipofectAMINE2000 reagent for 20 min at room temperature. The mixtures were overlaid onto monolayers of the various cell lines preincubated under serum-free conditions. After 4 h of incubation at 37 °C, the DNA-liposome complex was replaced with complete medium without antibiotics and cultivated for an additional 48 h at 37 °C. Cells were solubilized in 1× passive lysis buffer (Promega), scraped with a rubber policeman, and mixed with 50 μl of luciferase assay reagent (Promega). The firefly and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega) with a model TD-20/20 luminometer (EG & G Berthold,

Australia). Firefly luciferase activity value was normalized to *Renilla* activity value. Promoter activity was presented as the fold of relative luciferase unit (RLU) compared with the basic vector control. RLU indicates values of firefly luciferase unit/values of *Renilla* luciferase unit.

Preparation of Cytoplasmic and Nuclear Extract—After treatment, cells were resuspended in 400 μl of buffer A (containing 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A), lysed with 12.5 μl of 10% Nonidet P-40, and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μl of buffer B (20 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A) and agitated for 60 min at 4 °C, and the nuclear debris was spun down at 20,000 × *g* for 15 min. The supernatant (nuclear extract) was collected and stored at −80 °C until ready for analysis. Protein concentrations were determined with BCA protein assay kit.

EMSA—Double-strand DNA probes were labeled with 5 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Promega). The labeled oligonucleotides were separated from the free [γ-^{32}P]ATP using a column (Bio-Rad) according to the manufacturer's instructions. For EMSA, total reaction mixtures containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 4% glycerol, and 50 μg of poly(dI-dC)-poly(dI-C)/ml were incubated with 3 μg of nuclear extracts and various unlabeled competing oligonucleotides for 10 min at room temperature, followed by addition of 1 μl ((0.5–2) × 10^5 cpm) of ^{32}P-end-labeled oligonucleotides. Samples were separated by electrophoresis on 8% nondenaturing polyacrylamide gel, with detection of radioactive bands by autoradiography for 16–24 h at −80 °C.

siRNA Transfection—The siRNA duplexes consisted of 21 bp with a 2-base deoxynucleotide overhang (Proligo, Singapore). The sequences of the HSF1 siRNA were as follows (sense strand): siRNA 1, GAUGGCGGCGGCCAUGCUGdTdT. The control siRNA, GL2 (CGUACGCGGAAUACUUCGA), was directed against the luciferase gene. The cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions.

ChIP Assay—The ChIP assays were performed according to the protocol provided by the ChIP assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY). Briefly, cells were treated with 1% formaldehyde to cross-link proteins to DNA. After washing, the cell pellets were resuspended in lysis buffer and sonicated to yield an average DNA size of 500 bp. Sonicated extracts were subsequently clarified by centrifugation and diluted with ChIP dilution buffer. 20 μl of the diluted lysates was left as the input control. Other lysates were pre-cleared with protein A-agarose/salmon sperm DNA and then divided into two fractions and incubated with 5 μg of normal goat IgG or goat anti-human HSTF-1 antibody each. Protein A-agarose/salmon sperm DNA was added to each fraction and rotated at 4 °C. After thoroughly washing, immunoprecipitated products were eluted using elution buffer. The cross-linked DNA-protein complexes were reversed by heating at 65 °C. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Quantitation of the DNA from the *XAF1* promoter regions was determined by PCR using gene-specific primers as described in Table 1. Hotstart PCR amplification was performed by using either immunoprecipitated DNA, a control with goat IgG, or chromatin input that had not been immunoprecipitated. To ensure linear amplification of DNA, pilot PCRs were performed initially to determine the optimal PCR conditions. In general, samples were heated at 95 °C for 30 min, followed by 34 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. After cycling, samples were incubated at 72 °C for 7 min to permit completion of primer extension.

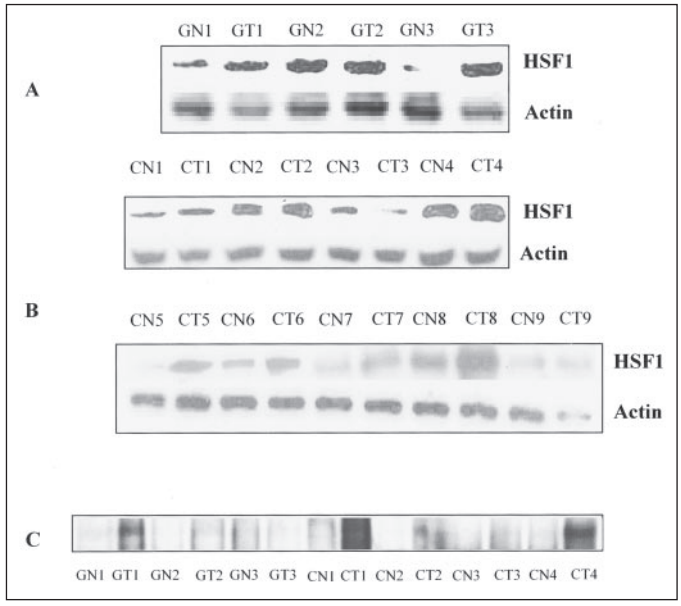

FIGURE 1. **GI cancer expressed higher level of HSF1 than normal tissues.** Immunoblotting for HSF1 expression in matched gastric (*A*) and colon (*B*) normal and tumor tissues. *GN1–GN3*, gastric normal tissue; *GT1–GT3*, gastric tumor tissue; *CN1–CN9*, colon normal tissue; *CT1–CT9*, colon tumor tissue. These figures are representatives of two independent experiments. *C*, HSE/consensus oligonucleotide was labeled with ^{32}P; EMSA was carried out to detect its binding to the whole cell lysate of tissue specimens. This figure is representative of three independent experiments.

Site-directed Mutagenesis—The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate constructs with mutation of HSF-binding elements. Briefly, the pLuc-920 construct was PCR-amplified in the elongation process by using *Pfu* DNA polymerase and primers (Table 1) with the mutation of the predicted HSF1-binding elements. The incorporation of oligonucleotide primers generated a mutated plasmid containing staggered nicks. The product was then treated with DpnI endonuclease, specific for methylated and hemimethylated DNA, and hence the parental DNA template was digested (because DNA originating from *Escherichia coli* is usually *dam* methylated). The nicked vector DNA carrying the desired mutations was proliferated in Epicurian coli XL1-Blue supercompetent cells. Plasmid DNA was isolated and sequenced to verify the prospective mutated sequence.

Statistical Analysis—Results obtained from triplicate luciferase experiments were expressed as the mean ± S.D. RLU with different treatments were compared using a two-tailed Student's *t* test and were considered significant if the *p* value was less than 0.05.

RESULTS

Gastric and Colon Cancer Expressed Higher Levels of HSF1 Than Normal Tissues—We detected HSF1 expression in three gastric cancer (Fig. 1*A*) and nine colon cancer (Fig. 1*B*) specimens and matched normal tissues by immunoblotting assay. All of the gastric cancer tissues and 7 of 9 colon cancer tissues expressed higher level of HSF1 than normal tissues. To evaluate the activity of HSF1, double-strand DNA probe consensus to the HSE sequence of human HSP70 promoter (HSE/consensus, Fig. 4*B*) was labeled with ^{32}P, and EMSA was carried out to detect its binding to the whole cell lysates of tissue specimens. It showed that 5 of 7 cancer tissues (GT1, GT2, CT1, CT2, and CT4) displayed higher binding activity than matched normal tissues (Fig. 1*C*).

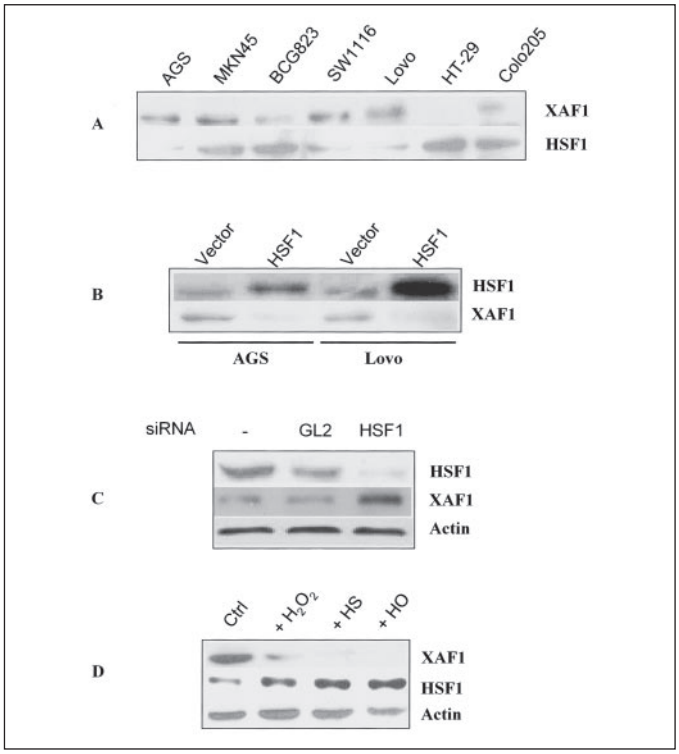

FIGURE 2. **XAF1 expression inversely correlated with HSF1 in GI cancer cells.** *A,* immunoblotting assay for HSF1 and XAF1 expression in GI cancer cell lines. *B,* AGS and Lovo cells were transfected with empty vector or pcDNA3.1-HSF1 construct for 48 h. HSF1 and XAF1 expressions were detected by immunoblotting. *C,* AGS cell was transfected without or with GL2 (control) or HSF1 siRNA for 48 h, and HSF1 and XAF1 expressions were detected by immunoblotting with actin as the internal control. This experiment was repeated twice in both AGS and Lovo cells with identical findings. *D,* Kato-III cell was treated with 200 μM of H_2O_2 for 24 h and HS (42 °C) or HO for 30 min followed by culture in standard medium for 24 h. HSF1 and XAF1 expressions were detected by immunoblotting. These figures are representative of two to three independent experiments.

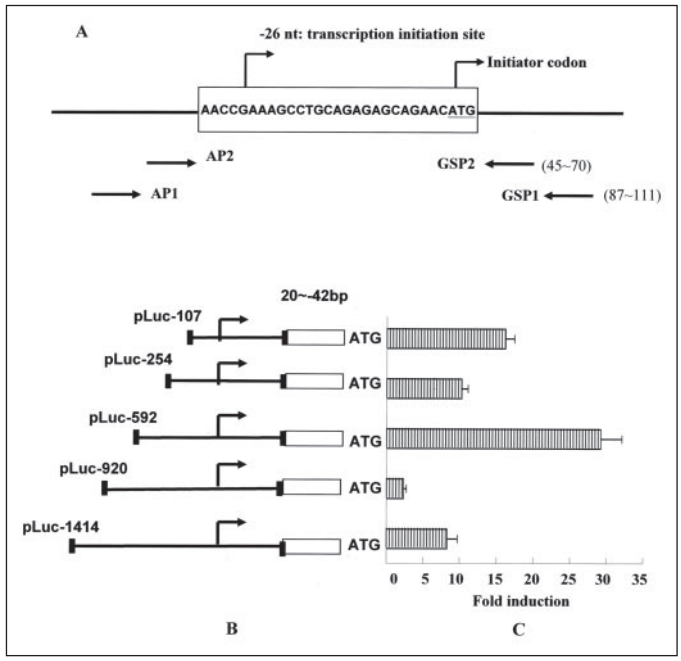

FIGURE 3. **Location of the regulatory promoter region of *XAF1* gene.** *A,* diagram of the location of *XAF1* GSP and TSS of *XAF1* gene determined by 5′-RACE assay. *B,* location of the truncated 5′-flanking DNA segments of *XAF1* gene cloned into pGL3 basic plasmid. The upstream nucleotide adjacent to the translation initiator ATG codon was defined as −1. *C,* AGS cells were transiently transfected with various pLuc constructs for 24 h. The firefly and *Renilla* luciferase activities were measured. Promoter activity was presented as the fold induction of RLU as compared with pGL3 basic vector. RLU = values of firefly luciferase unit/values of *Renilla* luciferase unit. This result is expressed as the mean of three independent experiments ± S.D.

Because the HSF1 expression and activity reflected cellular stress status, these results inferred that cancer cells have encountered higher stress pressure than normal cells.

XAF1 Expression Inversely Correlated with HSF1 in GI Cancer Cell Lines—To elucidate the correlation between XAF1 and HSF1 in cancer cells, we first checked their expression in GI cancer cell lines by immunoblotting. As shown in Fig. 2*A*, negative correlation was found between these two proteins in 6 of 7 cell lines except gastric cancer cell line MKN45. Second, to confirm the down-regulation of XAF1 by HSF1, we next transfected AGS and Lovo cells with pCDNA3.1-HSF1 expressing vector and detected XAF1 expression. We showed that overexpression of HSF1 down-regulated XAF1 expression in both cell lines (Fig. 2*B*). Third, we suppressed HSF1 expression by RNA interference (Fig. 2*C*). Consequently, XAF1 expression was up-regulated (Fig. 2*C*). These findings indicated that the low level expression of XAF1 in cancer cells might be attributed to the high expression of HSF1 and stress pressure.

To test the effect of HSF1 activator (stress stimuli) on XAF1 expression, we then treated gastric cancer cell Kato-III, which constitutively express XAF1, with oxidation (200 μM of H_2O_2), HO (for 30 min), or HS (42 °C for 30 min). We showed that stress stimuli up-regulated HSF1 and down-regulated XAF1 expression (Fig. 2*D*).

Location of the Regulatory Promoter of XAF1 Gene—To investigate the putative role of HSF1 in down-regulation of XAF1 expression through transcriptional regulation, the transcription starting site (TSS) of *XAF1* gene was determined by 5′-RACE assay. No visible band was found after the first round of touchdown amplification using AP1 and GSP1 primers, whereas a 96-bp fragment was obtained from nested PCR using AP2/GSP2 primer pair. After cloning the 96-bp PCR product into pGEM-T, 12 clones were sequenced using the vector primers, SP6 and T7 promoter sequences. Only three clones contained the adapter sequence and all of these clones mapped the 5′ end of the mRNA to an adenine 26 nucleotides upstream of the ATG initiator codon. A schematic diagram of the 5′-untranslated region of *XAF1* gene was shown in Fig. 3*A*. No typical TATA box was found within this region. Other clones contained the *XAF1* sequence but no adapter sequence, and for that reason we did not believe that these contained the true 5′ ends. The phenomenon was most likely the result of incomplete reverse transcription. Based on the above findings, we believed that *XAF1* has a single TSS located 26 bp upstream of the ATG initiator codon.

To determine the regulatory promoter region of the *XAF1* gene, truncated 5′-flanking sequences extending up to −1414 nt of the *XAF1* gene (Table 1 and Fig. 3*B*) were inserted in forward orientation upstream of a luciferase reporter gene (pGL3 vector) to generate pLuc constructs. The fold of RLU induced was evaluated after transient transfection into AGS/SW1116 cells. As shown in Fig. 3*C*, the RLU induction of pLuc-1414, pLuc-920, pLuc-592, pLuc-254, and pLuc-107 were 8.3 ± 1.4, 2.3 ± 0.4, 29.2 ± 2.9, 10.2 ± 0.9, and 16.2 ± 1.2, respectively. The highest RLU was observed for pLuc-592, indicating the presence of *cis*-enhancing element(s) between −26 to −592 nt. However, transfection of the longer *XAF1* 5′-flanking sequences, pLuc-920, resulted in a significant decrease in transcription activity, thus implicating the presence of a potential repressor element(s) between −592 and −1414 nt. Both cell lines have a similar pattern of transcription activities with different values for individual constructs.

Identification of HSF1-binding Sequence in XAF1 Promoter—The high affinity binding sequence for HSF1 comprises a minimum of two nGAAn/nTTCn elements arranged as an inverted dyad repeat (Fig. 4*A*).

TABLE 1
List of the oligonucleotide primers for amplification and mutation
GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.

Experiment	Name	Position or orientation	Sequence (5′–3′)
Luciferase construction	Reverse	−42 to −20	CC**GCTCGAG**TTCGGTTGAGTTTCGTTTCTTGC
	Forward 107	−90 to −107	GG**GGTACC**GATCTCCTCCCTCCCTGAA
	Forward 254	−235 to −254	GG**GGTACC**CAGCCTCAGGGAGGTAGATG
	Forward 592	−592 to −69	GG**GGTACC**AGGGTCTGGAAAAACTCTAAGGAC
	Forward 920	−920 to −896	GG**GGTACC**ATGCTTACATGAGGGATTAAAACGA
	Forward 1414	−1414 to 1391	GG**GGTACC**TTTTTAGTAGAGACGGGGTTTCAC
Site- directed mutagenesis	Wild type sequence	Sense	AACATAG**GAA**CAATGTT**GAA**ACAGTCT**TTCATT**CT**TTC**CCT
	Mutant 1	Sense	AACATAG**CCC**CAATGTTGAAACAGTCTTTCATTC**GGG**CCT
	Mutant 2	Sense	CAATGTT**CCC**ACAGTCT**GGG**ATTCTT
EMSA	−1008/−982	Sense	ATTTTCTCTTTTTTCATTTCATTTTTCTTT
	−862/−821	Sense	TGAACATAGGAACAATGTTGAAACAGTCTTTCATTCTTCCCT
RACE	Adapter 1	Forward	CCATCCTAATACGACTCACTATAGGGC
	Adapter 1	Forward	ACTCACTATAGGGCTCGAGCGGC
	XAFGSP1	Reverse	ACACTCCGGACACAGGACCAGGAAC
	XAFGSP2		CATGGAGGGTGAAGTTGGCAGAGACT
Reverse transcription-PCR	XAF1	Forward	GCTCCACGAGTCCTACTG
	XAF2	Reverse	ACTCTGAGTCTGGACAAC
	GAPDH	Forward	GACCACAGTCCATGCCATCAC
	GAPDH	Reverse	GTCCACCACCCTGTTGCTGTA
ChIP	−1021/−779	Forward	TCTCTGCCTCCATTTTCTCTTT
		Reverse	GAGAAGCAGTGTGTGGTGGT

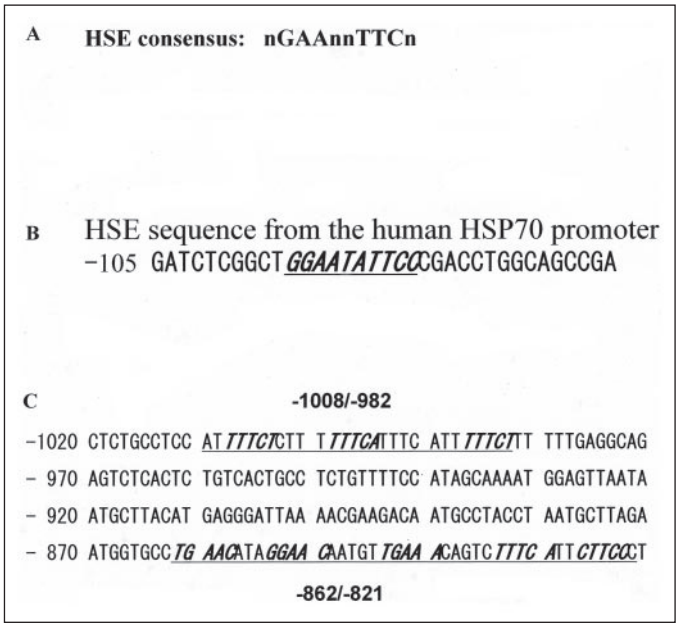

FIGURE 4. **Analysis of 5′-flanking sequence of *XAF1* gene.** *A,* consensus sequence of HSE. *B,* HSE sequence presented in human HSP70 promoter. *C,* two nGAAn (nTTCn)-rich sequences presented in the 5′-flanking region of the *XAF1* gene. The putative HSF1-binding elements are *underlined,* and the nGAAn (nTTCn) motifs are *italicized*.

Fig. 4*B* showed the sequence of the HSE from the human HSP70 promoter (HSE/consensus) (22). However, single nGAAn (nTTCn) elements are also functional in the transcription regulation of TNF-α and RANK ligand gene (13). Based on DNA sequence analysis, two putative HSE, −1008 to −982 and −862 to −821, which were rich in pentanucleotide nGGAn (nTTCn) sequences, were present between −592 and −1414-nt region of the *XAF1* 5′-flanking region (Fig. 4*C*).

To test the binding capacity of each nGAAn/nTTCn-containing sequence in the regulatory promoter/5′-flanking region of the *XAF1* gene, EMSA was performed using the following three-step strategy. First, the capacity of a 100-fold excess of unlabeled oligonucleotides (−1008/−982, −862/−821, and HSE/consensus) to compete and block binding to HSE/consensus was analyzed. Double-strand HSE/consensus was radiolabeled and used as probe with the nuclear extracts from AGS cells exposed to 42 °C for 30 min as a source of HSF1. It was found that −862/−821 but not −1008/−982 oligonucleotide blocked HSF1-specific binding. Competition for binding by −862/−821 oligonucleotide was as complete as that of a comparable concentration of HSE/consensus itself (Fig. 5*A*). Second, to confirm that HSF1 bound with high affinity to the −862/−821 sequence, we repeated the EMSA analysis using each −1008/−982 and −862/−821 oligonucleotide as a radiolabeled probe (Fig. 5*B*). Of the *XAF1* sequences studied, typical doublet bands were only observed with −862/−821 oligonucleotide and completely blocked by the excessive unlabeled cold probe, indicating the specificity of binding reaction. Third, to define further the role of the −862/−821 sequence in stress response, nuclear extracts of AGS cells with or without stress stimulations were extracted and bound to the radiolabeled −862/−821 oligonucleotide. As shown in Fig. 5*C*, HS, HO, and oxidative stress increased the binding capacity of the −862/−821 probe, and specific bands were completely blocked by unlabeled oligonucleotides. Therefore, we concluded that a high affinity HSE existed in the −862/−821 region. To substantiate the activity of this HSE *in vivo*, we performed ChIP assay by using specific antibody against HSF1. Normal goat IgG was used as the negative control. DNA associated with the chromatin immunoprecipitated by these antibodies was then amplified by PCR with primers specific for the putative HSE region of the *XAF1* promoter. As expected, no DNA fragments were detected when normal IgG was used (Fig. 5*D*, *lanes 1, 4, 7,* and *10*). In contrast, DNA fragments with the expected size were detected using anti-HSF1 antibody in AGS cells (Fig. 5*D*, *lane 2*). In addition, we showed that HS, HO, and oxidative stress increased the amount of immunoprecipitated DNA (Fig. 5*D*, *lanes 2, 5, 8,* and *11*). These findings suggested that the −862/−821 sequence of *XAF1* gene contained a high affinity HSE (HSE/XAF1) for HSF1.

Up-regulation of XAF1 Expression by Inactivation of HSF1 Binding—To clarify the function of the −862/−821 sequence in the repression of *XAF1* transcription, we abrogated this binding activity of HSE/XAF1 by introducing the GAA to CCC and TTC to GGG mutation into pLuc-920. As the typical HSF1-binding element consisted of inverted dyad repeats of the nGAAn/nTTCn motif, two mutant constructs were generated with type 1 mutating the outer pair of GAA/TTC elements and type 2 mutating the inner pair of GAA/TTC elements (Fig. 6*A*). After verifying the prospective mutation by DNA sequencing, the mutant

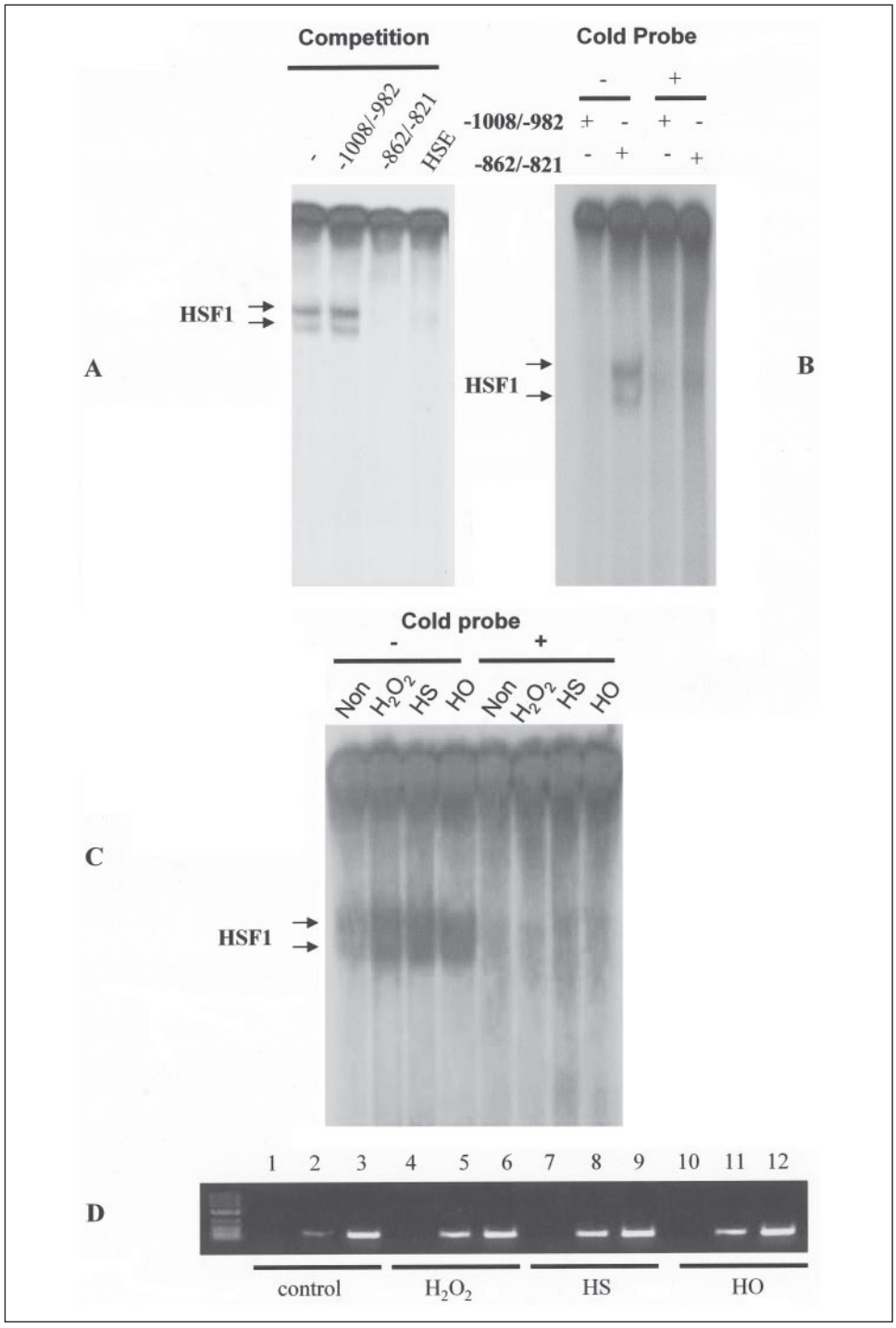

FIGURE 5. **Identification of HSF1-binding sequence in the *XAF1* gene.** *A,* double-strand HSE/consensus DNA probe was labeled with ^{32}P and bound to the nuclear extracts of heat-treated AGS cells with or without preincubation and with a 100-fold excess of each oligonucleotide (−1008/−982, −862/−821, and HSE/consensus). DNA binding activity was determined by EMSA. *B,* double-strand oligonucleotides consensus to the −1008/−982 and −862/−821 sequences were labeled with ^{32}P and bound to the nuclear extract of heat-treated AGS cells in the absence or presence of 100-fold excess of unlabeled probe (*cold probe*). *C,* double-strand −62/−821 oligonucleotide was labeled with ^{32}P and bound to the nuclear extract of AGS cells with various treatments. These figures represent one of three independent experiments with similar findings. *D,* ChIP analysis of HSE/*XAF1* element from untreated (*lanes 1–3*), H_2O_2- (*lanes 4–6*), HS- (*lanes 7–9*), and HO (*lanes 10–12*)-induced AGS cells using antibody specific for HSF1 (*lanes 2, 5, 8,* and *11*) or goat IgG control (*lanes 1, 4, 7,* and *10*). Input chromatins are presented in *lanes 3, 6, 9,* and *12*. This experiment was repeated twice in both AGS and Lovo cells, and the result was identical.

pGL3 constructs were transiently transfected into AGS and SW1116 cells. Transcription activity was evaluated and compared with the wild type construct. As indicated in Fig. 6*B*, RLU induction of wild type, type 1, and type 2 mutant pLuc-920 constructs were 3.04 ± 0.52, 2.74 ± 0.36, and 13.54 ± 0.36 in AGS cell and 3.15 ± 0.22, 3.08 ± 0.48, and 16.44 ± 0.33 in SW1116 cells. Type 2 but not the type 1 mutation increased the transcription activity of pLuc-920 significantly ($p < 0.05$ compared with the wild type control). To define further the binding capacity of the inner GAA/TTC sequence, EMSA was carried out to examine the specific binding of wild type and type 2 mutant oligonucleotides (Fig. 6*C*). Wild type but not the type 2 mutant probe bound to the nuclear extract of AGS cells effectively. These findings indicated that the inner GAA/TTC sequences in −862/−821 region contributed to HSF1 binding and repression of *XAF1* transcription.

Moreover, PFA, a novel defined inhibitor of HSF1 (24), was applied to examine its effect on the transcription activity of truncated *XAF1* promoter constructs. SW1116 cells were transiently transfected with pLuc-592, pLuc-920, and pLuc-1414 followed by incubation with 15 μM of PFA. Cells were lysed and assayed for luciferase activity. As shown in Fig. 6*D*, treatment with PFA did not or only slightly changed the transcription activity of pLuc-592 constructs. However, it increased the transcription activity of pLuc-1414 and pLuc-920 significantly ($p <$ 0.05). This finding proved the role of HSF1 binding in repression of *XAF1* transcription.

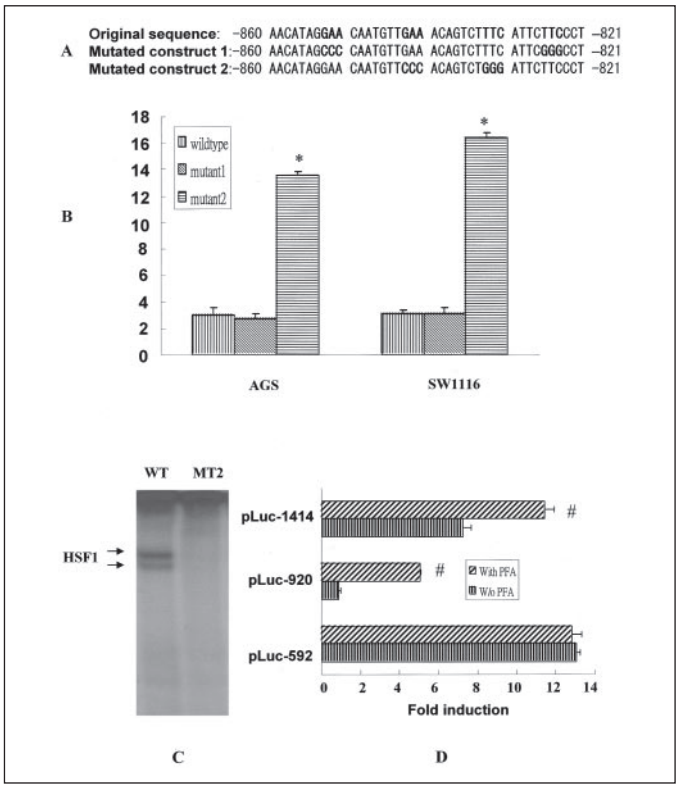

FIGURE 6. **Up-regulation of XAF1 expression by inactivation of HSF1 binding.** *A,* location of site-directed mutations of −862/−821 sequences of pLuc-920 constructs; the mutant bases are *highlighted*. *B,* wild type and mutant pLuc-920 constructs were transiently transfected into AGS and SW1116 cells. Luciferase activities were measured. The result was expressed as the mean of four independent experiments ± S.D. *, $p < 0.05$ *versus* wild type control. *C,* double-strand oligonucleotides consensus to wild type (*WT*) or mutant 2 (*MT2*) sequences were labeled with ^{32}P and bound to the nuclear extract of AGS cells. This experiment was repeated twice with identical findings. *D,* SW1116 cells were transiently transfected with pLuc-1414, pLuc-920, and pLuc-592 followed by incubation with 15 μM PFA or dissolvent control. Cells were lysed and assayed for luciferase activity. The result is expressed as the mean of three independent experiments ± S.D. #, $p < 0.05$ *versus* control.

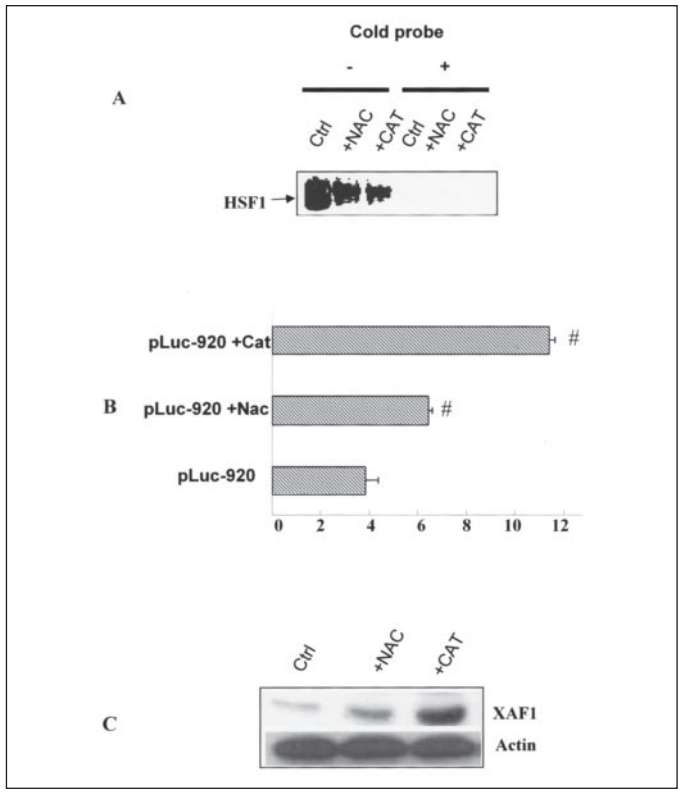

FIGURE 7. **Anti-oxidants restored XAF1 expression.** *A,* AGS cells were exposed to 200 μM of H_2O_2 in the presence or absence of antioxidants, NAc (20 mM) or catalase (*CAT*) (1000 units/ml), for 12 h. Nuclear extracts were incubated with a radiolabeled −862/−821 probe. This figure was the representative of two independent experiments. *B,* AGS cells transfected with pLuc-920 construct were exposed to NAc (20 mM) or catalase (1000 units/ml) and assayed for luciferase activity. The fold induction of RLU was expressed as the mean of three independent experiments ± S.D. #, $p < 0.05$ *versus* control. *C,* AGS cells were treated with or without antioxidants for 24 h, and XAF1 expression was detected by immunoblotting. This experiment was repeated twice with consistent findings.

Anti-oxidants Overcome the Suppression of XAF1 Expression—ROS has been implicated as an etiologic factor in numerous diseases, including cancer. ROS can originate exogenously from agents that generate oxygen free radicals and originate endogenously, for example, as a result of normal cellular metabolism, such as mitochondrial oxidative phosphorylation (25–27). It has been reported that H_2O_2 could stimulate binding of HSF1 to the HSE (28–30). To verify the role of HSF1 in suppressing XAF1 expression, AGS and SW1116 cells were exposed to 200 μM H_2O_2 in the presence or absence of antioxidants, NAc (20 mM) or catalase (1000 units/ml), for 12 h. Nuclear extracts were incubated with radiolabeled −862/−821 probe. EMSAs were carried out to examine the specific HSF1 binding activity. As shown in Fig. 7*A*, both NAc and catalase were able to suppress HSF1 binding activities. Regarding transcription activity, AGS cells transiently transfected with pLuc-920 were exposed to NAc or catalase to suppress the putative activation of HSF1. Transcription activities of pLuc-920 treated with dissolvent control, NAc, and catalase were 3.85 ± 0.5, 6.44 ± 0.16, and 11.4 ± 0.24, respectively. Antioxidants increased transcription activity of pLuc-920 by 50–150% (Fig. 7*B*, $p < 0.05$ comparing to nontreatment control). Moreover, pretreatment of AGS cells with antioxidants up-regulated XAF1 protein expression (Fig. 7*C*). These findings revealed that stress factors such as ROS suppressed transcription of XAF1 mediated by the interaction between HSF1 and HSE within the regulatory promoter region.

DISCUSSION

Bcl-2, HSP, and IAPs are three anti-apoptotic family proteins. The cross-talk between HSP and IAPs was confirmed by the finding that HSP90 positively modulated the expression and function of survivin in cancer cells via binding to the conserved baculovirus IAP repeat structure (32). Our present study provides new insight into the interaction between HSP and IAPs that HSF1 down-regulated IAP-interacting protein, XAF1, through transcription regulation.

It was well known that cancer cells have encountered higher level of stress pressure, both exogenous and endogenous (25, 26). Many cancers like sporadic colon cancers might have originated from inflammation such as inflammatory bowel diseases in which higher oxidative metabolites were produced by both infiltrated neutrophils and colon epithelial cells (33). The subsequent induction of stress-associated proteins, including HSPs and mitogen-activated protein kinases, will promote cell transformation (34, 35). As the pivotal transcription factor that stimulates HSP proteins, the expression profile of HSF1 in GI cancers has seldom been studied. By using matched normal and cancerous gastric and colon tissues, we showed cancer tissues expressed higher level of HSF1 than normal tissues. This result was consistence with the findings of Cen *et al.* (36) that HSF1 expression was increased in 86% (30/35) of sporadic colon cancer patients demonstrated by cDNA microarray assays. EMSA showed cancer tissues possessed stronger HSF1 binding activity than their normal counterparts. These data indicate that GI cancer cells had a higher level and active form of HSF1 protein than normal tissues.

Inhibitor of apoptosis proteins, which is partly attributable to adaptive stress response, is commonly found in cancer cells (1–3). XAF1, a newly identified antagonist of XIAP, has been identified as a tumor suppressor. Expression of XAF1 in cancer cells, including gastric cancers, was lower than that of normal tissues (16, 22). Our findings that HSF1 as well as oxidative, hypo-osmotic, and heat stress down-regulated XAF1 expression in GI cancer cell lines suggested that XAF1 was a stress-associated gene with its expression being negatively regulated during stress response.

Stress-activated survival response included the induction of anti-apoptotic proteins. Yet, if the exposure to a specific stress is excessive, cell death will occur, either by necrosis or apoptosis (3, 37–38). It is reported that when cells are exposed to low H_2O_2 concentration, they develop resistance to subsequent challenges with high concentrations of the same agent that would otherwise be lethal (38). In this study, the concentration of H_2O_2, the temperature of HS, and the osmolarity used were all within the tolerable or physiological range (39–41). Their effect on repression of XAF1 and/or induction of other IAP proteins such as survivin reflects a novel survival mechanism of cancer cells.

As a key stress-associated transcription factor, HSF1 exerts both an activating and suppressing effect on different target genes. Although the consensus HSF1-binding DNA sequence within the promoter of HSPs is the contiguous inverted dyad repeat of pentanucleotide nGAAn (HSE) (22), the capacities of the cis-formed dyad repeats of nGAAn or incontiguous nGAAn/nTTCn binding to HSF1 have been confirmed in all genes repressed by HSF1. These genes include *IL-1β*, c-*fos*, *TNF-α*, and RANK ligand (11–13). The HSE in RANK ligand is located in the −1275- to −2-kb region.

To search for the transcription regulatory element, we first demonstrated the transcription initiation site of the *XAF1* gene by 5′-RACE. It is located in the −26-nt adenosine upstream of the ATG initiator. By searching the DBTSS data base (dbtss.hgc.jp/index.html) where most genes possessed multiple TSS because of the different assay other than RACE utilized, we found the *XAF1* transcription starting region was located at −4 to −40 nt. Thus, we cloned the 5′-flanking sequence containing part of 5′-untranslated region sequence and defined them as the regulatory promoter of *XAF1*. A putative repressor or silencer sequence was eventually located between −592 and −920 nt by dual luciferase reporter assay. Two segments rich in nGAAn/nTTCn contigs between the −592- and −1414-nt region were found. Competitive EMSA excluded the specific HSF1 binding capacity of the −1008/−982 segment and hence demonstrated the −862/−821 segment as a high affinity HSE. In accordance with luciferase results, this segment is located at the proposed transcription silencer region. On the other hand, this region contained two pairs of inverted dyad repeats of nGAAn/nTTCn motifs. Further site mutagenesis strategy implicated the inner pair of the nGAAn/nTTCn contig as the functional HSF1-binding element (HSE/XAF1) that is responsible for repression of *XAF1* transcription.

To validate further the HSF1-binding mediated suppression of *XAF1* transcription, we used an HSF1 inhibitor (24), pifithrin-α, to inactivate cytosolic HSF1 protein. Pifithrin-α can also suppresses p53 activity; however, no typical p53-binding element is determined within the 5′-flanking region of *XAF1*. We showed that pretreatment with this inhibitor eliminated the effect of the transcription repressor within the *XAF1* regulatory promoter in unstressed cancer cells, suggesting endogenous intracellular stress pressure maintained the transcriptional inhibition of the *XAF1* gene in cancer cells.

ROS was the predominant endogenous stressor of cancer cells. Bittinger *et al.* (42) reported that melanoma cells produced large amounts of superoxide anions without stimulants, as implicated in metastasis by promoting endothelial injury. ROS also plays a central role in the modulation of HSF1 activation because it was not only one of the stressors that could activate HSF1 but could also be increased by many other cellular stresses that lead to HSF activation (28, 30, 43). Therefore, we evaluated the effects of antioxidants on XAF1 regulation. We found both *N*-acetyl-L-cysteine and catalase were able to suppress HSE/XAF1 binding activity, to abrogate transcription inhibition, and to induce XAF1 expression. Because XAF1 was a pro-apoptotic gene and its overexpression suppressed cell growth (data not shown), our findings were consistent with previous observations that a moderate level of intracellular ROS was important to maintain the appropriate redox balance and to stimulate cancer cell proliferation (44, 45).

In summary, GI cancer cells expressed high levels of HSF1. It mediated stress stimuli-induced down-regulation of XAF1 via interaction with an HSE within the 5′-flanking region. This mechanism may contribute to the low expression of XAF1 in cancer cells and prevent them from apoptosis. For the first time, our findings define XAF1 as a novel stress-associated gene that negatively modulates cancer cell growth.

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Genes: Structure and Regulation:

HSF1 Down-regulates XAF1 through Transcriptional Regulation

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