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ORIGINAL PAPER

Transcriptional analysis of the *hsp70* gene in a haloarchaeon *Natrinema* sp. J7 under heat and cold stress

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Abstract Although ubiquitous in all haloarchaea, little is known about the transcription and regulation of the haloarchaeal *hsp70*. The purpose of this study is to investigate the transcription of the haloarchaeal *hsp70* gene in *Natrinema* sp. J7 under the temperature and osmotic stress. The *hsp70* gene was found to be both temperature- and osmotic-induced, while the response of *hsp70* to cold shock was stronger than that to heat shock. Western blot analysis corroborated the similar results at the level of Hsp70 protein. Northern blot and primer extension analyses indicated that the *hsp70* was transcribed into a monocistronic transcript and the thermal stress had no effect on the transcription initiation sites choice. The deletion analyses showed that two putative elements, TATA-box (TTTAAAA) and BRE (AGTAAC) located −27 bp upstream of the transcription initiation site played an essential role for the basal transcription of P_{hsp70}. The results suggested that there are some special regulators of *hsp70* gene in *Natrinema* sp. J7.

Keywords *hsp70* · Hsp70 · Stress · Transcription · Haloarchaea

Introduction

Cells have developed a lot of protective mechanisms to deal with the stresses, one of which is the activation of the stress genes. The products of the stress genes, named heat shock proteins (Hsps), accumulate greatly and transiently in response to the stresses. The Hsps are highly conserved both in structure and function, and their induction is generally regulated at the transcription level. Many stress proteins are also called molecular chaperones. These molecular chaperones also play an essential role under normal physiological conditions (review in Macario et al. 1999).

Among the Hsps, the Hsp70 (DnaK) family is a well-studied group of the chaperoning systems. The Hsp70 (DnaK) system is constituted of the proteins Hsp70 (DnaK), Hsp40 (DnaJ), and GrpE in prokaryotes, but in eukaryotes GrpE is replaced by other molecules (Bukau and Horwich 1998). The Hsp70 system is present in all bacterial and eukaryotic organisms. It is surprising that some archaea possess the molecular chaperone machine, but others do not (Macario et al. 2004), while the Hsp70 system occurs in all extreme halophiles with no exception reported till date.

Hsp70 protein is one of the most highly conserved proteins. The regulation of *hsp70* expression has been extensively characterized in domains Bacteria and Eucarya. In *Escherichia coli*, the *dnaK* operon was regulated by sigma32 (Narberhaus 1999; Nonaka et al. 2006). In *Bacillus subtilis*, the control of *dnaK* operon expression is governed by the widespread HrcA/CIRCE system (Zuber and Schumann 1994). While in eukaryotic organisms, the *hsp70* gene expression is transcribed as a monocistron from a typical promoter located immediately upstream of a HSE element (Horowitz and Robinson 2007). Compared with these two domains, very little is known about the molecular mechanisms regulating *hsp70* gene expression in the domain Archaea, especially in haloarchaea (Hickey et al. 2002).

It was demonstrated that the genes in the triad 5′-*grpE-hsp70-hsp40*-3′ in the methanogenic archaeon

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Methanosarcina mazeii S-6 are transcribed individually from a different promoter for each gene, rather than as an operon from a single promoter as in many bacteria (Clarens et al. 1995). Recently, microarray-based studies have provided a global transcriptional profiling of the haloarchaeon *Halobacterium* sp. NRC-1 and demonstrated that the *hsp70* gene was upregulated under heat shock (Coker et al. 2007). The expression of another major heat shock protein gene *cct* was previously identified in *Haloferax volcanii* (Thompson and Daniels 1998). The regulation of small heat-shock protein gene *hsp5* was also discovered in *Halobacterium* sp. NRC-1 (Lu et al. 2008). However, more details about the regulation of heat shock genes need to be elucidated, especially *hsp70* gene.

In haloarchaea, putative promoter sequences were found upstream of the three genes *grpE*, *hsp70*, and *hsp40*, suggesting that the mode of transcription of the three genes is likely to be monocistron as the genes of locus in *M. mazeii* (Macario et al. 2006). In addition, although the three genes are also arranged in the order 5′-*grpE-hsp70-hsp40*-3′, they are not sitting adjacent to each other. One or more predicted genes are scattered in the intergenic region of 5′-*grpE-hsp70*-3′ or 5′-*hsp70-hsp40*-3′. These locus diversity parallels other kinds of variations observed in haloarchaeal genomes, implying that the mode of transcriptional regulation for the genes is different in haloarchaea from that of other archaea (Macario et al. 2006). We have previously cloned a 5.2-kb fragment containing Hsp70 chaperone machine gene locus from a haloarchaeon *Natrinema* sp. J7, which arranged unidirectionally in the order of *grpE*, *hsp70* and *hsp40* (Zhang et al. 2007). The function of the haloarchaeal Hsp70 has been partially characterized, but the transcriptional patterns and the response of *hsp70* gene to thermal stress are still unknown. Whether *hsp70* locus is transcribed in a manner of polycistron or monocistron needs to be elucidated. In this study, we report a detailed investigation of the transcriptional analysis of the haloarchaeal *hsp70* responsive to thermal stress. A transcription initiation site was determined by primer extension. Using a haloarchaeal *bgaH* reporter system, the putative basal transcriptional elements and the 5′ terminus of the *hsp70* promoter were identified. Northern blot and quantitative real-time PCR analyses demonstrated that the *hsp70* promoter is sensitive to both high and low temperatures in *Natrinema* sp. J7.

Materials and methods

Strains, plasmids, and culture conditions

Natrinema sp. J7 strain was grown in 20% (salt, w/v) MGM containing 5 g of peptone, 3 g of yeast extract, 160 g of NaCl, 4.67 g of KCl, 23.33 g of $MgSO_4 \cdot 7H_2O$, and 20 g of $MgCl_2 \cdot 6H_2O$ per liter at 45°C. *Haloferax volcanii* DS52 used as a haloarchaea host was cultivated at 37°C in 18% (salt, w/v) MGM (Woods and Dyall-Smith 1997). 10, 15, or 25% (salt, w/v) MGM were prepared according to Dyall-Smith (2008). *Escherichia coli* strains, DH5α and JM110, were grown aerobically at 37°C in LB broth. The *Hfx. volcanii–E. coli* shuttle plasmid pSY1, containing *bgaH* reading frame, was used for constructing deletion mutants of haloarchaeal *hsp70* promoter in β-galactosidase activity assay (Yang et al. 2003).

RNA purification of haloarchaeal cells under stressors

To investigate the expression of the thermal stress-responsive *hsp70* gene, cells of *Natrinema* sp. J7 were grown at 45°C until mid-logarithmic growth phase, and then shifted to declined (25°C, 37°C) or elevated (60°C) temperatures for cold or heat shock for 1 h. The total RNA of the thermal-shocked cells was immediately isolated and purified by the method of Dyall-Smith (2008). To study the effect of reduced (10%, 15%) or elevated (25%) salt concentrations on *hsp70* gene expression, 20 ml of a *Natrinema* sp. J7 culture ($A_{600} = 0.6–0.8$) in 20% MGM was divided into four culture tubes. A 5 ml of 45°C pre-incubated complex media containing salt concentration of 0, 10, 20, or 30% MGM was mixed with the four aliquots, respectively. After 1 h incubation at 45°C, the total RNA of the salt-stressed cells were immediately isolated.

Northern blot hybridization

RNA samples (20 μg) were electrophoresed on a 1.2% formaldehyde–agarose denaturing gel and then transferred overnight to an N-Hybond nylon membrane, as previously described (Clarens et al. 1995). Hybridization was performed with random primer PCR-based probes prepared by a commercial kit (TaKaRa, China). The template for random probes was amplified with primer pairs KNR/KNF (Table 1) and all the probes were labeled with [α-^{32}P]-dCTP. After washed at high stringency, the membranes were autoradiographed for 24–48 h at −70°C in a light-proof cassette equipped with an intensifying screen.

RT-PCR and quantitative real-time PCR

Five hundred nanograms of total RNA treated with DNase I was used in a PrimeScript RT-PCR system (TaKaRa, China). cDNA was synthesized by reverse transcriptase with random hexamers. Real-time PCR amplification was performed using gene-specific primers (Table 1) with product sizes of approximately 130 bp and was run on a Rotorgene system (Corbett Research, Australia), using the

Table 1 Oligonucleotides used in the study

Name	Sequence(5′–3′)[a]
KF	TGAGGTACC AGATCTATTCAGAACGCCCAGG
KR	TTGAAGCTTGCCATATGGGACGGGTATTGTGC
KF1	GGAAGATCTCGAAGCCGCCGAAAACG
KF2	TTGAGATCTATGCGACGGACGCCGAGGA
KF3	TGCAGATCTAGCGGACGAGTAACACACG
KF4	CCGAGATCTGACGCTGATTTTTACTGC
KF5	CCGAGATCTTAACCTTTAAAACAAAGTG
*bgaH*R	TTGCCATGGTCACTCGGACGCGAGTC
DEFM1	GGTATTGTGCGCACTTTGGGTTACTAGCTGAC
DERM1	CAGCTAGTAACCCAAAGTGCGCACAATACCCGT
DEFM2	TAGCTGACCGGCGAAGCCGCGCGCGTCTGCCG
DERM2	TACGCCGGCAGACGCGCGCGGCTTCGCCGGTCA
DEFM3	CGCGACAGTTGGCTCGCAGCAGCGTCACTCCG
DERM3	CGGAGTGACGCTGCTGCGAGCCAACTGT
KNF	GCAGCGGACAACCAAACCACG
KNR	GGG AAGCTTCTCCTCGTCGTCCTCGTC
PE	ATCGCCGCCTTCCATCACC
KRTF	GCTCGGCGAGTTCCACCTGA
KRTR	TGGTGCCGCTGCCTTTGTC
16sRTF	CCGTGAGGCGTCCTGTTA
16sRTR	ACCTACCGTTGCCCGTTCC

[a] Restriction sites introduced are underlined

SYBR PrimeScript™RT-PCR kit (TaKaRa) according to the manufacturer's instructions. Reaction conditions were 95°C for 2 min (1 time), then 95°C for 30 s, 60°C for 30 s, and 72°C for 10 s (40 times). The PCR efficiency of each primer set was determined with a standard curve using serially diluted cDNA. 16S rRNA was amplified to serve as an internal reference for normalization. The formation of nonspecific products was excluded using the melting curve analysis. Data analysis was conducted with the RotorGene 6.0 software (Corbett Research) calculating C_T values from the intersection of a threshold line with the early exponential interval of the fluorescence curve. Relative expression levels were determined by the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Primer extension experiment

The transcription initiation site of the haloarchaeal *hsp70* gene was determined by primer extension method (Sambrook and Russell 2001). A 19-mer oligonucleotide (primer PE, 5′-ATCGCCGCCT TCCATCACC-3′) complementary to nucleotides 57–75 of the *hsp70* encoding sequence was 5′ end labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. A concentration of 0.1 pmol of this probe was hybridized to 10 mg of total RNA from *Natrinema* sp. J7 cells and was extended at 42°C for 90 min with reverse transcriptase. The primer-extended products were analyzed on a 6% polyacrylamide–7 M urea sequencing gel. The same oligonucleotide served as a primer for a sequencing ladder that was run simultaneously. Sequencing was carried out by the dideoxynucleotide-chain termination method.

Constructs used for transformation of *Hfx. volcanii* and reporter gene analysis

For the analysis of P_{hsp70} activity in vivo, a haloarchaeal β-galactosidase-based reporter system was employed in *Hfx. volcanii* DS52 (Holmes and Dyall-Smith 2000). The 396-bp promoter region of *hsp70* was amplified by PCR using the primer pair KF/KR, with the genomic DNA of *Natrinema* sp. J7 as template. The primer KR contained an *Nde*I site that overlapped the ATG start codon of *bgaH*. This PCR product was digested with *Kpn*I/*Nde*I, and ligated with the *Nde*I/*Nco*I-digested *bgaH* reading frame obtained from plasmid pSY1. The resulting P_{hsp70}-*bgaH* fragment was then inserted into the *Kpn*I/*Nco*I-digested pSY1. The resulting plasmid, named pNK, was used for constructing the P_{hsp70} deletion mutants.

For 5′ flanking deletion, the P_{hsp70} mutants pNKF1 through pNKF5 were amplified with pNK as template using primers KF1 through KF5 together with *bgaH*R, respectively. For three putative TATA-boxes deletion, the P_{hsp70} mutants were amplified by the overlapping PCR using complementary primers including the deletion mutations, and the pNK as template. The P_{hsp70} mutants pNKT1 through pNKT3 were amplified by the respective mutation primer DEFM1, DEFM2, or DEFM3 plus KF for the first PCR, and the mutation primer DERM1, DERM2, or DERM3 plus KR for the second PCR. Two PCR products contained the deletion mutations in the overlapping end. The full-size fragments were finally amplified by KF/KR primers.

Each of these amplified fragments was purified by gel electrophoresis, and the correct mutation and fusion were determined by DNA sequencing analysis. All the primers used in this study are listed in Table 1.

Transformation of *Hfx. volcanii* and β-galactosidase activity assay

Prior to the transformation of *Hfx. volcanii* DS52, each construct was passaged through the *E. coli* dam$^-$ strain JM110 to avoid a halobacterial restriction barrier. Transformation was done as described by Dyall-Smith (2008). Transformants were selected on agar plates containing 0.2 μg ml^{-1} novobiocin.

The BgaH activity of the various *Hfx. volcanii* P_{hsp70}-*bgaH* transformants was measured by ONPG (*ortho*-nitro-

phenol β-D-galactoside) assay as described by Dyall-Smith (2008). The specific activity (SA) was calculated as $SA = 1{,}000 \times (\Delta A/\Delta t)/(\text{culture volume} \times OD_{600})$ (culture volume referred to volume, in ml, of culture added to the assay).

Western blotting analysis

Cells were grown at 45°C until mid-logarithmic growth phase, and then shifted to declined (25°C, 37°C) or elevated (60°C) temperatures for cold or heat shock for 15, 30, 60, 120, 180, or 240 min, respectively. Western blotting was performed by transferring the amount of 10-μg total proteins, after electrophoresis on 10% acrylamide gels, to a nitrocellulose membrane using the Transblot cell from BioRad, and then incubating the membrane with polyclonal anti-NnmHsp70 rabbit antiserum diluted 1:10,000. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Immu-Club) was used in 1:5,000 dilution as a secondary antibody. The immunoblots were developed with SuperSignal West Pico Substrate (PIERCE, USA).

Results

Expression of *hsp70* at different temperatures

In order to determine whether *hsp70* is expressed in response to stress, we determined the level of the *hsp70* mRNA in *Natrinema* sp. J7 cells incubated at 25, 37, 45, and 60°C by northern blot analysis. The control growth temperature was set to be 45°C (OTG optimal temperature for growth). Cells of *Natrinema* sp. J7 were grown at 45°C until mid-logarithmic growth phase, and then shifted to declined (25°C, 37°C) or elevated (60°C) temperatures for cold or heat shock for 1 h. As shown in Fig. 1b, a 2.1 k-nt mRNA transcript was identified predominantly, which could correspond by length to the complete *hsp70* gene. This demonstrated that the *hsp70* gene is transcribed in a manner of monocistron in *Natrinema* sp. J7. The *hsp70* was expressed under different temperatures. Cold shock at 25 and 37°C both caused accumulation of *hsp70* mRNA (Fig. 1b). The cold induction showed a higher level at 25°C than at 37°C, while the heat shock of *hsp70* is not distinct. Thus, Hsp70 may play a role in optimal and stress conditions.

Quantitative real-time RT-PCR assays were performed to accurately investigate the extent to which the transcription of *hsp70* is modulated by temperature stress. cDNA was synthesized by reverse transcriptase with random hexamers and the PCR efficiency of each primer set was determined with a standard curve using serially diluted cDNA. As shown in Fig. 2a, a cold shock response of the

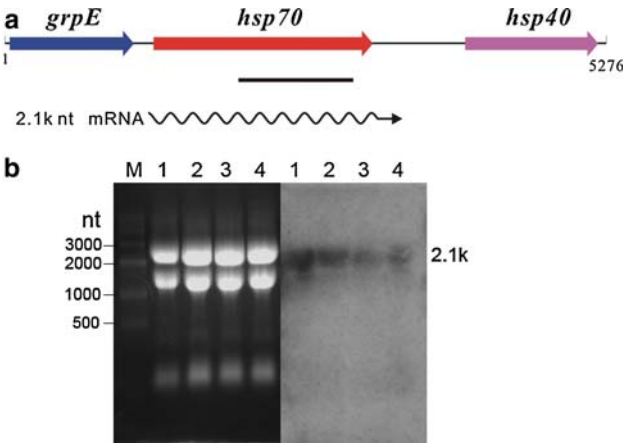

Fig. 1 Transcriptional analysis of *hsp70* gene under thermal stress. **a** Transcriptional maps of *hsp70* gene locus. The probes targeting the *hsp70* gene are shown by *solid lines*. The direction and size of the transcript are indicated by *wave lines*. **b** Northern blot analysis of *hsp70* gene under thermal stress. Total RNAs (20 μg per lane) were isolated from cultures under optimal growth temperature (45°C, *lane 3*) or thermal stress conditions at 25°C, 37°C, or 60°C (*lane 1*, *2*, *4*) for 1 h, respectively. The left half shows the gel stained with ethidium bromide, while the right half shows the corresponding Northern blot. The size of RNA Marker (*lane M*) is shown to the left and the approximate transcript size (in kilonucleotide) is indicated on the right

hsp70 in *Natrinema* sp. J7 was observed when cells were transferred from 45°C to declined temperatures. And the maximum level (about 5.5-fold) of transcripts occurred at 25°C. These results corresponded to what were observed in northern blot analysis. Furthermore, the *hsp70* expression was induced by a shift in reduced salt concentrations (Fig. 2b). A 9.7-fold increment of *hsp70* transcripts under 10% salt concentration was detected, which suggested an important role of Hsp70 protein under low osmotic stress. It was consistent with our previous finding that the Hsp70 protein of *Natrinema* sp. J7 remained low ATPase activity even without NaCl and KCl (Zhang et al. 2007).

Expression levels of intracellular Hsp70 in *Natrinema* sp. J7 under thermal stress

In addition to mRNA levels, the protein levels of Hsp70 in *Natrinema* sp. J7 under thermal stress were detected by western blot analysis. Cells grown at 45°C until mid-logarithmic growth phase were shifted to declined (25°C, 37°C) or elevated (60°C) temperatures for cold or heat shock for 15, 30, 60, 120, 180, or 240 min, respectively. Cytosol isolated from seven aliquots taken at various times during thermal shock was analyzed. As shown in Fig. 3, the immunoblots revealed a basal expression at normal physiological conditions and an increasing signal with time under both cold and heat shock before 1 h. The peak of Hsp70 levels under 25°C shock was reached at 60 min and

began to decline after 180 min, suggesting a rapid and transient cold shock induction pattern, whereas under 60°C a persistent increase till 240 min was observed.

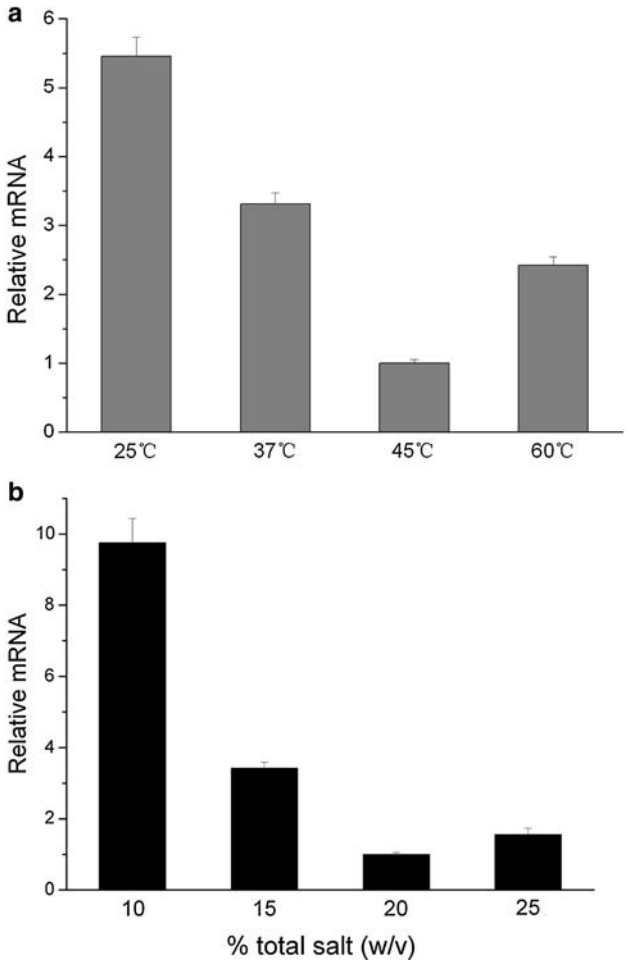

Fig. 2 Quantitative analysis of *Natrinema* sp. J7 *hsp70* gene expression induced by thermal shock (**a**) and saline stress (**b**). Total RNAs were isolated from *Natrinema* sp. J7 following exposure to various extremes of osmolarity and temperature for 1 h, and analyzed by quantitative real-time RT-PCR assays. The relative mRNA level for each gene is normalized with the 16S ribosomal RNA and the signal intensity for optimal growth conditions (45°C or 20% salt concentration) was defined as 1.0. Different temperatures (**a**) or salt concentrations (**b**) are indicated under respective histograms. In each case, the mean value from three independent experiments (each in triplicate) is shown

Identification of the transcription initiation site of *hsp70* gene

Next, we turn to the mechanisms underlying the stress expression of the *hsp70* gene, especially at low temperatures. In order to accurately identify the transcriptional start sites from which the *hsp70* transcription takes place at 25, 37, 45, and 60°C, the primer extension analysis with RNA from *Natrinema* sp. J7 cells incubated at various temperatures for 1 h was performed. A single and identical 5′-end was detected in the *hsp70* mRNA, independent of the incubation temperature. The 5′-end was mapped 4 bp upstream of the translational start codon (Fig. 4). Further analysis of the putative promoter region of *hsp70* revealed a canonical TATA-box (TTTAAAA) located −27 bp upstream of transcription initiation site. A purine-rich region as BRE (AGTAAC) was also detected immediately upstream of the TATA-box.

In vivo analysis of P_{hsp70} promoter activity in *Haloferax volcanii*

The haloarchaeal *β*-galactosidase gene derived from *Haloferax lucentense* was employed as reporter gene and the 396 bp full length of *hsp70* promoter region was inserted immediately upstream of *bgaH* reading frame into the *Hfx. volcanii–E.coli* shuttle plasmid pSY1 (Yang et al. 2003). The resulting plasmid, named pNK, was used for P_{hsp70} promoter activity analysis throughout growth phase in *Hfx. volcanii* DS52.

As shown in Fig. 5, under optimal growth conditions, the *Hfx. volcanii* transformants harboring the P_{hsp70}-*bgaH* construct exhibited large amount of BgaH activities, the peak of which was achieved in samples derived from the exponential growth phase. A slight reduction of the BgaH activity was observed during the stationary growth phase. Compared with the native promoter P_{bgaH} (pMLH32, SA = 45.4 ± 3 on the exponential growth phase), P_{hsp70} showed the high promoter activity (about 7.4 fold), which indicated a constitutive basal expression level of *hsp70* in *Natrinema* sp. J7 under normal physiological conditions.

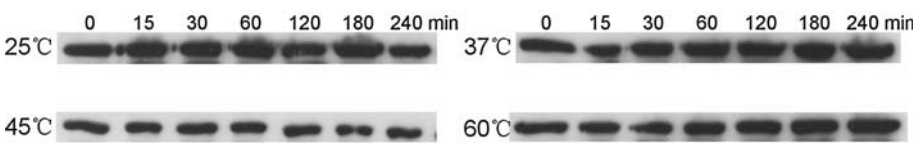

Fig. 3 Western blot analysis of expression levels of intracellular Hsp70 in *Natrinema* sp. J7 under thermal stress. Cytosol of *Natrinema* sp. J7 was prepared at various points of time during thermal stress, subjected to Western blotting, and detected using a 1:10,000 dilution of polyclonal antibodies against NnmHsp70

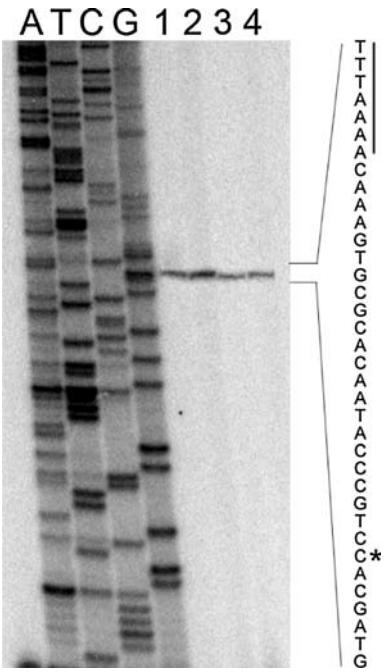

Fig. 4 Identification of the transcription initiation site for *Natrinema* sp. J7 *hsp70* by primer extension analysis. Total RNAs isolated from cultures under optimal growth temperature (45°C, *lane 3*) or thermal stress conditions at 25°C, 37°C or 60°C (*lane 1*, *2*, *4*, respectively) for 1 h were subjected to primer extension analysis by primer PE. The sequence of partial P_{hsp70} was indicated at the right. The *asterisk* denotes the transcription initiation site of *hsp70* (C located 4 bp upstream of the ATG) and the putative TATA-box is indicated by a *solid line*. Lanes ATCG, standard dideoxynucleotide sequencing reactions

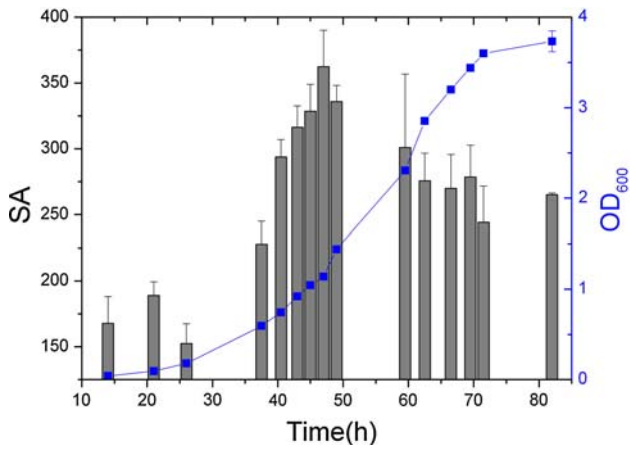

Fig. 5 Growth curves and β-galactosidase activities determined for P_{hsp70}-*bgaH* transformants. Growth curves were shown by dot lines and the specific β-galactosidase activities were indicated by histograms. The left *Y*-axis refers to the β-galactosidase activities, while the right *Y*-axis indicates the optical density of P_{hsp70}-*bgaH* transformants. β-galactosidase activities were determined by the ONPG assay and calculated as SA. More details were shown in "Materials and methods"

Identification of the 5′ terminus of P_{hsp70} by series deletion mutagenesis

To identify the 5′ boundary of the *hsp70* promoter region containing full functional activity, a series of sequential 5′ deletion mutants with the 3′ terminus corresponding to position +4 in the *hsp70* coding region were generated by PCR amplifications (Fig. 6). The P_{hsp70} mutants pNKF1 through pNKF5 were introduced into *Hfx. volcanii* DS52 and the promoter activity of each deletion mutant in vivo was measured by β-galactosidase assay. As shown in Fig. 6, with the deletion of 5′ terminus in the *hsp70* promoter region, the promoter activities of P_{hsp70} deletion mutants were decreased gradually. Specially, when 5′ flanking regions were deleted to position −34 where dinucleotide transversion in the putative BRE was generated (AGTAAC to CTTAAC), only 5.8% of full-size promoter activity remained. It seemed that the putative BRE was an essential transcriptional element and breaking the BRE sequences dramatically affected the activity of P_{hsp70}. Furthermore, because of the similar level of promoter activity between pNK and the deletion mutant pNKF1, it was believed that the promoter region of pNKF1 extending from position −324 to +4 was the minimal segment sufficient for transcription.

Identification of the basal transcriptional elements by deletion analysis

To explore the regulatory mechanism for *hsp70*, we have searched for regulatory elements of heat shock stress up- and downstream of the putative core elements. However, neither the bacterium-type regulatory elements such as CIRCE or ROSE (Narberhaus 1999; Narberhaus et al. 1998; Segal and Ron 1996; Yuan and Wong 1995; Zuber and Schumann 1994), nor the eucaryote-type HSE (Fernandes et al. 1994; Sorger 1991) was found in the *hsp70* promoter of *Natrinema* sp. J7, which suggested a novel regulatory mode distinctive from those found in the other two domains. Furthermore, two conserved regulatory elements (5′-CGAA-3′ and 5′-CAAaC-3′) of the heat shock *cct* family member genes in *Hfx. volcanii* (Kuo et al. 1997; Thompson and Daniels 1998) were aligned with P_{hsp70}, neither of them was detectable. Intriguingly, using the approach described by Brenneis et al. (2007), two AT-rich elements more like the consensus sequence of TATA-box in haloarchaea were identified (Fig. 7a). The two AT-rich elements, named TATA-box2 or TATA-box3, respectively, were located upstream of the putative core transcription element (i.e., TATA-box1). In order to elucidate whether these conserved domains played an important role in the basal and induced transcription of *hsp70* gene under thermal stress, five P_{hsp70} mutants containing distinct

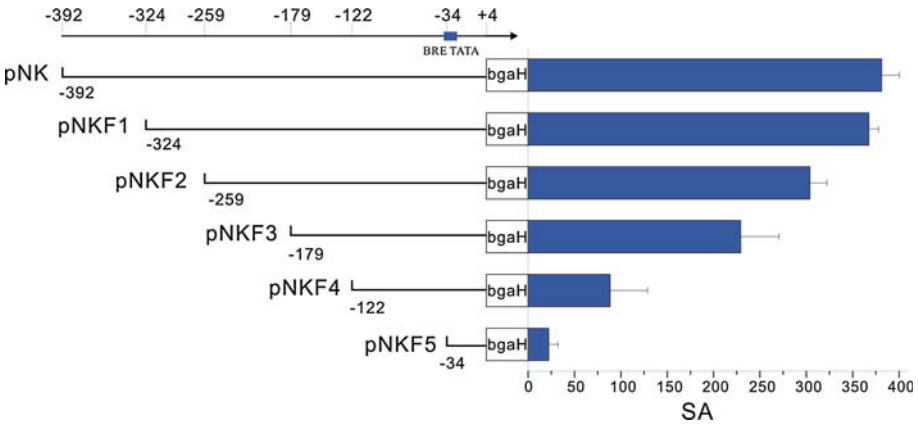

Fig. 6 Identification of the 5′ terminus of P_{hsp70} by series deletion mutagenesis. The P_{hsp70} mutants pNKF1 through pNKF5 were amplified using the respective primers KF1 through KF5 together with *bgaH*R and pNK as template. The promoter activity of each deletion mutant was measured by *β*-galactosidase assays. The size of respective promoter region in deletion mutants was indicated by the *solid lines* immediately upstream of *bgaH* reading frame. The putative BRE and TATA-boxes were shown by the *solid box*

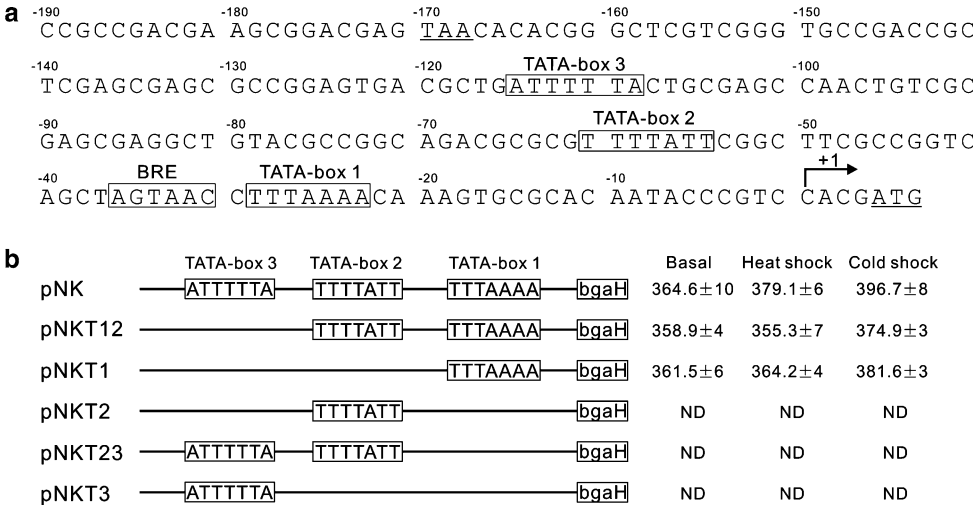

Fig. 7 Deletion mutagenesis of three putative TATA-boxes in P_{hsp70}. **a** Nucleotide sequence of the *hsp70* promoter. Three putative TATA-boxes (*boxed*), the putative BRE (*boxed*), transcription initiation site (*arrow*), and the translation start codon (ATG, *underlined*) are indicated. The positions in digit refer to the transcription initiation site. **b** Schematic representation of three putative TATA-boxes deletion in P_{hsp70}. The BgaH activities of each deletion mutant under basal or thermal shock conditions are given in SA. *ND* no detectable BgaH activities

TATA-box deletion mutagenesis were generated by the overlapping PCR method. The promoter activity of each deletion mutant under normal and thermal stress conditions was measured by *β*-galactosidase assay. As shown in Fig. 7b, the effects of thermal stress on BgaH activity of P_{hsp70}-*bgaH* transformants were measured. Surprisingly, only a slightly activated promoter activity was observed under both heat and cold shock conditions, no matter the duration of exposure (data not shown). When TATA-box1 was not deleted, no matter whether TATA-box2 or TATA-box3 existed or not, the transcription efficiency under normal or thermal shock conditions did not decrease dramatically. On the contrary, once TATA-box1 was deleted in the promoter region, neither basal transcription nor the promoter activity under thermal shock conditions was detectable, regardless of the combination of TATA-box2 and TATA-box3. These results suggested an essential role of TATA-box1 which was indeed responsible for the basal transcription of P_{hsp70}. Both the putative TATA-box2 and TATA-box3 have no effect on the basal and regulatory expression of *hsp70* in *Hfx. volcanii*. The reason may be that a putative regulatory activator functioning under thermal stress in *Natrinema* sp. J7 was absent from *Hfx. volcanii*.

Discussion

Haloarchaea investigated till now showed that all have the stress gene *hsp70* that encodes the molecular chaperone Hsp70, which is always present in bacteria and eukaryotes (Macario et al. 2004). In this study, we investigated the expression of *hsp70* gene from halophilic archaea *Natrinema* sp. J7 under the different conditions. A predominant 2.1 k-nt mRNA was identified by Northern blot analysis (Fig. 1b). The result indicated that the mode of the transcription of the *hsp70* gene is monocistronic in *Natrinema* sp. J7, because *hsp70* is located in the middle of gene locus immediately adjacent to the other two heat shock genes,

grpE and *hsp40*, and no other sizes of hybridization bands were detected, it is postulated that *grpE* and *hsp40* are transcribed as a monocistronic mRNA, respectively. This mode was the same as that found in another archaeon *Methanosarcina mazeii* and demonstrated an eucaryal-type transcription in a bacterial-like gene locus (Clarens et al. 1995).

Under both optimal growth conditions and thermal stress temperatures, a same major extension product was identified by primer extension analysis and there were no effects of thermal stress on initiation site choice. The *hsp70* transcripts were initiated from nucleotide C located 4 bp upstream of the translational start site of *hsp70* gene (Fig. 4), which was the same as previously we had performed by 5′ RLM-RACE PCR analysis. The identification of the transcription initiation site indicated a leadless transcript of *hsp70* and no Shine–Dalgarno (SD) motif was involved in the translation, which was a predominant mode for translation initiation in haloarchaea (Brenneis et al. 2007).

Northern blot and quantitative real-time RT-PCR analyses revealed that both thermal and osmotic stress could stimulate the up-regulation of *hsp70*, implying that the *hsp70* was part of the general stress response of *Natrinema* sp. J7. The transcription of *hsp70* gene was dramatically induced under cold shock stress, the maximum level (about 5.5-fold) of which occurred at 25°C, whereas only the weakly activated transcripts were found in cells exposed to heat shock stress (Fig. 2a). Western blot analysis corroborated that the peak of Hsp70 levels under 25°C shock was reached at 60 min and began to decline after 180 min (Fig. 3), suggesting a rapid and transient cold shock induction pattern.

Recently, the existence of the heat shock genes (especially the *hsp70* gene) involved in cold shock response has been documented in several organisms, including *E. coli*, yeast, soybean, *Arabidopsis*, spinach, fish, and so on (Aguilera et al. 2007; Anderson et al. 1994; Cabane et al. 1993; Lelivelt and Kawula 1995; Li et al. 1999; Place et al. 2004; Sung et al. 2001; Zhang and Guy 2006). However, so far none of them has been found in Archaea. To our knowledge, this is the first report that an archaeal heat shock gene could response to cold shock stress. Yet the reasons why Hsp70s are induced at low temperature remain unclear. It was reported that low temperatures could affect the structural and functional properties of cellular components (Cavicchioli et al. 2000). A decrease in membrane fluidity and diffusion rates, alterations in molecular topology or modifications in enzyme kinetics occur as a consequence of low temperatures. Upregulation of Hsp70s under cold shock stress may be related to an increasing demand for molecular chaperone function at low temperature, the same way as operating under heat shock stress. It was reported that Hsc66, an Hsp70 homolog in *E. coli*, is induced under cold shock and could function as a cold shock molecular chaperone (Lelivelt and Kawula 1995), which indicates a versatile role of *hsp70*(*dnaK*) family under thermal stress. The predicted amino acid sequence of the functional ATPase domain in the Hsp70 of *Natrinema* sp. J7 has 62% similarity and 41% identity to the *E. coli* cold-inducible heat shock protein Hsc66, while the substrate-binding domain and the carboxyl-terminal domain are less conserved. Probably they perform the similar molecular chaperone functions under cold shock stress using the conserved ATPase domains, whereas bind distinct unfolding components in respective cells by less-conserved substrate-binding domains.

Using a haloarchaeal β-galactosidase reporter system, the promoter activity of P_{hsp70} was analyzed. With respect to growth-phase-dependent transcription analysis of P_{hsp70} in *Hfx. volcanii*, the transformants revealed a high constitutive activity during the exponential growth phase. These results were unexpected since the regulatory promoter P_{hsp70} appeared to have strong activity even at normal physiological conditions. Deletion mutagenesis demonstrated that the 5′ flanking region extending from position −324 to +4 was sufficient for transcription (Fig. 6). Moreover, the putative BRE identified that even dinucleotide transversion in it could dramatically influence the activity of P_{hsp70}, and the putative TATA-box1 was identified as the basal transcriptional core element for *hsp70*.

Surprisingly, no stress shock response of the *hsp70* promoter from *Natrinema* sp. J7 was observed in *Hfx. volcanii* transformants. We investigated the *bgaH* expression of *Hfx. volcanii* transformants on mRNA levels under thermal stress, however, no difference among the amount of *bgaH* transcripts under various temperatures was detected (data not shown). These results suggested that the regulatory factor of *hsp70* in *Natrinema* sp. J7 is distinctive from that of *Hfx. volcanii.* Neither of the two putative TATA-boxes (i.e., TATA-box 2 and TATA box 3), located upstream of the putative core transcription element, was responsible for the basal and regulatory expression of the *hsp70* in *Hfx. volcanii* transformants. However, it should not be eliminated since TATA-box 2 and TATA box 3 could be involved in the regulation of the transcription in *Natrinema* sp. J7 under thermal or osmotic stress because *Hfx. volcanii* and *Natrinema* sp. J7 may not possess the same regulatory mechanism.

To find out the regulatory elements responsible for cold shock stress, a comparison of the haloarchaeal *hsp70* promoter region with those of related cold shock genes was performed. Intriguingly, a cold-inducible box (i.e., Y-box element in *csp* of *E.coli*), which was conserved in the promoters of cold-induced genes and was responsible for cold shock gene regulation, was identified (Jones et al.

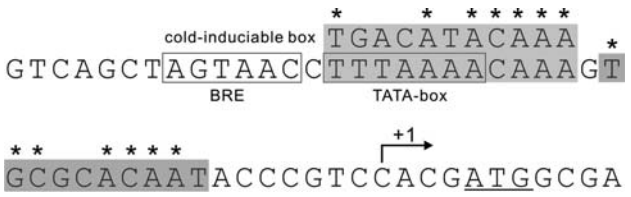

Fig. 8 Alignment of the cold-inducible box from *Methanococcoides burtonii* with *hsp70* promoter region. The TATA-box and BRE elements are boxed and the transcription initiation site is indicated by *arrow*. The two putative cold-inducible boxes are shaded in *gray*. The identical nucleotides are indicated by *asterisks*

1992; La teana et al. 1991; Phadtare et al. 1999). Especially, it was reported that an archaeal RNA helicase gene (*deaD*) from *Methanococcoides burtonii* was cold inducible within which presented an 11 bp sequence that closely matched the cold-inducible box (Lim et al. 2000). The alignment of this 11 bp cold-inducible box with the haloarchaeal *hsp70* promoter region was shown in Fig. 8. Two putative cold-inducible boxes were detected in *hsp70* promoter region, the more conserved one of which was overlapping with the TATA-box. It is hypothesized that a transcriptional activator (or dimer) could bind to this two regulatory elements and enhance or recruit the general transcription factor TBP to interact with TATA-box under cold shock stress. However, more details need to be elucidated.

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