Journal of Cellular Biochemistry 84:401–407 (2002)

# Genomic Cloning and Promoter Analysis of the *GAHSP40* Gene

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**Abstract** The new heat shock protein (GAHSP40), which binds to Gadd34, is a member of the Hsp40 family gene and has a J domain, which is similar to bacterial DNAJ. We have isolated and sequenced the mouse *GAHSP40* gene including 1.6 kb of the 5′-flanking region. Primer extension analysis revealed that the transcription initiation site was located 36-bp upstream of the ATG translation initiation codon. In order to identify the heat-responsive regions in the *GAHSP40*, NIH3T3 cells were transiently transfected with a series of 5′ terminus-truncated mutants of the *GAHSP40* promoter linked to the luciferase reporter gene. We found that the region of −284 to −184 bp from initiation start site responded to heat shock treatment. By the gel shift analysis, we found the heat shock elements (HSEs) located in this region from −257 to −225. This HSEs has five 5 bp motifs. The transfection studies using HSEs mutant vectors revealed that those 3′ two 5 bp motifs are essential for heat responsive transcription. J. Cell. Biochem. 84: 401–407, 2002.


**Key words:** GADD34; heat shock factor; heat shock element

Cells, subjected to stress, respond by synthesizing a group of evolutionarily conserved proteins called heat shock proteins (HSPs). These stress-induced proteins perform many essential roles in cell survival, including prevention of protein aggregation and facilitation of protein folding. HSP40 family is one of the HSPs containing the DNAJ homologous region of *Escherichia coli*, J-domain of DNAJ. The main future of DNAJ function is the ability to directly interact with DNAK to stimulate ATPase activity and act as a chaperon in conjunction with DNAK [Georgopoulos, 1992]. DNAJ like HSP40 proteins have been isolated in yeast (SEC63/NPL1, SCJ1, YDJ1, SIS1, Zuotin), plant (ANJ1), fruit fly (Csp29, Csp32), mammalian (Csp1, Csp2, MTJ1, and ZRF1), and human cells (HDJ-1, HDJ2, HSJ1, HSDJ, HSPF1, and HLJ1) [Sadler et al., 1989; Ohtsuka et al., 1990; Zinsmaier et al., 1990; Blumberg and Silver, 1991; Caplan and Douglas, 1991; Luke et al., 1991; Atencio and Yaffe, 1992; Zhang et al., 1992; Zhu et al., 1993; Brightman et al., 1995; Hughes et al., 1995; Chamberlain and Burgoyne, 1996; Hoe et al., 1998], and their functions are under investigation [Raabe and Manley, 1991; Cheetham et al., 1992; Chellaiah et al, 1993; Oh et al., 1993; Ohtsuka, 1993; Hata et al., 1996; Kelley, 1998].

Previously, in order to examine the function of GADD34, we used the yeast two-hybrid system to clone the protein that interacts with the murine GADD34. We have cloned new HSP40 family gene, *GAHSP40* [Hasegawa et al., 2000]. *GAHSP40* is about a 1.4-kb gene and the N-terminal 70 amino acids correspond to the J-domain, which has been demonstrated to be the HSP70 interaction region and conserved among all proteins defined as DNAJ family. GAHSP40 was induced not only by heat shock but also by MMS (methyl methanesulfonate), that is a DNA alkylating agent. One of the hallmarks of heat shock genes is the presence of

Grant sponsor: Fund for Comprehensive Research on Aging and Health, Ministry of Health and Welfare of Japan and in part Grant-in-Aid for Scientific research.

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Received 15 June 2001; Accepted 5 September 2001


DOI 10.1002/jcb.10029

heat shock elements (HSEs) in their 5′-flanking regions to which heat shock transcription factor (HSF) binds stimulating transcription. HSE has been defined as an array of adjacent inverted pentamers with the sequence 5′-nGAAn-3′ [Amin et al., 1988; Xiao and Lis, 1988]. HSE sequences in Human HSP40 gene were composed of eight contiguous nGAAn motifs and were essential for heat shock response. In this paper, we clone the 5′ flanking region of *GAHSP40* gene and examine the regulation of its expression responded to heat shock. We show that GAHSP40 also has HSE element, which is essential for heat shock response.

## MATERIALS AND METHODS

### Oligonucleotides

To determine the nucleotide sequence of clones, the following oligonucleotides were used: *Bca*BEST Sequencing Primer RV-M5′-GAGCGGATAACAATTTCACACAGG-3′, *Bca*BEST Sequencing Primer M13-47 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′. To determine the transcription initiation site, the following oligonucleotides were used in primer extension experiments: 5′-CTGTAGCTCCTTTGTCAATTCCCA-3′.

### Cell Culture

NIH3T3 cells from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in a 37°C humidified atmosphere containing 5% $CO_2$ with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

### Cloning and DNA Sequencing of Genomic DNA for GAHSP40

Screening of the Lambda FIX II library (Stratagene) isolated genomic clones with the mouse *GAHSP40* cDNA probe under high-stringency hybridization conditions.

Two isolated clones were cloned into pUC18 vector. The nucleotide sequence of each clone was determined by the ABI 377 autosequencer with *Bca*BEST Sequencing Primer RV-M and *Bca*BEST Sequencing Primer M13-47 (Takara). If necessary, oligonucleotides were chemically synthesized and were used as sequencing primers.

### Primer Extension

Total cellular RNA was extracted from NIH3T3 cells at 42°C for 1 h by the method described elsewhere. A 25-mer primer, which was complementary to the region downstream of the ATG codon, was chemically synthesized. The 5′ end of the primer was labeled with [$\gamma$-$^{32}$P] and hybridized with 50 μg of total RNA for 16 h at 45°C in solution containing 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, and 0.4 M NaCl. The RNA and primer were ethanol-precipitated and re-dissolved in a solution for primer extension. The primer was extended for 1 h at 42°C in a 9 μl reaction mixture containing 50 mM Tris-HCl, 50 mM KCl, 10 mM MgCl, 10 mM dithiothreitol, 1 mM each dNTP, 0.5 mM sperimidine, 6.2 mM sodium pyrophosphate, and 300 U AMV reverse transcriptase. After incubation, the reaction was stopped by adding 1 μl of 0.5 M EDTA, and 1 μl of RNase A (1 mg/ml) was added to digest RNA. The digestion was carried out for 30 min at 37°C. The reaction product was ethanol-precipitated and redissolved in 4 μl of Loading dye containing 98% formamide, 10mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue. The samples were analyzed by a gel electrophoresis under denaturing conditions, followed by autoradiography. The nucleotide positions were cumulatively determined with references to a sequencing reaction in which the same primer was used (Primer Extension System-AMV Reverse Transcriptase, Promega, Madison WI).

### Plasmid Construction

GAHSP40 promoter-containing reporter constructs were made as follows: The phage positive clone was cut by EcoRI and subjected to southern hybridization. The 1.6 kb of EcoRI digested band was positively stained by GAHSP cDNA probe. This fragment was inserted into pUC18 EcoRI site. Then this construct was re-inserted into pGL3-basic luciferase vector (pGL3b-1557). The 5′-deletion mutants were obtained by making deletions in pGL3b-1557 by using an exonuclease III based system (Nippon Gene, deletion kit). To create a panel of site-directed mutants, mutagenesis of pGL3b-284 was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotides used in these mutagenesis reactions are as follows: pGL3b-m1, 5′-GTTCGCTGTTAACTAAAGTACAGAG-3′ and 5′-TCTGTACTTTAGTTAACAGCGAA-3′; pGL3b-m2, 5′-GTAGCGTTCGTTAACTCGCTGTTCTAG-3′ and 5′-TAGAACAGCGAGTTAACGAACGCTA-3′; pGL3b-m3 5′-GTCCTGTAGCGTTA-

ACTCGTTCGCTG-3′ and 5′-AGCGAACGAGTTAACGCTACAGGA-3.

### DNA Transfection and Luciferase Assay

NIH3T3 cells were plated onto 12-well plates at a density of 40,000 cells/plates. Cells were transfected with 2.5 μg/plates of plasmid by SuperFect Transfection Reagent (Qiagen, Hilden. Germany) according to the manufacturer's instructions. The test plasmid (2.25 μg/plates) and internal control plasmid (0.25 μg/plates) were co-transfected into cells. After 20 h incubation, the medium was replaced with fresh cell growth medium and the cells were grown for a further 20 h before heat shock treatment at 42°C for 6 h. The cells were harvested and lysed after heat shock treatment. The cell lysate was subjected to the assay for protein expression. The transfection efficiency was estimated via co-transfection with thimidine kinase promoter-driven *Renilla* luciferase reporter vector (pRL-TK plasmid, dual luciferase assay system, Promega, Madison, WI). The intensity of chemiluminescence was measured by a luminometer (Lumut LB9501, Berthold).

### Gel Mobility Shift Assay

Nuclear extracts from either proliferating or heat shock treated NIH3T3 cells were prepared as described [Hasegawa et al., 1997]. A total of 5 μg of nuclear extract was incubated with 5 fmol of $^{32}$P end labeled double-stranded oligonucleotides with a sequence corresponding to the region from −257 to −225 bp from initiation start site in the GAHSP40 promoter. Incubation was carried out in 1 volume of 10 μl at room temperature for 20 min. All binding reactions contained 10 mM Tris-HCl (pH 7.5), 4% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, and 0.5 mg/ml of poly (dI–dC). For competition, 1 pmol of unlabeled oligonucleotides was incubated in reactions. In supershift experiments, 1 μl of antibody was added 10 min before the labeled probe. The electrophoretic mobility shift assay products were separated on 4% polyacrylamide 0.5 × Tris-borate EDTA gel at room temperature at 150 V for 1.5 h. The gel was dried and subjected to autoradiography. The oligonucleotides used on these experiments were as follows: 5′-GTAGCGTTCGTTCGTTCGCTGTTCTAGAAAGTACAG-3′ and 5′-TGTACTTTCTAGAACAGCGAACGAACGAACGCTA-3′. The polyclonal antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA).

## RESULTS

### Cloning of the Gene for the GAHSP40

The 0.2-kb fragment that codes the regions of J domain was used as a probe to screen the mouse genomic library. Of approximately $1 \times 10^6$ clones screened, one positive clone was isolated. The complete nucleotide sequence of the region containing 1.6 kb genomic DNA was determined (Fig. 1).

### Determination of Transcriptional Initiation Site of the *GAHSP40* Gene

The transcription initiation site of *GAHSP40* was determined by primer extension. Figure 2 shows the nucleotide sequence of the DNA near the 5′ end of *GAHSP40* mRNA. Alignment of nucleotides with genomic sequence indicated that the majority of transcription originates at or near c residue 36 nt upstream of the ATG initiation codon (Fig. 3). Typical TATA box was not present in the 1.6 kb region of the *GAHSP40* promoter.

### Determination of the Promoter Region of the *GAHSP40* Gene

In order to identify the heat-responsive regions in the *GAHSP40*, NIH3T3 cells were transiently transfected with a series of 5′ terminus-truncated mutants of the *GAHSP40* promoter linked to the luciferase reporter gene. The transfected cells were either kept in DMEM medium or heat shocked (42°C, 6 h) before luciferase assay. As shown in Figure 4, heat shock induced more than a 25-fold expression compared to basal level in the cells transfected with the pGL3b-1557. Until the deletion to −284, all the constructs responded to heat shock (approximately 10–15-fold compared to basal level). The luciferase activity of pGL3b-184 was dramatically decreased in basal and heat shock state. These results showed that the region of −284 to −184 bp from the initiation start site responded to heat shock treatment.

### Identification of the Nuclear Factors Involved in Heat Shock Response

In order to identify the proteins that bind to this region (from −284 to −184), Gel shift assay was performed with $^{32}$P-labeled oligonucleotides and nuclear extracts from cultured

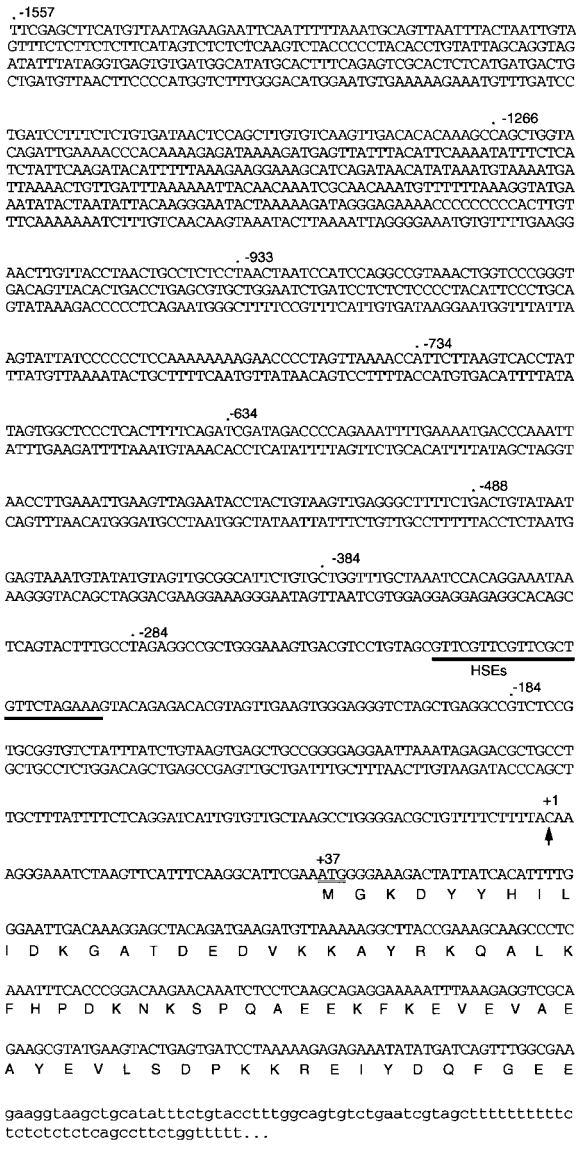


**Fig. 1.** Nucleotide sequence of the 5′ upstream region of the *GAHSP40* gene. Translation initiation codon (ATG) is indicated by the double underline. Arrowhead indicates the main transcription initiation site, which was denoted as + 1. The intron sequences are indicated by lowercase letters. Dots indicate the deletion points of the constructs used in the reporter assay.

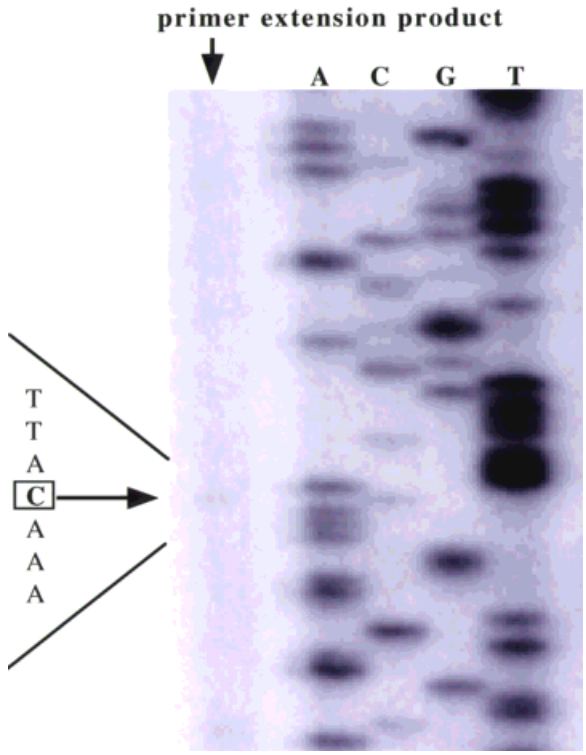


**Fig. 2.** Primer extension analysis of GAHSP40 mRNA. The major primer extension product is shown by an arrow. The DNA sequence of the GAHSP40 is shown on the left, and the nucleotide corresponding to the primer extension product is boxed.

NIH3T3 cells. (The probes used were −284 to −258, −257 to −225, and −224 to −185.) The positive bands were detected only by using the probe −257 to −225 (data not shown). According to database analysis, we noticed that there is a complex of HSEs (GTTCGTTCGTTCGCTG-TTCTAGAAA) in this region. We used −257 to −225 probe for Gel shift assay. Specific DNA–protein complexes were detected (Fig. 5). The specificity of these complexes was confirmed by competition, using excess amounts of unlabeled HSE-probe (lane 5), or with unrelated sequences (lane 6). The factors bound to HSE are

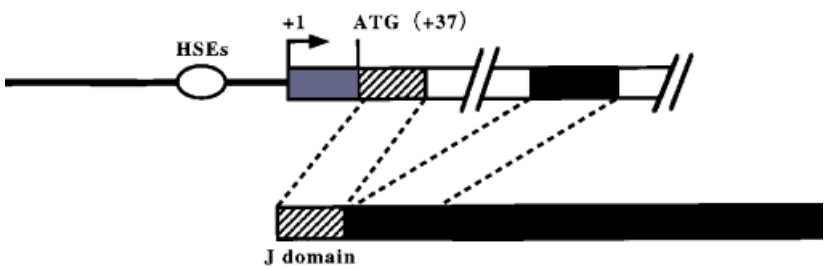


**Fig. 3.** Schematic structures of genomic and cDNA clones of GAHSP40. Filled boxes represent coding sequences. Open boxes represent noncoding sequences. Shaded boxes represent J domain motifs.

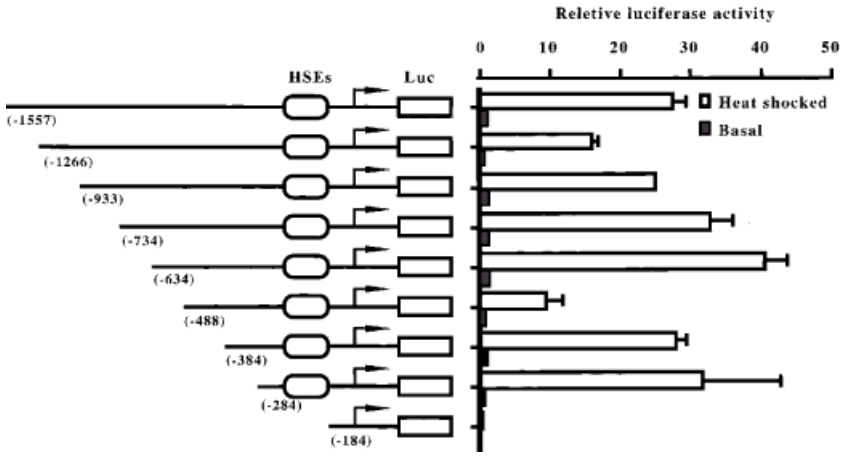


**Fig. 4.** Reporter assays on 5′ upstream region of *GAHSP40* gene. A 0.2 μg of wild type or 5′ terminus-truncated mutant *GAHSP40* promoter-firefly luciferase reporter constructs was transiently transfected into NIH3T3 cells. At 32 h after transfection, cells were either kept in DMEM medium or heat shocked (42°C, 6 h). Results were correlated with renilla luciferase activity that came from co-transfected pRL-TK and expressed as relative luciferase activity. The results are the mean and SD of six transfections from two separate experiments. The error bars indicate the SEM.

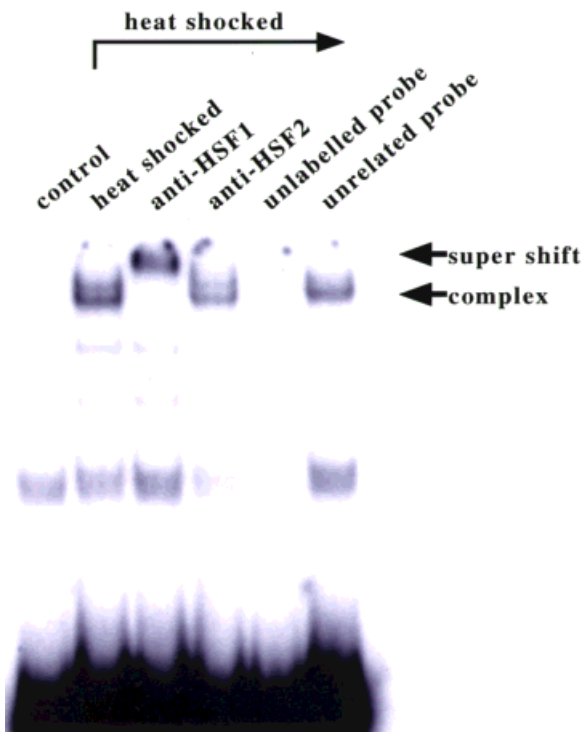


**Fig. 5.** Gel mobility assay. Nuclear extracts from NIH3T3 cells were incubated with $^{32}$P-labeled HSE-probe correspond to −222 to −257 of wild-type GAHSP40 promoter sequence. **Lane 1**, extract from control cells; **lanes 2–6**, extract from heat shocked (42°C, 1 h) cells; pre-incubated with anti-HSF1 antibody; lane 4, pre-incubated with anti-HSF2 antibody; lane 5, 100-fold molar excess of unlabeled probe was added; lane 6, 100-fold molar excess of unrelated competitor (consensus SP1 binding sequence) was added.

known as HSF. To establish whether the DNA binding activity corresponds to HSF1, an antibody super-shift experiment was performed. As shown in Figure 5, supershifted band was detected only with the anti-HSF1 antibody (lane 3), not with anti-HSF2 antibody (lane 4). These results suggest that HSF is concerned with the expression of *GAHSP40* gene.

### Effects of Mutations in HSE and Its Related Sequences on Promoter Activity of the *GAHSP40* Gene

To examine the role of these HSEs in *GAHSP40* promoter activity more precisely, site-directed mutations were introduced into the region from −284 to −184 bp of the *GAHSP40* promoter (Fig. 6). While other mutations did not significantly change the heat responsiveness, two mutations in HSE1 and HSE2 (pGL3-m2 in Fig. 6) decreased both basal and heat induced transcription. These results strongly suggest that the HSE1 and HSE2 located in the *GAHSP40* promoter are the *cis*-responsive elements for heat-induced transcription of *GAHSP40*.

## DISCUSSION

We have cloned the 5′ region of the *GAHSP40* gene. Although several mammalians HSP40 cDNA have been reported, there exists no analysis of 5′ region of transcriptional regulation except human HSP40. Human HSP40 has TATA box, which lies 30 bp upstream of

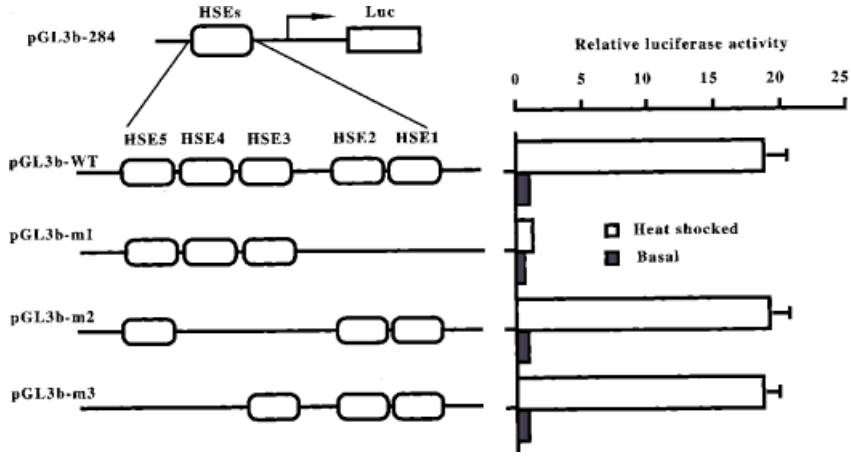


**Fig. 6.** Effects of mutations on promoter activity. A 0.2 μg of wild type or site-directed mutant *GAHSP40* −284 promoter constructs were transiently transfected into NIH3T3 cells. Transfected cells were kept in DMEM or heat shocked (42°C, 6 h). Luciferase activity was normalized and expressed as described in Figure 4. The results are the mean and SD of six separate transfections from two experiments. The SEM are indicated by error bar.

initiation start site. In contrast, *GAHSP40* has no TATA box. Human *HSP40* has GC-box and CAAT box, but *GAHSP40* has neither GC-box nor CAAT box.

Promoter analysis using deletion mutants defined that heat inducibility depends on the sequences from −284 to −184 position from major transcription start site. According to database analysis, we noticed that there are five 5-bp HSE motifs in this area. The protein factor bound to the HSE motifs is shown to be a HSF1 by gel mobility supershift analysis. In the case of human *HSP40*, GC-box and CAAT box had little effect to basal and heat inducible promoter activity. HSEs were both basic and heat inducible elements for human *HSP40*. This correlates to our *GAHSP40*. Both basal and heat inducible elements were laid in the HSEs. The difference between human *HSP40* and *GAHSP40* is that in human HSEs the first three 5 bp units are essential for promoter activity, whereas in *GAHSP40* last two 5 bp units are essential. HSEs in human *HSP40* is GGAAGGTTCTGGAGGGGGCTGGCGGGCTCTGGAAGCTTCC. HSEs in *GAHSP40* is GTTCGTTCGTTCGCTGTTCTAGAAA. By introducing mutation to *HSP70* HSEs, it has been shown that possibly the trimeric structure of proteins bind to HSEs [Cunniff et al., 1991]. Heat shock elements are best described as contiguous arrays of variable numbers of the 5 bp-sequence nGAAn arranged in alternating orientation. At least two nGAAn units are needed for high affinity binding of heat shock factor in vitro [Sorger, 1991]. In our *GAHSP40* HSEs 3′ two 5 bp motifs are essential for transcription (Fig. 6). Taken together, these data show that different sequence HSEs in different promoters work as heat inducible *cis*-elements. In our *GAHSP40*, HSEs works not only as a heat-inducible *cis*-element but also works as a basal *cis*-element.

Although in many HSP promoter analyses such as human HSP40, HSP70 or HSP105, duration of heat shock treatments are 1 h (42°C), 6 h (42°C) are needed to induce *GAHSP40* promoter activity. This may come from the fact that the duration of mRNA expression of GAHSP40 is shorter than other HSPs [Hasegawa et al., 2000].

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