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Dissection of the regulatory mechanism of a heat-shock responsive promoter in Haloarchaea: a new paradigm for general transcription factor directed archaeal gene regulation

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ABSTRACT

Multiple general transcription factors (GTFs), TBP and TFB, are present in many haloarchaea, and are deemed to accomplish global gene regulation. However, details and the role of GTF-directed transcriptional regulation in stress response are still not clear. Here, we report a comprehensive investigation of the regulatory mechanism of a heat-induced gene (*hsp*5) from *Halobacterium salinarum*. We demonstrated by mutation analysis that the sequences 5′ and 3′ to the core elements (TATA box and BRE) of the *hsp*5 promoter (P_{hsp5}) did not significantly affect the basal and heat-induced gene expression, as long as the transcription initiation site was not altered. Moreover, the BRE and TATA box of P_{hsp5} were sufficient to render a nonheat-responsive promoter heat-inducible, in both *Haloferax volcanii* and *Halobacterium* sp. NRC-1. DNA–protein interactions revealed that two heat-inducible GTFs, TFB2 from *H. volcanii* and TFBb from *Halobacterium* sp. NRC-1, could specifically bind to P_{hsp5} likely in a temperature-dependent manner. Taken together, the heat-responsiveness of P_{hsp5} was mainly ascribed to the core promoter elements that were efficiently recognized by specific heat-induced GTFs at elevated temperature, thus providing a new paradigm for GTF-directed gene regulation in the domain of Archaea.

INTRODUCTION

Archaea are prokaryotic microorganisms similar to bacteria in many aspects of morphology and metabolism, but are more closely related to eukarya in the genetic information processing system (1,2). The archaeal basal transcription machinery is fundamentally related to the core components of the eukaryotic RNA polymerase (RNAP) II apparatus, possessing a multi-subunit RNAP and two general transcription factors (GTFs). These GTFs, termed TBP and TFB, are homologues of the eukaryal TATA-box binding protein and transcription factor IIB (TFIIB), respectively (3,4). In the process of transcription initiation, TBP first recognizes and binds to the TATA box, resulting in bending of DNA at the promoter region. Then TFB binds to the TBP–DNA complex, making sequence-specific contact with the BRE (TFB recognition element) upstream of the TATA box. This contact directs RNAP to the promoter, thus specifically initiating transcription at an initiator sequence located about 25 bp downstream of the TATA box (5).

Intriguingly, although the archaeal transcription apparatus is eukaryotic-like, many putative transcription regulators encoded by archaea are homologous to those in bacteria (6). Several instances of negative control of archaeal transcription by such regulators have been described. The metal-dependent repressor 1 (MDR1) from *Archaeoglobus fulgidus* (7) and LrpA from *Pyrococcus furiosus* (8), were found to bind to the operator sequences overlapping the transcription start sites, whereas the Lrs14 from *Sulfolobus solfataricus* (9,10) and TrmB from *Thermococcus litoralis* (11) bind to the sites overlapping the BRE/TATA elements. Thus, these regulators could inhibit transcription initiation through occlusion of RNAP or TBP–TFB recruitment. On the other hand, there are fewer known mechanisms of positive control of archaeal transcription. GvpE, resembling eukaryal basic leucine-zipper protein, has been identified as an activator in the gas vesicle synthesis in haloarchaea (12,13), but the exact mechanism has yet to be elucidated. One of the best

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characterized archaeal transcriptional activators is Ptr2 from *Methanococcus jannaschii*. It could bind to the sequences upstream of the core promoter elements of ferredoxin A (*fdx*A) and rubredoxin 2 (*rb*2) genes, and activate transcription through direct recruitment of TBP to these promoters (14). It should be mentioned that multiple TBPs and TFBs are present in several archaea, including *Halobacterium* sp. NRC-1 (15–18). This raises another possibility that particular TBP–TFB combinations may recognize different promoters and therefore regulate different genes (19). Recently, microarray-based studies have provided evidence that certain GTFs (TBPs/TFBs) interact with specific groups of promoters and are likely involved in global gene regulation (20), and TBPd and TFBa co-regulate, either directly or indirectly, a subset of genes that account for over 10% of the *Halobacterium* sp. NRC-1 genome (21).

Heat-shock response is a widespread physiological phenomenon in all three domains of life and an attractive process for investigation of gene expression regulation. Current genome projects have identified numerous heat-shock proteins in archaea, such as HSP70 (DnaK), HSP60 (GroEL), HSP40 (DnaJ), GrpE and many small heat-shock proteins (sHSP) (18,22,23), but no homologues of eukaryotic-type heat-shock transcription factors (HSF) or heat-shock response elements (HSE) have been identified. To date, only a few studies on heat-shock response have been reported in the domain of Archaea. Among the thermophilic archaea, it has been proposed that the Phr from *P. furiosus* (24,25) and HSR1 from *A. fulgidus* (26) might specifically bind to the promoters of some heat-shock genes under optimal growth temperature, and release from them in response to heat shock. Intriguingly, one of the two TFB-related genes in *P. furiosus* is transcriptionally heat-inducible, implying it may be involved in heat-shock regulation (27). For extremely halophilic archaea, Daniels and co-workers have studied a heat-responsive promoter of the chaperonin-containing Tcp-1 gene (*cct*1) in *H. volcanii*, and revealed that the 5′-CGAA-3′ element upstream of the *cct*1 TATA box and other two sites downstream of the TATA box are necessary for both basal and heat-shock transcription (28,29). *Halobacterium volcanii* possesses multiple genes encoding TBP and TFB proteins, among which the *tfb*2 gene was transcriptionally induced during heat shock at 60°C (30), suggesting that TFB-modulated heat-shock response might exist in haloarchaea. Noteworthily, knock-out of *tbp*D and/or *tfb*A genes in *Halobacterium* sp. NRC-1 downregulates many genes including two heat-shock genes, *hsp*1 and *cct*A (21).

In this study, we report a comprehensive investigation of transcriptional control of the *hsp*5 gene that encodes a sHSP in *Halobacterium*. Using in-depth genetic and biochemical approaches, we demonstrated, for the first time, that alternative GTFs, rather than bacterial-type regulators, specifically modulated the heat-shock inducibility of the *hsp*5 promoter in both *H. volcanii* and *Halobacterium* cells. Therefore, our results establish a new paradigm of GTF-modulated transcriptional regulation in the domain of Archaea.

MATERIALS AND METHODS

Strains, plasmids and primers

Escherichia coli JM109 was used as a host for the cloning experiments and *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) for over expression of recombinant proteins. All *E. coli* strains were grown in Luria–Bertani (LB) medium at 37°C (31). When needed, ampicillin and kanamycin were added to a concentration of 100 and 50 μg/ml, respectively. Unless otherwise noted, *H. salinarum* CGMCC 1.1959, *Halobacterium* sp. NRC-1 and *H. volcanii* DS70 (32) were cultivated at 37°C in CM medium (per liter, 7.5 g Bacto casamino acids, 10 g yeast extract, 3.0 g trisodium citrate, 200 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2.0 g KCl, 50 mg $FeSO_4 \cdot 4H_2O$ and 0.36 mg $MnCl_2 \cdot 4H_2O$, pH 7.2). When required, mevinolin was added to a concentration of 5 or 10 μg/ml for *H. volcanii* or *Halobacterium* sp. NRC-1, respectively. The plasmid pNP22 (33) was used as the source for *bga*H gene, while the *H. volcanii*–*E. coli* shuttle vector pWL102 (34) was used for constructing the *bga*H reporter module. The primers used in this study are listed in Table 1.

Cloning the *hsp*5 gene from *H. salinarum* CGMCC 1.1959

Using the sequence information of the *hsp*5 gene (VNG_6201G, in GenBank AE004438) of *Halobacterium* sp. NRC-1 (18), primers hspF82 and hsp5R were designed to amplify the corresponding gene of *H. salinarum* CGMCC 1.1959 and its promoter region. The hspF82 primer located 101 bp upstream of the *hsp*5 start codon, while hsp5R was complementary to an 18 bp DNA region in the 3′ terminus of the *hsp*5 open reading frame (ORF). The resulting PCR product was ligated into the vector pUCm-T (Sangon, China) and sequenced.

Constructs used for transformation of haloarchaea and reporter gene analysis

For analysis of P_{hsp5} activity *in vivo*, we used a plasmid-based transcriptional reporter system as described previously (33,35). The P_{hsp5} region was amplified by PCR using primers hspF82 and hspRNdeI, with CGMCC 1.1959 genomic DNA as template. The primer hspRNdeI was complementary to a DNA region including the first three codons of the *hsp*5 gene. This PCR product was purified and cleaved with NdeI and ligated to the NdeI/NcoI-digested *bga*H fragment derived from plasmid pNP22. The resulting NdeI-fused fragment was used as a template to amplify the P_{hsp5}-*bga*H fragment using primer hspF82 and bgaHRNcoI, which is complementary to the 3′-terminal sequence of *bga*H. Then, the PCR product was cloned into the pWL102 at the BamHI and NcoI sites. The resulting plasmid, named pL82, was used for constructing the 5′ flanking deletion mutants of P_{hsp5}, named pL52, pL42, pL37 and pL32, using forward primers hspF52, hspF42, hspF37 and hspF32, respectively. The 3′ flanking deletion mutant (Mdel) was constructed in a similar way to pL82, except that the forward primer hspF37 and reverse primer hspR10 were used to acquire the 3′ flanking deleted-P_{hsp5} fragment. In order to generate site-specific mutants,

Table 1. Primers used in this study

Name	Sequence (5′-3′)[a]
hspF82	CTAGGATCCCCACGCCACACCAATGTA
hsp5R	TCAGGCCTGGACCTCGAT
hspRNdeI	ACTCCATATGAAGGGGCATGGCTAATCA
hspF52	GCGGGATCCTATTTGTGTTTTGATAGAAAATTTTTTA
hspF42	GCGGGATCCTTGATAGAAAATTTTTTA
hspF37	GCGGGATCCAGAAAATTTTTTACGAAATGCG
hspF32	GCGGGATCCATTTTTTACGAAATGCGGAC
hspF24	GCGGGATCCCGAAATGCGGACATAGTTT
hsppF	ACTGTTCGAAACCGGTCG
hsppR	TGGGCCTCGATATCGTCG
bgaHpF	TCGGGAAGTTCGGTATGA
bgaHpR	AGGTCGGCAACGCTCTCA
7SF	CCAACGTGGAAGCCTCGTC
7SR	GGTGGTCCGCTGCTCACTTC
bgaHRNcoI	ACACCATGGTCACTCGGACGCGAGTCC
hsp5seq	GAACAGTCGGCTCGGGAATC
bgaHseq	AGCCTCGGCCATCTGACTGA
FM1	GCGGGATCCAGAAAATTTTTTA**AT**AAATGCGG
FM2	GCGGGATCCAGAAAATTTTTTACG**GG**ATGCGGAC
FM3	GCGGGATCCAGAAAATTTTTTACGAA**GCAT**GGACATAGTT
FM4	GCGGGATCCAGAAAATTTTTTACGAAATGC**TT**ACATAGTTTT
FM5	GCGGGATCCAGAAAATTTTTTACGAAATGCGG**CA**ATAGTTTTGG
FM6	GCGGGATCCAGAAAATTTTTTACGAAATGCGGAC**CG**AGTTTTGGC
FM7	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACAT**CT**TTTTTTAGGGAGTCAAGG
FM8	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACATAG**GGCC**GGCTGGAGTC
FM9	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACATAGTTTT**TTAG**GGAGTCAAGG
FM10	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACATAGTTTTGGCT**TTCT**TCAATTTGAT
FNM10	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACATAGTTTTGGCTG**TCT**TCAAGGTGAT
FM11	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACATAGTTTTGGCTGGAG**GACC**GGTGATTA
FM12	GCGGGATCCAGAAAATTTTTTACGAAATGCGGACATAGTTTTGGCTGGAGTCAA**TT**TGATTAG
hspR10	ACTCATATGCCTTGACTCCAGCCAAAA
bopF	GCGGGATCCTCGTAGAGTTACACACATATCC
hBbopF	GCGGGATCCAGAAAAAGTTACACACATATCCT
hTbopF	GCGGGATCCTCGTAGTTTTTTACACATATCCTCGTTA
hBTbopF	GCGGGATCCAGAAAATTTTTTACACATATCCTCGTTAGGT
bBhspF	GCGGGATCCTCGTAGTTTTTTACGAAATGC
bThspF	GCGGGATCCAGAAAAAGTTACACGAAATGCGGACATA
bBThspF	GCGGGATCCTCGTAGAGTTACACGAAATGCGGACATAGT
boppF	GTTAGACCTCGCGTTGCTCGTT
boppR	ACAGCAGCGTCTCGATGT
tfbBF	GGAGGATCCAGTGACAGTACAATCAGAACATACAG
tfbBR	ATAAAGCTTTCAGGCGGCTGCTTCGGT
tfbGF	GGAGGATCCACACGGTCCACCCGCCAG
tfbGR	ATAAAGCTTTCAGCCGTGAATACCCAT
tfb2F	GGAGGATCCAGCGACACGATAACCACC
tfb2R	ATAAAGCTTTTACGCGAGCAGGGTGCC

[a]Restriction sites are underlined; the substituted nucleotides in primers FM1 to FM12 are indicated by bold characters.

specific forward primers carrying the desired mutated nucleotides (FM1-FM12, Table 1), and the reverse primer bgaHRNcoI were used to amplify the P_{hsp5}-*bga*H fusion fragments from pL37. The resulting PCR products were inserted into pWL102 to generate the desired constructs.

Similarly, the P_{bop}–*bga*H fusion was generated by PCR amplification using the primers bopF and bgaHRNcoI with the plasmid pNP22 as the template. To generate P_{bop}-P_{hsp5} chimeras (bBhsp, bThsp and bBThsp), primers containing the BRE or/and TATA box sequence of P_{bop} (Table 1) were used with plasmid pL37 as the PCR template. The P_{hsp5}-P_{bop} chimeras hBbop, hTbop and hBThsp were acquired by PCR amplification using primers containing the BRE or/and TATA box sequence of P_{hsp5} (Table 1), and using plasmid pNP22 as template. The PCR products of the promoter chimera-*bga*H fusion were cloned into pWL102 at BamHI and NcoI sites. The fidelity of PCR-amplified products in these recombinant plasmids was confirmed by DNA sequencing. *H. volcanii* DS70 and/or *Halobacterium* sp. NRC-1 cells were transformed with plasmid DNA isolated from *E. coli* JM109 as described by Cline *et al.* (36).

Isolation of RNA from cells under heat shock

Cells of *Halobacterium* sp. NRC-1, *H. salinarum* CGMCC 1.1959 or *H. volcanii* were grown at 37°C until mid-logarithmic growth phase, and then shifted to elevated temperatures (45, 48, 55 or 58°C) for heat shock for 15 min. The heat-shocked cells (5 ml) were immediately collected for RNA extraction using TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions, with cells remaining at 37°C as the controls.

Northern blot and primer extension analyses

Activities of all the promoters in this study were measured by northern blot analysis. For monitoring the gene expression of *hsp*5, *bga*H and *bop*, the *hsp*5 probe (228 bp), *bga*H probe (340 bp) and *bop* probe (341 bp) were amplified with primer pairs hsppF/hsppR, bgaHpF/bgaHpR and boppF/boppR (Table 1), respectively. The 7S RNA was monitored as an internal control by a specific probe (110 bp) amplified with the primers 7SF and 7SR (Table 1). All the PCR products used for probes were labeled with [α-^{32}P]-dCTP and subjected to northern blot analysis as described previously (37). The northern hybridization signal of the *hsp*5 or reporter gene (*bga*H) was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA) by scanning the exposed X-ray films, and normalized against the signal of the internal control (7S RNA). Heat-shock induction folds were determined by taking the ratio of the normalized heat-shock to nonshock *hsp*5 or *bga*H signals. Quantification of these transcript levels and heat-shock induction folds were based on the results of two or more independent experiments for each promoter.

To determine the transcriptional start sites of the P_{hsp5}-controlled *hsp*5 in CGMCC 1.1959 and *bga*H reporter gene in *H. volcanii*, the primer hsp5seq hybridizing to 20 nt within the *hsp*5 gene and the primer bgaHseq complementary to a 20 bp DNA region within the *bga*H gene were used. These primers were labeled at the 5′-end with [γ-^{32}P]-ATP and were used for both DNA sequencing and primer extension as previously described (37).

Overexpression and purification of TFBs

The *tfb*B and *tfb*G genes were cloned from *Halobacterium* sp. NRC-1 by PCR with primer pairs tfbBF/tfbBR and tfbGF/tfbGR, respectively, and the *tfb*2 gene was amplified from *H. volcanii* with primers tfb2F and tfb2R (Table 1). All the PCR fragments were sequenced and cloned into the expression vector pET28a at the BamHI/HindIII sites. The recombinant plasmids were then introduced into *E. coli* BL21 (DE3). The *E. coli* recombinants were cultured until mid-logarithmic phase and then induced with 1 mM IPTG for an additional 4 h. All the histidine-tagged proteins were purified by a Ni-NTA agarose column (Novagen) according to the manufacturer's instructions. The eluted solution containing TFB was identified by SDS–PAGE and subsequently pooled and dialyzed against buffer A [50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 10% glycerol, 0.1 mM $ZnCl_2$, 50 mM $MgCl_2$, 2M KCl, and 0.2 g/l of PMSF] and subsequently concentrated by ultrafiltration using an Amicon Ultra-15 centrifugal filter device with 10 kDa molecular-weight cutoff (Millipore, Bedford, MA, USA). The concentrations of purified proteins were determined by using the BCA™ protein assay kit (Pierce, Rockford, IL, USA).

Determination of the interaction between P_{hsp5} and TFBs by EMSA

In order to test the specificity of DNA binding of TFB2, TFBb and TFBg, the following DNA fragments were prepared for EMSA: FW (−37 to +10 region of the wild-type P_{hsp5}), FM (BRE and TATA box of P_{hsp5} in FW were replaced by corresponding parts of P_{bop}) and FD (BRE and TATA box of P_{hsp5} in FW were deleted) were generated by PCR, using [γ-^{32}P]-ATP-labeled primer pairs hspF37/hspR10, bBThspF/hspR10 and hspF24/hspR10 (Table 1), respectively. These [γ-^{32}P]-ATP-labeled PCR products were purified with UNIQ-10 Column (Sangon, China). Interaction between P_{hsp5} and TFBs was performed as described by Ken and Hackett (38) with minor modifications. Briefly, TFBs (0–4 μM) were incubated with 20 fmol ^{32}P-labeled DNA in a 20 μl reaction mixture containing 0.5 M NaCl, 25 mM EDTA and 3 μg poly (dI/dC) at 37 or 50°C for 30 min. The resulting complexes were run on a 5% polyacrylamide gel (acrylamide/bisacrylamide weight ratio of 60 : 1) in 100 mM sodium phosphate buffer (pH 6.0, preheated to 37 or 50°C). The gels were electrophoresed at 3 V/cm for 5–6 h.

RESULTS

Cloning and transcriptional analysis of *hsp*5 gene in *H. salinarum*

It has been reported that the *hsp*5 is one of the most highly upregulated genes under heat shock in *Halobacterium* sp. NRC-1 (39,40). To determine whether the corresponding gene was also present and heat shock-inducible in some other *Halobacterium* strains, we have cloned the *hsp*5 gene and its promoter region from the genome of *H. salinarum* CGMCC 1.1959. Interestingly, pairwise sequence comparisons showed that *hsp*5 of CGMCC 1.1959 exhibited 100% identity with that of *Halobacterium* sp. NRC-1. Moreover, when the CGMCC 1.1959 cells were grown to the mid-logarithmic phase at 37°C and then shifted to elevated temperatures (45, 48, 55 or 58°C) for 15 min, northern blotting clearly revealed that the *hsp*5 transcripts increased upon temperature rising (up to ~12-fold at 58°C), exhibiting a typical pattern of heat-shock response (Figure 1A).

The transcription initiation site of *hsp*5 was then demonstrated by primer extension. Under both normal growth temperature and heat-shock conditions, the *hsp*5 transcripts were initiated from the same residue (G) located 19 bp upstream of the ATG start codon (Figures 1B and 2A). Further analysis of the *hsp*5 promoter (P_{hsp5}) sequence identified a typical TATA box (−31 TTTTTTA −25) located 25 bp upstream of the transcription initiation site, and a putative BRE (−37 AGAAAA −32) immediately upstream of the TATA box (Figure 2A). Interestingly, just 2 bp upstream of these putative core promoter elements, there was the stop codon (−41 TGA −39) of the upstream gene. To ascertain if any regulatory elements exist adjacent the BRE/TATA box, we defined the DNA sequence from −82 to +19 as the full-length promoter region (Figure 2A) for investigation of expression regulation.

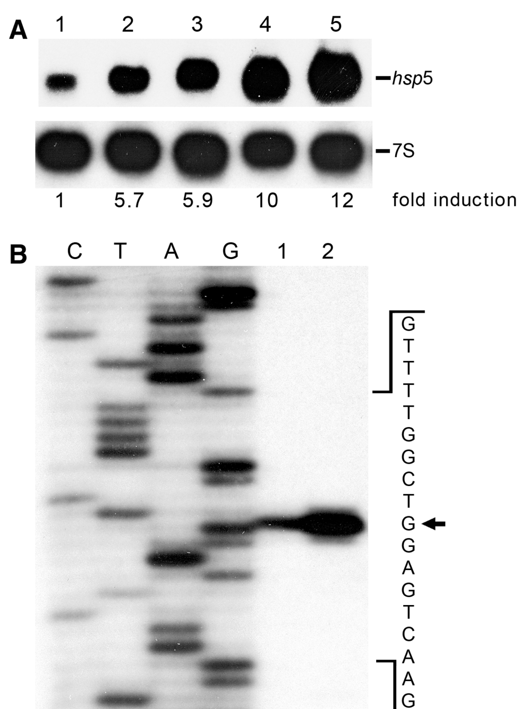

Figure 1. Transcriptional induction of the *hsp*5 gene in *H. salinarum* CGMCC 1.1959. (**A**) Northern blot analysis of heat-induced gene expression of *hsp*5. Total RNAs (10 μg) were isolated from mid-logarithmic phase cells of CGMCC 1.1959 under normal growth temperature (37°C) (lane 1) and heat-shock conditions at 45, 48, 55 or 58°C for 15 min, respectively (lanes 2–5). The internal control (7S RNA, 305 nt) is provided separately due to its similar size with the *hsp*5 transcripts (~370 nt). The heat-shock induction folds are indicated and calculated as described in Materials and methods section. (**B**) Mapping of the transcriptional start site of *hsp*5 by primer extension. The relevant sequence is shown on the right. The transcriptional start site is indicated with an arrow. Lanes CTAG, standard dideoxynucleotide sequencing reactions to size the mapping signals. RNAs (5 μg) isolated from cells under normal growth (37°C, lane 1) or heat shock (58°C for 15 min, lane 2) conditions were used as the templates for primer extension analysis.

In vivo analysis of P_{hsp5}-controlled transcription under heat shock

In order to establish a well-defined *in vivo* system to dissect the regulation mechanism of P_{hsp5}, the β-galactosidase gene (*bga*H) from *Haloferax lucentense* (previously *Haloferax alicantei*) (33,41) was placed immediately downstream of P_{hsp5} region, and cloned into the shuttle vector pWL102. The resulting vector, named pL82 (with full-length P_{hsp5}, Figure 2A), was introduced into *H. volcanii* DS70, a widely used strain that lacks detectable *bga*H transcripts as well as β-galactosidase activity (32,41). Since the BgaH enzyme was likely unstable at high temperatures (data not shown), and the *bga*H expression could only be detected on mRNA levels in the case of weak promoters (33), hence the activities of P_{hsp5} and its derived promoters were evaluated by a direct assay of the *bga*H transcripts with northern blot analysis. First, a time course induction of P_{hsp5}-controlled *bga*H transcription was investigated under both 48 and 58°C. The peak for transcription induction occurred at 58°C for 15 min (Figure 2B), resembling that of *hsp*5 in the wild-type strain (Figure 1A). Therefore, we selected the treatment of 58°C for 15 min as a standard heat-shock stress in all the following experiments.

To confirm whether the transcription initiation site was altered by fusing the reporter gene to P_{hsp5}, primer extension analysis was performed on cellular RNA extracted from the *H. volcanii* transformants harboring pL82 under both 37°C and 58°C. As shown in Figure 2C, the transcription initiation site from the P_{hsp5}-*bga*H fusion was exactly the same as the native *hsp*5 gene for both basal and heat-shock transcription, demonstrating that the transcription start site controlled by P_{hsp5} was not affected by either the reporter gene or the alternative host strain.

Mapping the 5′ boundary of the P_{hsp5} by deletion analysis

To determine the minimal region of the promoter P_{hsp5} for both basal and heat-inducible function, we created a set of promoters with different 5′-deletions (from −82 to −32) and the same 3′ terminus (+28 within the *hsp*5 coding region) by PCR amplification (Figures 2A and 3A). The full-length and shortened promoters fused with *bga*H gene were cloned into plasmid pWL102. These constructs, named pL82, pL52, pL42, pL37 and pL32 (Figure 3A), respectively, were introduced into *H. volcanii*. The relative activity of each promoter was tested under both normal growth temperature (37°C) and heat shock (58°C), by measuring the levels of *bga*H transcripts. It was revealed that the full-length promoter in pL82, and 5′ flanking-shortened mutants in pL52, pL42 and pL37 exhibited similar transcription activities, with about 8 to 11-fold upregulation under heat shock (Figure 3), resembling the native promoter in *H. salinarum* CGMCC 1.1959. However, when the 5′-end of P_{hsp5} was shortened to −32 bp where the putative BRE (−37 AGAAAA −32) was deleted, both the basal and heat-induced transcription activities became hardly detectable (Figure 3B). Moreover, the putative TATA box (−31 TTTTTTA −25) was also extremely important. Substituting three of the six nucleotide 'T' with 'G' made the promoter completely inactive (data not shown). These results demonstrated that the 5′ terminus of the functional P_{hsp5} extends to the position −37, which was exactly the 5′ boundary of the core promoter elements, the BRE and TATA box.

Mutational analysis of the sequence downstream of the TATA box in P_{hsp5}

Since the sequence upstream of the BRE and TATA box was not involved in the transcriptional regulation of the P_{hsp5}-controlled genes, we then analyzed to determine whether the downstream sequence accounted for the heat-shock response. PCR-based scanning mutagenesis was performed to alter the targeted nucleotides downstream of the TATA box. The resulting mutants (M1 to M12, and Mdel), based on pL37, were introduced into *H. volcanii*. The transcription efficiency of each mutated promoter was determined by northern blot analysis, and was compared with that of the intact functional promoter in pL37. It was shown that both basal transcription and heat induction (12 ± 4 fold) were not significantly changed for these

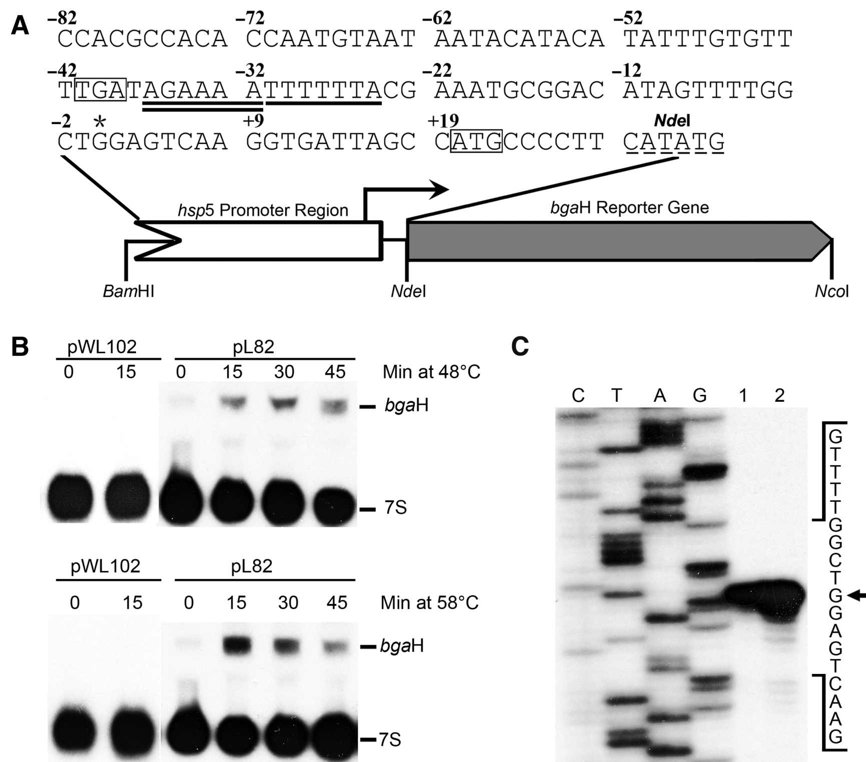

Figure 2. Northern blot and primer extension analyses of P_{hsp5}-*bga*H reporter fusion. (**A**) Schematic representation of the P_{hsp5}-*bga*H fusion of pL82 (not to scale). Putative BRE (double underlined), TATA-box (single underlined), transcription initiation site (asterisk) of P_{hsp5}, translational start codon (ATG, boxed) of *hsp*5 and stop codon (TGA, boxed) of the upstream gene, as well as the NdeI restriction site (dashed) are indicated. (**B**) The *bga*H expression in *H. volcanii* transformant harboring plasmid pWL102 (negative control) or pL82. *H. volcanii* cells were grown to mid-logarithmic phase at 37°C and exposed to heat shock at 48 or 58°C for 0, 15, 30 or 45 min. Hybridization signals corresponding to *bga*H transcripts and 7S RNA (the internal control) are indicated. (**C**) Primer extension analysis of the P_{hsp5}-controlled *bga*H reporter gene (performed as in Figure 1B). The transcriptional start point is indicated with an arrow. RNAs (5 µg) isolated from the *H. volcanii* cells harboring plasmid pL82 under normal growth (37°C, lane 1) or heat-shock (58°C for 15 min, lane 2) conditions were used as the templates for primer extension analysis.

mutated promoters, except for mutant M10 that completely lost transcriptional activity (Figure 4). Further analysis of the mutations within M10 revealed that the transcription initiation point was altered; thereby, the transcription initiation was inhibited. When the transcription initiation residue (G) was restored in the mutant NM10, it acquired the similar basal and heat-inducible transcription activities as the native promoter (Figure 4).

Noteworthily, there was a large inverted repeat (IR) sequence (-5 TGGCT-N4-TCA-N3-TGA-N2-AGCCA + 20), which overlapped the transcription start site (Figure 4), and was likely to be a regulatory element for heat-shock response. However, point mutations of the 5′-half (M9 to M12) or even deletion of the 3′-half (Mdel) of this IR did not significantly affect the transcription activity of P_{hsp5} under either normal growth temperature or heat-shock conditions, implying that this region was not involved in heat-shock regulation in *H. volcanii*. These results suggested that there were likely no heat-shock response elements within the region between the core promoter elements (BRE and TATA box) and the translational start codon.

BRE and TATA box are responsible for both basal and heat-induced transcription of P_{hsp5}

The earlier results suggested that only the core promoter elements, the BRE and TATA box, were the likely candidates for regulation of the detectable basal as well as the strong heat-inducible transcriptional activity of P_{hsp5}. To confirm this, we constructed a set of chimeric promoters by recombining the BRE, TATA box and downstream sequences between the P_{hsp5} and a nonheat-inducible promoter of the bacterio-opsin gene (P_{bop}) (42). These promoters were then ligated with *bga*H ORF and inserted into pWL102. The *bop* promoter (in the construct bopW) consisted of the TATA box and six upstream nucleotides (we assigned it as the putative BRE in this article) as well as the sequences between the TATA box and the *bop* ATG start codon, while the wild-type P_{hsp5} (in construct hspW) used the same sequence as that in pL37. The chimeric promoters bBhsp, bThsp or bBThsp were constructed by substitution of the BRE, TATA box or both elements of the P_{hsp5} with the counterparts of P_{bop}. Similarly, the chimeric promoters hBbop, hTbop or hBTbop were derived from P_{bop}, by substitution with the BRE, TATA box or both elements of the P_{hsp5} (Figure 5A).

Each of these constructs was introduced into *H. volcanii* for transcriptional analysis. Significantly, while P_{hsp5} was heat-inducible (hspW, Figure 5B) and P_{bop} was not (bopW, Figure 5C) as expected, it was clearly shown that when the BRE/TATA elements of P_{bop} were replaced by the counterparts of P_{hsp5}, it rendered the nonheat-inducible

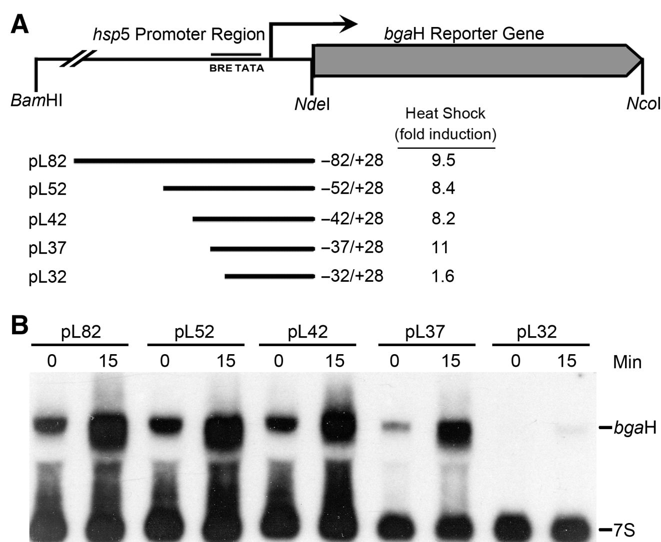

Figure 3. Deletion analysis of the 5′ flanking region of P_{hsp5}. (**A**) Schematic representations (not to scale) of the constructs pL82, pL52, pL42, pL37 and pL32, showing P_{hsp5} and 5′ flanking sequence-shortened promoter mutants (solid lines, $-82/+28$ to $-32/+28$) that were fused with the *bga*H reporter gene (filled gray arrow). The heat-shock induction fold for each promoter determined by northern blot analysis of the *bga*H signals (**B**) is indicated. (B) Northern blot analysis of the *H. volcanii* transformants harboring pL82, pL52, pL42, pL37 and pL32, respectively. Cellular RNAs (10 µg) were extracted from *H. volcanii* transformants under nonshock (zero time point) and heat-shock (58°C for 15 min) conditions. Hybridization signals corresponding to *bga*H transcripts and 7S RNA (the internal control) are pointed.

promoter P_{bop} completely heat-inducible in the resulting chimeric promoter (hBTbop, Figure 5C). On the contrary, if the BRE/TATA elements of the P_{hsp5} were substituted by those of P_{bop}, the resulting chimeric promoter bBThsp lost heat-inducible activity, and the transcript level of the reporter gene became too low to be detectable by northern blotting (Figure 5B). These results reinforced the conclusion that only the BRE and TATA elements of P_{hsp5} accounted for the heat-inducible feature of this promoter in *H. volcanii*.

Interestingly, it is likely that both the BRE and TATA box of the P_{hsp5} are heat responsive elements, since retaining either the TATA box or BRE in the chimeric promoters derived from P_{hsp5} (bBhsp and bThsp, Figure 5B), or substitution with either the BRE or TATA box of P_{hsp5} in the P_{bop}-derived chimeras (hBbop and hTbop, Figure 5C), the resulting chimeric promoters acquired higher transcriptional activities (~2- to 5-fold) at elevated temperature than at normal growth temperature. Thus, both the BRE and TATA box of P_{hsp5} are important for heat-shock response, while their combination provided the most significant contribution to transcriptional activation under heat shock (hspW and hBTbop, Figure 5B and C).

Transcriptional analysis of the chimeric promoter hBTbop in *Halobacterium* sp. NRC-1

Considering that the promoter P_{hsp5} was acquired from *Halobacterium* and our above investigations were mainly

BRE TATA box IR1 * IR2 IR2 IR1

	Sequence	Relative Transcription Activity: Basal (%wt)	Relative Transcription Activity: Heat Shock (fold induction)
pL37	AGAAAATTTTTTACGAAATGCGGACATAGTTTTGGCTGGAGTCAAGGTGATTAGCCATGCCCCTT	100	12
M1	-------------AT--	82	10
M2	---------------GG--	70	13
M3	-----------------GCAT--	63	11
M4	---------------------TT--	72	15
M5	-----------------------CA--	98	10
M6	-------------------------CG--------------------------------------	86	9
M7	---------------------------CT------------------------------------	88	10
M8	-----------------------------GGCC--------------------------------	112	12
M9	---------------------------------TTAG----------------------------	98	13
M10	-------------------------------------TTCT------------------------	0	0
NM10	--------------------------------------TCT------------------------	54	14
M11	---GACC--------------------	123	8
M12	---TT------------------	58	9
Mdel	---TGATTAGCCATGCCCCTT	57	11

Figure 4. Mutagenesis of sequence downstream of the TATA box in P_{hsp5}. The DNA sequence of wild-type P_{hsp5} is given at the top (pL37). BRE (double underlined), TATA-box (single underlined), transcription initiation site (asterisk) and the translational start codon of *hsp*5 (boxed) are indicated. A large IR sequence (IR1 + IR2) is indicated by the opposing arrows. The mutated nucleotides of different mutants (M1–M12) are shown below the wild-type promoter sequence. Dashes in the mutant sequences indicate nucleotides that are identical to the wild-type P_{hsp5}. The deleted sequences are indicated by the dots (Mdel). The basal (37°C) and heat-induced (58°C) transcript levels of *bga*H controlled by these promoters in *H. volcanii* were determined by northern blotting. The basal transcription activities of the mutant promoters are expressed as a percentage of the wild-type activity (set as 100%wt) and the heat-shock induction folds are calculated as described in Materials and methods section.

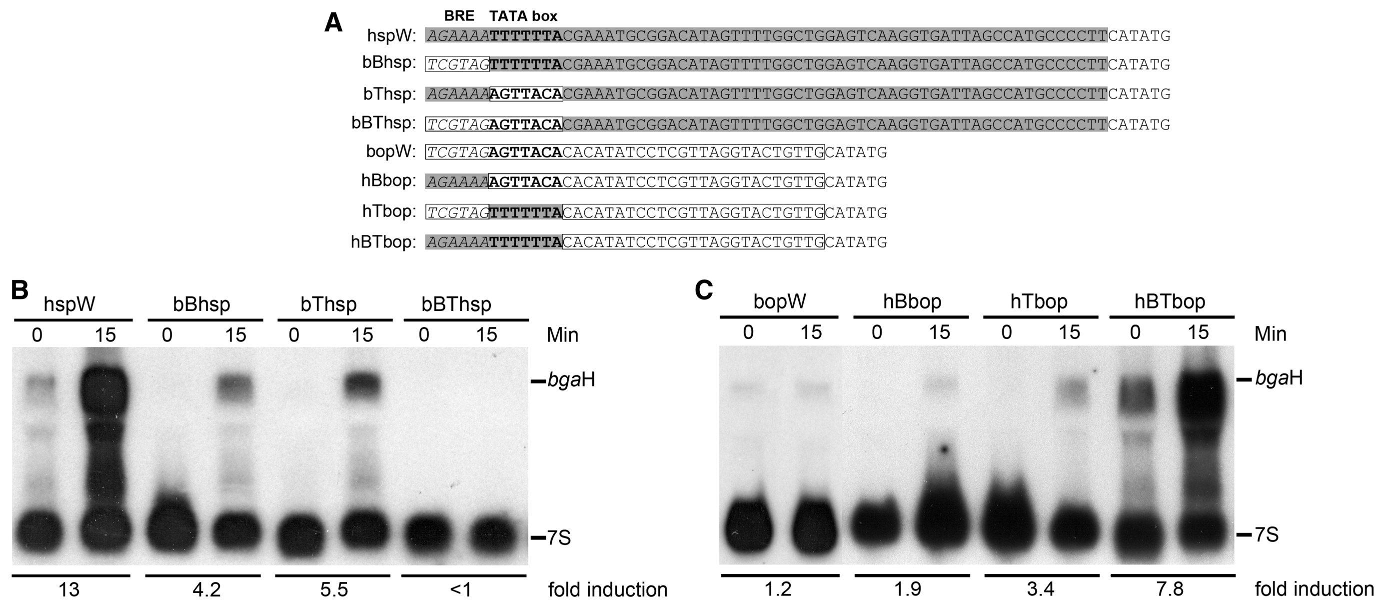

Figure 5. Transcriptional analysis of the promoter chimeras between P_{hsp5} and P_{bop} in *H. volcanii*. (**A**) Sequences of the wild-type P_{hsp5} (hspW), P_{bop} (bopW) and the chimeras between P_{bop} and P_{hsp5}. The sequences from P_{hsp5} are shaded in gray, and the sequences from P_{bop} are boxed. Putative BRE (italic) and TATA-box (bold) elements of P_{hsp5} and P_{bop} are indicated. The sequence of the NdeI restriction site (CATATG), downstream of the promoters, is also presented. (**B**) Northern blot analysis of the *H. volcanii* transformants harboring the pWL-based reporter system with the BRE and/or TATA box of P_{hsp5} substituted by that of P_{bop}. (**C**) Northern blot analysis of the *H. volcanii* transformants harboring pWL-based reporter system with the BRE and/or TATA box of P_{bop} substituted by that of P_{hsp5}. Cellular RNAs (10 µg) were extracted from *H. volcanii* transformants under nonshock (zero time point) and heat-shock (at 58°C for 15 min) conditions. The hybridization signals corresponding to *bga*H transcripts and 7S RNA (the internal control), and the calculated heat-shock induction folds (see Materials and methods section) are indicated.

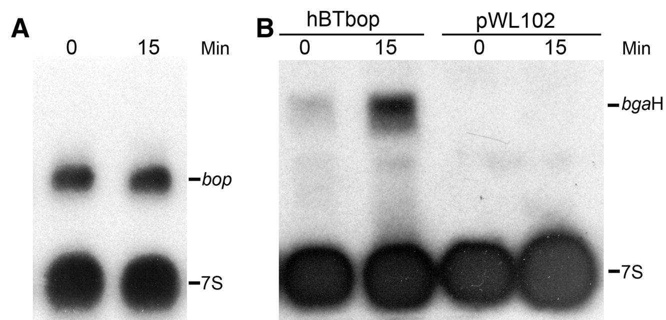

Figure 6. Transcriptional analysis of *bop* gene and the chimeric promoter-controlled *bga*H gene in *Halobacterium* sp. NRC-1. (**A**) Northern blot analysis of the *bop* transcripts under nonshock (zero time point) and heat-shock (58°C for 15 min) conditions. (**B**) Northern blot analysis of the *bga*H expression driven by chimeric promoter hBTbop in *Halobacterium* sp. NRC-1, under nonshock (zero time point) and heat-shock (58°C for 15 min) conditions (left two lanes). The *Halobacterium* sp. NRC-1 harboring vector pWL102 was used as the negative control for the same treatment (right two lanes). The hybridization signals corresponding to the *bop* and *bga*H transcripts, and 7S RNA (the internal control) are indicated.

performed in *Haloferax*, we then further asked whether the conclusion made sense in another model haloarchaeon *Halobacterium* sp. NRC-1, which is phylogenetically closely related to *H. salinarum* CGMCC 1.1959. First, we analyzed the mRNA levels of *bop* in *Halobacterium* sp. NRC-1, and confirmed that the *bop* promoter was not heat-inducible (Figure 6A). Then, the construct hBTbop was introduced into *Halobacterium* sp. NRC-1 and the transcript levels of the reporter gene (*bga*H) under both nonshock and heat-shock conditions were determined. Our results confirmed again that the BRE and TATA box were indeed the determinants of the heat-inducible activity of P_{hsp5}. The chimeric promoter in hBTbop, with the BRE/TATA elements from P_{hsp5} and downstream sequence from P_{bop}, acquired strong heat-inducible transcriptional activity (Figure 6B).

Transcriptional profiling of GTF genes of *Halobacterium* sp. NRC-1 in response to heat shock

Previous investigations have demonstrated that the gene encoding the transcription factor TFB2 in *Haloferax* is upregulated under heat shock (30). In order to determine which GTF genes in *Halobacterium* sp. NRC-1 were responsive to heat-shock stress (if any), we have analyzed our microarray database (39, and unpublished data). As shown in Table 2, the expression level of the six *tbp* genes (*tbp*A-F) were scarcely altered after heat shock, ranging from about −1.05 to 1.25-fold. However, among the seven *tfb* genes (*tfb*A∼G), *tfb*B and *tfb*G were significantly upregulated, with fold changes of about 1.68 and 2.41, respectively. Therefore, it was of interest to determine whether these heat-induced GTFs, TFB2 from *Haloferax* and TFBb or TFBg from *Halobacterium*, were involved in transcriptional regulation of P_{hsp5} by recognition of the promoter elements.

Table 2. DNA microarray analysis of transcriptional changes of the GTF genes under heat shock in *Halobacterium* sp. NRC-1[a]

Gene ID	Gene Name	Fold change values	$\log_2(\times)$ ratio	Standard deviation of $\log_2(\times)$ ratio
5039	*tbp*A	−1.024	−0.033	0.018
5052	*tbp*B	1.253	0.316	0.173
5142	*tbp*C	1.096	0.121	0.178
5163	*tbp*D	−1.050	−0.073	0.306
2243	*tbp*E	1.029	0.036	0.171
6438	*tbp*F	−1.005	−0.008	0.109
2184	*tfb*A	1.197	0.253	0.136
734	*tfb*B	1.681	0.592	0.658
6351	*tfb*C	1.102	0.125	0.306
869	*tfb*D	1.093	0.113	0.325
6389	*tfb*E	1.006	0.008	0.122
315	*tfb*F	−1.074	−0.094	0.201
254	*tfb*G	2.412	1.199	0.450

[a]Microarray data processing and statistical analysis were carried out as previously described (39).

TFBb and TFB2 specifically bind to P_{hsp5} at elevated temperature

To test whether the P_{hsp5} was recognized by the heat-induced general transcription factors, TFB2 from *H. volcanii*, and TFBb and TFBg from *Halobacterium* sp. NRC-1, they were overproduced and purified in *E. coli*, and were subjected to electrophoretic mobility shift assay (EMSA) to determine their interactions with the P_{hsp5} DNA and its mutants (Figure 7). Interestingly, TFB2 could efficiently bind to the wild-type P_{hsp5} (FW), with even higher binding efficiency at 50°C than at 37°C, as more DNA-protein complex and less proportion of free FW DNA appeared at 50°C when same concentration of TFB2 was included in the reaction (Figure 7B). This binding appears to be specific, since interaction between TFB2 and the BRE/TATA-deleted fragment (FD) was not detectable in the same EMSA. A relatively weak interaction between TFB2 and FM (BRE/TATA of P_{bop}) was detectable; however, it only occurred at 50°C when high concentrations of TFB2 (e.g. 4 μM) were available (Figure 7B). These results may help explain the heat-inducibility of P_{hsp5} in *Haloferax*, as the TFB2 was upregulated under heat shock (30), and could efficiently bind to P_{hsp5} at high temperature.

Significantly, when TFBb and TFBg were incubated with the P_{hsp5} DNA (FW) and P_{hsp5}-derived mutants (FM and FD), only TFBb but not TFBg could specifically bind to the P_{hsp5} DNA at the high temperature (50°C), and no detectable interactions were observed for either of the TFBs at the lower temperature (37°C) (Figure 7C and D). Moreover, TFBb and TFBg could not interact with the P_{hsp5}-derived mutants (FM and FD) in EMSA under the same conditions, suggesting that the interaction of TFBb and P_{hsp5} is specific and likely temperature-dependent. These results indicated that TFBb, but not TFBg, might regulate the *hsp*5 gene expression at elevated temperature in *Halobacterium*.

Taken together, our results have established a new paradigm for archaeal gene regulation in response to environmental changes. Under heat shock, a few heat-inducible GTFs, such as TFB2 in *Haloferax* or TFBb in *Halobacterium*, together with the corresponding TBPs, yet to be identified, could immediately modulate a group of downstream target genes, including the small heat-shock gene *hsp*5, to cope with the environmental stress.

DISCUSSION

Multiple GTFs are present in haloarchaea and have been speculated to regulate differential gene expression for years (19), and systems approach has provided supports that the GTFs in *Halobacterium* sp. NRC-1 likely accomplish large-scale regulation of transcription (20,21). However, detailed studies of the role of GTF-directed transcriptional regulation of specific genes in response to environmental signals in archaea are limited. In this article, we demonstrated that the BRE and TATA box of the P_{hsp5} play a critical role in both basal and heat-induced gene expression, which was confirmed by both genetic and biochemical approaches. Therefore, our work has established a new paradigm for TFB–TBP modulated gene regulation in the domain Archaea.

The *hsp*5 gene and its homologs, encoding sHSPs, are present in numerous haloarchaeal genomes including *Halobacterium* sp. NRC-1, *Haloarcula marismortui* and *Haloquadratum walsbyi* (15,17,18). These proteins belong to the Hsp20/α-crystallin family (43), and act as molecular chaperones to protect cellular proteins against irreversible aggregation during stress conditions (44). The *hsp*5 gene is upregulated under heat shock in both *Halobacterium* sp. NRC-1 (39,40) and *H. salinarum* CGMCC 1.1959 (Figure 1A), and the *hsp*5 promoter also exhibited similar heat-inducibility in *H. volcanii* (Figure 2B). Deletion analysis demonstrates that the 5′ boundary of the functional promoter of *hsp*5 is exactly at the position of the putative BRE and TATA box (Figure 3). Therefore, there is no upstream activation sequence (UAS) adjacent the BRE/TATA box in the defined full-length promoter P_{hsp5}. It is noteworthy that there is an IR overlapping the transcription initiation site in P_{hsp5} (Figure 4). This IR resembles the heat-shock regulatory elements usually presented in many bacterial (45–47) and some archaeal heat-shock genes (26). For instance, a conserved palindromic motif, CTAAC-N5-GTTAG, located downstream of the BRE/TATA elements of the promoter P_{hsr1} and $P_{hsp20\text{-}2}$ in *A. fulgidus*, is involved in heat-shock regulation by binding of the heat-shock repressor HSR1 (26). However, the IR in P_{hsp5} was not found to be involved in the P_{hsp5}-controlled heat-shock response in *H. volcanii*, since mutagenesis of the sequences downstream of the TATA box including this IR did not significantly change the promoter activity, as long as the transcription initiation site was not altered (Figure 4). Moreover, replacement with the BRE/TATA box of P_{hsp5}, rendered the nonheat-inducible promoter (P_{bop}) heat-inducible, in both *H. volcanii* and *Halobacterium* sp. NRC-1 (Figures 5 and 6). Therefore, there is also no heat-shock response element downstream of the core promoter elements, and the BRE and TATA box of P_{hsp5} are likely the only

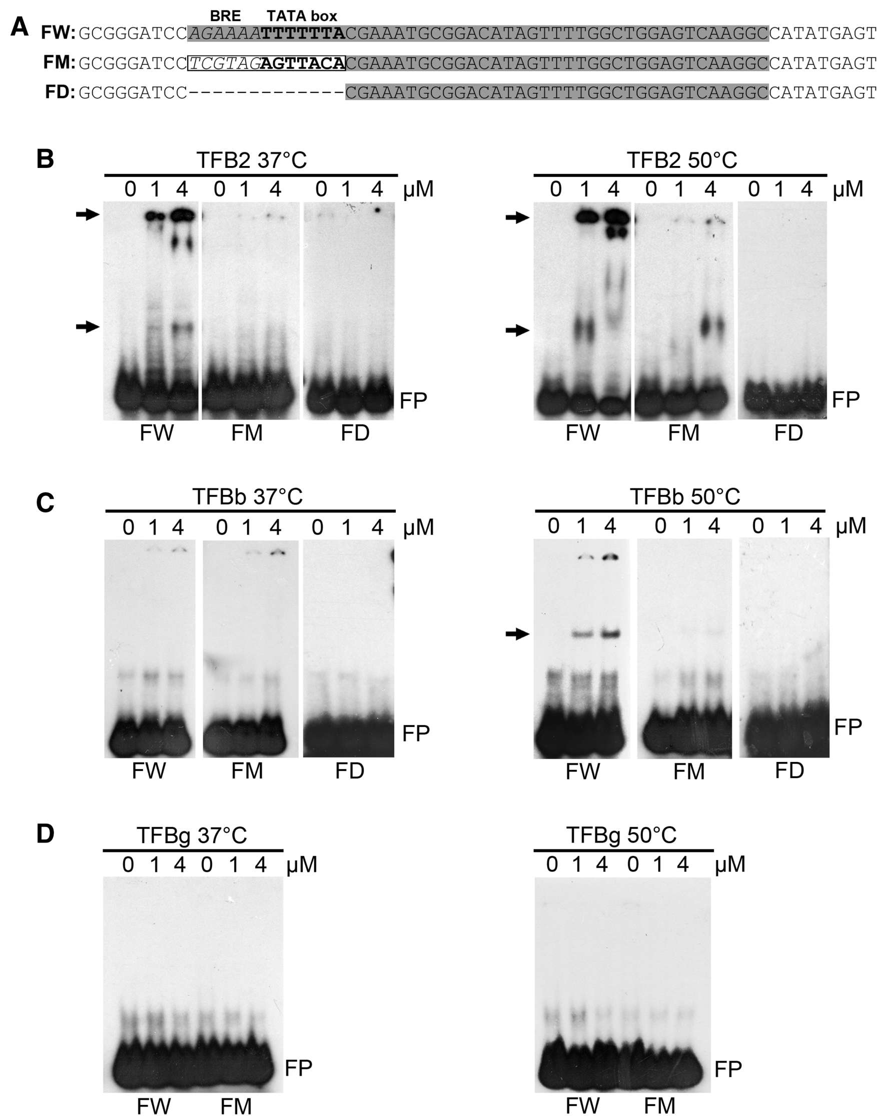

Figure 7. EMSA analysis of interaction between TFBs and double-stranded DNA fragments of wild-type and mutant P_{hsp5}. (**A**) Nucleotide sequences of the fragments used in EMSA. FW, DNA fragment of wild-type P_{hsp5}; FM, BRE/TATA-substituted mutant; FD, BRE/TATA -deleted mutant. The sequences from P_{hsp5} are shaded in gray; the sequences from P_{bop} are boxed; the deleted nucleotides are indicated in dash. Putative BRE (italic) and TATA-box (bold) elements of P_{hsp5} and P_{bop} are indicated. The sequences up- and down-stream of the promoters generated by PCR primers are also presented. (**B–D**) EMSA performed on wild-type (FW) and mutant (FM and FD) P_{hsp5} DNA fragments (20 fmol) with TFB2 (B), TFBb (C) and TFBg (D) at low (37°C) or high (50°C) temperature. The amount of proteins (0–4 μM) in each lane is indicated. The free probes (FP) and DNA–protein complex (arrows) are indicated.

elements accounting for both basal of heat-inducible transcription in these haloarchaea. These results are slightly different from the earlier observations for the P_{cct1} in *H. volcanii*, where the heat-responsiveness of P_{cct1} is mapped to the TATA box and surrounding sequences, including the putative BRE and two downstream sites (29). Nevertheless, it is most likely that the sequences surrounding the TATA box in P_{cct1} are also the contact sites of TFB or TBP; hence both P_{cct1} and P_{hsp5} might use the same mechanism of GTFs directed strategy in response to heat shock.

This novel strategy of gene expression regulation for P_{hsp5} was further supported by direct biochemical evidence that P_{hsp5} was recognized by specific heat-inducible GTFs, TFB2 from *Haloferax*, and TFBb from *Halobacterium* (Figure 7). Our EMSA results indicated that both TFB2 and TFBb were able to recognize the corresponding core promoter without the assistance of TBPs, at least *in vitro* when high concentration of TFBs was supplied (Figure 7B and C). It was observed that the binding efficiency of TFB2 was likely higher than that of TFBb. Since *H. volcanii* has a lower salt optimum than *Halobacterium* strains and both proteins were over expressed in *E. coli*, this different affinity is likely due to the presence of more properly folded molecules of TFB2, compared to TFBb, in the purified samples. The high molecular weight DNA–protein complexes appeared around the loading wells (Figure 7B) are likely the aggregation of sufficient

TFB2/P_{hsp5} complexes, which might occur when the complexes were transferred from the EMSA binding buffer (high salt concentration) to the electrophoresis buffer (low salt concentration). However, the formation of these DNA–protein complexes is obviously due to the specific interaction of TFB2 and the P_{hsp5} DNA but not nonspecific DNA–protein co-aggregation, as such a complex was never generated between TFB2 and the P_{hsp5} mutants in the same EMSA experiments (FM and FD, Figure 7B). Interestingly, although TFBg is also upregulated in *Halobacterium* sp. NRC-1 under heat shock (Table 2), amino acid sequence analysis revealed that TFBb shared more homology with TFB2 than TFBg (TFB2/TFBb, 71%; TFB2/TFBg, 62%). Moreover, microarray data has shown that under low temperature the *tfb*G gene is also upregulated, whereas the expression of *hsp*5 is highly inhibited (39). All these results indicated that TFBb, but not TFBg, selectively modulates the transcription of *hsp*5 and probably other heat-shock genes. A recent study on *Halobacterium* sp. NRC-1 has demonstrated that most of the TFBs, including TFBb, could interact with a single TBP (TBPe) (20), and most TBPs are not significantly upregulated under heat shock (Table 2). Meanwhile, multiple TFBs but only one TBP are found in the genomes of some other haloarchaea, e.g. *H. marismortui* (17) and *Natronomonas pharaonis* (16). Thus, it is likely the expanded family of TFBs plays a much more important role in heat-shock response in these investigated haloarchaea. However, the heat adaptability of TBP in interactions with the TATA box of the heat-shock promoter should not be underestimated. It was observed that the TATA box of P_{hsp5} itself could slightly increase gene expression under heat shock (Figure 5), implying that the corresponding TBP interacts more efficiently with the P_{hsp5} at elevated temperature. This temperature-dependent interaction manner of GTFs with heat-shock promoters was also observed in other archaea, e.g. the TBP and TFB of *Methanosarcina mazeii* were suggested to interact more strongly with stress-gene promoters during heat shock (48). Therefore, it is evident that both TFB and TBP contribute significantly to the upregulation of *hsp*5 under heat shock.

It is noteworthy that specific transcriptional repressor modulated heat-shock response has also been reported recently in some thermophilic archaea, such as *P. furiosus* (24,25) and *A. fulgidus* (26); however, these kinds of heat-shock regulators are still not identified in the extremely halophilic archaea. Interestingly, while many haloarchaea encode multiple TBPs and TFBs (19,20,30), some other archaea only harbor one or two TBPs and TFBs. So it is reasonable that haloarchaea have developed an additional sophisticated strategy of gene transcriptional regulation by selection of alternative TFBs and TBPs, as we have revealed in the *hsp*5 regulation. This regulatory strategy is conceptually similar to the alternative sigma factors directed transcriptional activation of several heat-shock genes in bacteria (49), and is reminiscent of the HSFs stimulated transcription in eukaryotes (50). Notably, haloarchaea flourish in extremely hypersaline environments and are confronted with many environmental stresses, including frequent changes of temperature. Transcriptional regulation of the important genes including those for sHSPs by GTFs, but not other secondary regulators, would help haloarchaeal cells respond quickly to the environmental challenges, and thereby adapt more efficiently to the harsh environments.

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Conflict of interest statement. None declared.

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