A Novel Archaeal Transcriptional Regulator of Heat Shock Response*

Received for publication, September 10, 2002, and in revised form, October 9, 2002
Published, JBC Papers in Press, October 14, 2002, DOI 10.1074/jbc.M209250200

Gudrun Vierke, Afra Engelmann, Carina Hebbeln, and Michael Thomm‡
From the Institut für Allgemeine Mikrobiologie, Am Botanischen Garten 1-9, 24118 Kiel, Germany

Archaea have a eukaryotic type of transcriptional machinery containing homologies of the transcription factors TATA-binding protein (TBP) and TFIIB (TFB) and a pol II type of RNA polymerase, whereas transcriptional regulators identified in archaeal genomes have bacterial counterparts. We describe here a novel regulator of heat shock response, Phr, from the hyperthermophilic archaeon Pyrococcus furiosus that is conserved among Euryarchaeota. The protein specifically inhibited cell-free transcription of its own gene and from promoters of a small heat shock protein, Hsp20, and of an AAA+ ATPase. Inhibition of transcription was brought about by abrogating RNA polymerase recruitment to the TBP/TFB promoter complex. Phr bound to a 29-bp DNA sequence overlapping the transcription start site. Three sequences conserved in the binding sites of Phr, TTTA at −10, TGGTAA at the transcription start site, and AAAA at position +10, were required for Phr binding and are proposed as consensus regulatory sequences of Pyrococcus heat shock promoters. Shifting the growth temperature from 95 to 103 °C caused a dramatic increase of mRNA levels for the aaa’ atpase and phr genes, but expression of the Phr protein was only weakly stimulated. Our findings suggest that heat shock response in Archaea is negatively regulated by a mechanism involving binding of Phr to conserved sequences.

All living organisms have developed molecular mechanisms to protect themselves from the harmful effects of elevated temperatures and other stress factors. The overwhelming majority of information on these heat-induced heat shock proteins (Hsps) is and heat shock genes comes from bacteria and eukaryotes. In bacteria, several independent mechanisms of regulation of heat shock promoters have been elucidated. Regulation in Escherichia coli is brought about by the use of alternative sigma factors that direct RNA polymerase to heat shock promoters differing from the standard consensus promoter sequence (1). A different mechanism operates in Gram-positive bacteria, which involves binding and dissociation of a repressor to a DNA control element upstream of the groEL and dnaK operons (2). Recent analyses suggest that bacteria have evolved sophisticated regulatory networks often combining positive and negative control mechanisms to allow a time-tuned expression of heat shock genes (3). In eukaryotes stress-induced transcription requires activation of a heat shock factor, HSF, which binds as a trimer to the heat shock DNA element, thereby stimulating transcription. The monomeric form of HSF lacks both promoter binding and transcriptional activity (4).

Regulators of the heat shock response in the domain of Archaea have not yet been identified, but studies of stress genes in archaeal genomes revealed the presence of homologues of Hsp70/DnaK, Hsp60, Hsp40, GrpE, and of small heat shock proteins (5). Hsp70 is only present in about 50% of the archaeal species inspected; GroEL and GroES seem not to exist in Archaea (5). Their function in peptide folding is apparently performed by the archaeal thermosome, which has been studied in some molecular detail in Thermoplasma (6), Methanopyrus (7), and Pyrococcus (8).

We are exploring the mechanism and regulation of gene transcription in Pyrococcus furiosus growing optimally at 100 °C (9), using a cell-free transcription system (10). The transcriptional machinery of Archaea is eukaryotic-like. It consists of a pol II-type of RNA polymerase, a TATA-box-binding protein (TBP), and a homologue of the transcription factor TFIIB, TFB (11, 12).

Archaeal TBP and TFB interact with the archaeal promoter elements TATA-box and B-recognition element (BRE) of an archaeal promoter in a similar manner as their eukaryotic counterparts (13, 14). The information on the regulation of transcription in Archaea is scarce but in contrast to the eukaryotic nature of the basal archaeal transcriptional machinery many homologues of bacterial regulators have been identified in archaeal genomes (15, 16). A transcriptional activator of gas vesicle proteins containing a leucine zipper motif has been identified in the extreme halophilic archaeon Halobacterium salinarum (17), although this protein did not show significant sequence similarity to eukaryotic activators. Archaeal homologues of the general bacterial regulator leucine responsive regulatory protein, Lrp, have been shown to inhibit transcription from their own promoters in vitro (18, 19). Since archaeal cells do not have sigma factors or homologues of eukaryotic heat shock factors (HSF) or sequences similar to heat shock factor elements (HSE) the mechanism of control of heat shock response and the components involved in this process are completely unclear.

We describe here an archaeal transcriptional repressor that shows, with the exception of the region of its helix-turn-helix motif, no sequence similarity to eukaryotic or bacterial regulators. It binds specifically to the transcriptional start site of an archaeal heat shock gene thereby inhibiting RNA polymerase recruitment. We also show that mRNA levels of this regulator are induced after heat shock and during stationary growth.

* This work was supported by a grant from the Deutsche Forschungsgemeinschaft priority program and from the Fonds der Chemischen Industrie (to M. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Universität Regensburg, Universitätstr. 31, 93040 Regensburg, Germany. Tel.: 49-941-943-2403; E-mail: mthomm@ifam.uni-kiel.de or Michael.Thomm@Biologie.Uni-Regensburg.de.
1 The abbreviations used are: Hsps, heat shock proteins; pol II, polymerase II; EMSA, electrophoretic mobility shift assay; TBP, TATA-box-binding protein; BRE, B-recognition element; RNAP, Pyrococcus RNA polymerase; TFB, transcription factor IIB (TFIIB); Phr, Pyrococcus heat shock regulator.
promoters were amplified by PCR using primers ATPF1 (5′-GTTGAATCTCTCTCTGGTG-3′) and ATPR1 (5′-CTCAACACACTCTCTGGTG-3′) for the aa’’ atpase promoter, 20F3 (5′-GCTCGTGAGAAGATTCTG-3′) and 20R1 (5′-GTCGGCTGTGGAGATATTCTG-3′) for the hsp20 promoter and PRP1R (5′-GCTCGTCTGAAGATTCGTTGCTG-3′) and PRP1I (5′-GCTCGTCTGAAGATTTGCTGTTGCTG-3′) for the phr promoter. The PCR products were ligated into Smal-digested pUC18 (pUC19 in the case of cloning the phr promoter). This resulted in the plasmid constructs atpase, hsp20 and phr. The sequences of the archaeal DNA in the plasmids were confirmed by DNA sequencing. Plasmid DNA was purified by centrifugation in CsCl density gradients as described previously (22).

In Vitro Transcription—In vitro transcription assays were performed as described previously (10). Assays were composed of 500 ng of linearized plasmid DNA (Xhol-digested uph20 or uphpr or KpnI-digested upatpase), 0.012 μM Pyrococcus RNA polymerase (RNAP), 0.076 μM recombinant TFB, 0.68 μM recombinant TBF, and different amounts (0.37–1.5 μM) recombinant Phr in a 25-μl reaction mixture. The control template was Xhol-digested plasmid pUC19 containing the P. furiosus gdh promoter sequence from −95 to +163 (10). The reactions were assembled in vitro and then incubated at 70 °C for 30 min. The transcripts were analyzed by denaturing polyacrylamide gel electrophoresis as described previously (22). Transcriptional activities were quantified using a PhosphorImager (FLA 5000 Fuji, Aida Imaging Software, Raytest).

Primer Extension—For analyses of the transcriptional start sites in vivo, cell-free transcription reactions with unlabeled precursors (0.44 mM each) were performed. The end-labeled primers used for the reactions were: ATPR1 (5′-CTCAACACACTCTCTGGTG-3′), complementary to nucleotides +139 to +159 of the aa’’ atpase gene, 20RI (5′-GCTCGTCTGAAGATTTGCTGTTGCTG-3′), complementary to nucleotides +116 to +136 of the hsp20 gene and PRP1R (5′-GCTCGTCTGAAGATTTGCTGTTGCTG-3′), complementary to nucleotides +107 to +126 of the phr gene. Primer extension reactions were performed as described previously (10). For in vivo RNA analyses primer extension was performed with 30 μg of total RNA of P. furiosus. The RNA was isolated from cells cultured under different growth conditions as described above. Primer extension products were quantified using a PhosphorImager.

Preparation of Probes for EMSA—DNA fragments were generated by PCR using the end-labeled primers ATPF2 (5′-GCTTTCTATATCATATAATTTCACACAGG-3′) and M13/pUCrev (5′-GAGCGGATAACAATTTCACACAGG-3′), and plasmid upatpase as template. The PCR products were excised from a 6% native polyacrylamide gel, eluted in TE buffer (10 μM Tris-HCl, pH 7.0, 0.1 mM EDTA) and precipitated with ethanol.

Oligonucleotides (MWG Oligo) were hybridized as follows: 500 pmol of complementary oligonucleotides (sequence of the noncoding strand shown in Fig. 6B) dissolved in hybridization buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.2 mM NaCl) were heated to 95 °C and allowed to cool to room temperature overnight. Double-stranded DNA was purified by centrifugation in 10 mM EDTA. Double-stranded DNA was excised from the gel, the gel slices were immersed in gel elution buffer (1 mM Tris-HCl, pH 7.0, 0.5 mM NaCl, and 0.7 mM EDTA), and allowed to cool to room temperature overnight. Double-stranded DNA fragments were radiolabeled using T4 polynucleotide kinase and [γ-32P]ATP (Promega). For analyses of the transcriptional start sites in vivo, cell-free transcription reactions with unlabeled precursors (0.44 mM each) were performed. The end-labeled primers used for the reactions were: ATPR1 (5′-CTCAACACACTCTCTGGTG-3′), complementary to nucleotides +139 to +159 of the aa’’ atpase gene, 20RI (5′-GCTTTCTATATCATATAATTTCACACAGG-3′), and plasmid upatpase as template. The PCR products were excised from a 6% native polyacrylamide gel, eluted in TE buffer (10 μM Tris-HCl, pH 7.0, 0.1 mM EDTA) and precipitated with ethanol.

EMSAs—The 10-μl binding reaction mixture contained 40 μM HEPES-NaOH, pH 7.3, 7.325 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 5% polyethylene glycol (PEG) 8000, 5 μg of bovine serum albumin, and 1 μg of calf thymus DNA (nonpecific competitor DNA).

Each reaction contained 1–10 ng of labeled DNA. Binding reactions were incubated at 70 °C for 30 min and contained 0.23 μM recombinant TBP, 0.13 μM recombinant TFB, 0.008 μM RNAP, and 0.74, 1.9, or 3.7 μM recombinant Phr. DNA protein complexes were analyzed on nondenaturing 5% acrylamide gels buffered in Tris borate/EDTA buffer (TBE) (24).
Archaeal Heat Shock Regulator

Fig. 1. Purification of the archaeal heat shock regulator, Phr. 12% SDS-PAGE of purified N-terminal histidine-tagged Phr (lane 2) stained with Coomassie Blue. Molecular weight standards are shown in lane 1.

Preparation of Antibodies Against Recombinant Pyrococcus Phr—Purified native His-tagged Phr (500 μg) was used for immunization of a rabbit using the standard protocol (Eurogentec, Seraing, Belgium). The preimmune and anti-Phr IgG fractions were isolated from the serum by affinity chromatography on protein A-Sepharose (Amersham Biosciences).

RESULTS

Phr Is a Specific Regulator of Archaeal Heat Shock Genes—A putative heat shock regulator from P. furiosus, Phr, is composed of 202 amino acids encoded by an open reading frame of 609 nucleotides (GenBank™ accession no. Q8U030). It has a predicted pI of 7.68, a molecular mass of 24,034, and forms a dimer in solution (see “Experimental Procedures”). The His-tagged protein showed an apparent molecular mass of 26,000 under denaturating conditions (Fig. 1). Comparison of the amino acid sequence of Phr with putative heat shock regulators from other Archaea revealed that the related Pyrococcus strains P. abyssi and P. horikoshii have the highest percent identity and similarity to Phr (Table I). The putative regulator of a moderate thermophilic methanogenic archaean Methanothermobacter thermoautotrophicus shows lower percent identity and of the hyperthermophilic archaean sulfate reducer Archaeoglobus the lowest percent identity. The availability of a cell-free transcription system for P. furiosus allowed us to investigate the function of Phr as transcriptional regulator.

To characterize these putative archaeal heat shock genes, the promoter regions of the Pyrococcus aaa- atpase and hsp20 gene and of the Phr were cloned by PCR and used as templates in cell-free transcription reactions. The in vitro transcription start sites were identified by primer extension analyses of RNA products synthesized by the cell-free Pyrococcus system. All these templates showed typical archaeal TATA-boxes and BRE-elements (Fig. 2B). As expected, they were expressed in the cell-free system (Fig. 2A). Transcription initiation at a distance of 24, 23, and 20 nucleotides downstream of the TATA-box at a G, T, or A residue, respectively.

To study the effect of Phr on the expression of these genes the protein was added to cell-free transcription reactions containing different putative heat shock genes as templates, and the labeled RNA products were analyzed directly in denaturing gels. When increasing amounts of Phr were added to the templates encoding hsp20, the aaa-atpase or to the gene encoding the regulator Phr itself, synthesis of the run-off transcripts was reduced at lower concentrations and strongly or completely inhibited at higher concentrations of Phr (Fig. 3, A and C). The inhibitory activity of Phr was highest at the aaa-atpase promoter (complete inactivation at a molar ratio DNA: regulator of 1:25; addition of 500 ng) and lowest at the phr promoter (no complete inhibition at a molar ratio of 1:250; addition of 1000 ng). The effect of Phr on cell-free transcription of the P. furiosus glutamate dehydrogenase promoter (Fig. 3B) was studied as a control. Addition of Phr did not affect the rates of transcription from this promoter. These findings suggest that Phr is a negative regulator of heat shock response.

Phr Inhibits Transcription by Abrogating RNA Polymerase Recruitment—To investigate the interaction of Phr with the archaeal transcriptional machinery in more detail Phr was added to DNA binding reactions containing the aaa-atpase promoter, TBP/TFB, and RNAP. Electrophoretic mobility shift analyses showed that Phr bound to a labeled DNA fragment encoding the DNA region from position −95 to +225 (Fig. 4, lanes 1 and 2). No shift was observed when a DNA fragment containing the gdh promoter region was used (data not shown), indicating that binding of Phr to the aaa-atpase promoter was specific. TBP/TFB formed a complex with lower electrophoretic mobility (Fig. 4, lane 3). Addition of RNA polymerase (RNAP) to binding reactions containing TBP/TFB resulted in the formation of a third, slowly migrating complex (lane 5). We have recently shown the presence of TBP/TFB and of RNAP in similarly migrating complexes formed at the lrpA promoter of P. furiosus by serological analyses (19). We therefore assume that these components are also present in the corresponding complexes observed at the aaa-atpase promoter. When Phr was added to binding reactions containing TBP/TFB, binding of the archaeal transcription factors was not affected, but a third complex of lower mobility was observed (lane 4). This finding and the decrease in intensity of the band corresponding to the Phr-DNA complex in lane 4 suggest that this third complex consisted of TBP/TFB and Phr. When Phr was added in low concentrations (molar ratio RNAP:Phr of 1:92) to binding reactions containing both TBP/TFB and RNAP, the Phr-DNA, the

Table I

| Organism               | Gene name | Identity | Similarity |
|------------------------|-----------|----------|------------|
| Pyrococcus horikoshii  | PH1744    | 81       | 92         |
| Pyrococcus abyssi      | PAB0208   | 84       | 91         |
| Methanothermobacter    | MTH1288   | 40       | 54         |
| thermosudetrophicus    |           |          |            |
| Archaeoglobus fulgidus | AF1298    | 33       | 50         |

Percent identity and percent similarity in comparison to P. furiosus Phr are shown. Alignments and calculations were performed using HUSAR Heidelberg.
TBP/TFB-DNA, the TBP/TFB-Phr-DNA, and the TBP/TFB-RNAP-DNA complexes were observed (lane 6). However, addition of increasing amounts of Phr to the binding reaction containing TBP/TFB and RNAP decreased the amount of the TBP/TFB-RNAP-DNA complex and increased the amount of the TBP/TFB-Phr-DNA complex (molar ratio RNAP:Phr of 1:237 in lane 7 and 1:462 in lane 8). This finding supports the conclusion that binding of Phr to DNA did not affect binding of TBP/TFB, but inhibited association of RNAP with the TBP/TFB promoter complex.

To analyze the DNA binding region of Phr and of Pyrococcus transcriptional components at the aaa’ atpase promoter in molecular detail, DNase I footprinting experiments were performed. Phr protected the DNA region from position –15 to +14 on the non-template (Fig. 5A, left panel, lanes 1–4) and from –17 to +12 on the template DNA strand (Fig. 5A, right panel, lanes 1–4) from DNase I digestion. The TBP/TFB footprint extended from position –41 to –17 on the non-template (Fig. 5A, left panel, lanes 5 and 6) and from –36 to –19 (Fig. 5A, right panel, lanes 5 and 6) on the template DNA strand. The TBP/TFB-binding site was centered at the TATA-box and Phr bound to the DNA region overlapping the transcription start site (Fig. 5B). This DNA segment downstream of the TBP/TFB binding site has been shown to be bound by the archaeal RNAP (25). This finding suggests that Phr and TBP/TFB can bind simultaneously to the same DNA molecule. To investigate this both Phr and TBP/TFB were added to binding reactions. Phr and TBP/TFB induced a footprint both on the nontemplate and template DNA strand (Fig. 5A, left and right panel, lanes 7 and 8). The extension of the protected DNA regions was the same as with individual components. On the non-template DNA strand a hypersensitivity site located at position –6 was observed in complexes containing both TBP/TFB and Phr (left panel, lanes 7 and 8). Therefore, binding of Phr to the region of the transcription start site prevents the association of RNAP with the TBP/TFB promoter complex.

Identification of Cis-acting Sequences Required for Phr Binding—To identify the structural determinants of archaeal heat shock promoters required for operator recognition by Phr, binding experiments with short double-stranded oligonucleotides containing the wild-type aaa’ atpase DNA binding region and mutated sequences were performed. Inspection of sequences in

---

**Fig. 2. Analyses of the transcription start sites at P. furiosus heat shock promoters and at the promoter of the heat shock regulator.** A., primer extension reactions were performed using the products of in vitro transcription assays. The position of the primer extension products are indicated by arrows. A, T, G, and C lanes correspond to the dideoxynucleotide sequencing reactions carried out using the same oligonucleotide primers as in primer extension reactions. The sequences of the template strands are shown on the right side of the figures with the transcription start sites shown in bold letters. B, summary of the primer extension data, showing the sequences of the aaa’ atpase, hsp20, and phr promoters. The transcription start sites (+1) are shown in bold with arrows. The positions of the TATA elements and BRE are boxed. Sequence similarities among the three promoters are underlined.
Archaeal Heat Shock Regulator

**Fig. 3.** Phr inhibits cell-free transcription of archaeal heat shock genes and of its own promoter. Linearized plasmid DNA containing the aat promoter, hsp20, and gdh promoter regions of *P. furiosus* were used as templates in cell-free transcription experiments. The run-off transcripts are indicated by arrows. The reactions contained 0, 250, 500, or 1000 ng of Phr as indicated on the top of the lanes, which correspond to 0, 0.37, 0.75, and 1.5 μg, respectively. Cell-free transcription assays performed with the aat promoter and hsp20 promoters (A), the gdh promoter (B), and the phr promoter (C) as templates. Transcriptional activities were quantified using a PhosphorImager. Transcription activity in the absence of Phr equals 100%.

**Fig. 4.** Phr specifically binds at the aat promoter and prevents RNP recruitment. EMSA was performed with the radiolabeled aat promoter DNA using 0.23 μM TBP, 0.13 μM TFB, 0.006 μM RNAP, and 0.74 (+), 1.9 (+), or 3.7 μM (++) Phr, as indicated. Free DNA and protein-DNA complexes are indicated at the left.

detail we carried out a mutational analysis of binding motifs within the 36-bp fragment. Mutation of three nucleotides in a nonconserved DNA region located at position −17 to −20 decreased the binding ability of Phr only slightly from 66 to 56% (Fig. 6B, lanes 2 and 3, compare wild type and control). Mutation of three nucleotides of the −10 motif (mutV), of three nucleotides of the initiator site motif (mutM), and three nucleotides of the AAAAA motif (mutH) reduced the binding activity of Phr to 2.5, 9, and 29% (Fig. 6B, lanes 4–6, respectively). This result identified the −10 region as most important for operator recognition by Phr and therefore, the effects of single point mutations in this DNA segment were analyzed. Each of the T residues of the TTAT motif was replaced by a C, the A residue by a G (Fig. 6B, mut1–mut 4). Analysis of the binding ability of these transition mutants revealed that each nucleotide of this position contributed significantly to DNA binding (Fig. 6B).

The first and third T residues were more important for complex formation than the second T residue and the A residue. These studies provide biological evidence for the importance of the three conserved binding motifs in the regulatory region of Phr and identify the TTAT motif at −10 as crucial for Phr binding.

**Transcription of the aat gene and phr Is Induced under Heat Shock Conditions and Stimulated during Stationary Growth Phase** — *In vitro* data provided in this study demonstrate inhibition of transcription of putative archaeal heat shock genes by an archaeal regulatory protein and suggest that Phr is a negative regulator of heat shock response in *Pyrococcus*. To provide additional experimental evidence that the investigated genes are real heat shock genes and that the function of the regulator is related with stress, the expression of the aat gene and of the phr gene was investigated under various physiological conditions. *P. furiosus* cultures were grown in a 100-liter fermentor at 95 °C, cells were heated to 103 °C for 30, 60, and 90 min, and the mRNA levels were analyzed by primer extension experiments. In a separate set of experiments the mRNA levels of the same genes were analyzed during different growth phases.

Analysis of aat mRNA showed that the RNA levels in *Pyrococcus* cells were increased after 30 min of heat shock treatment by a factor of 25 (Fig. 7A). After 60 and 90 min of heat shock treatment, the mRNA levels were still elevated by a factor of 11 and 13, compared with cells grown at 95 °C. Also the mRNA levels of the phr gene were dramatically induced by treatment of cells at 103 °C. After 30 min, the mRNA level was 42 times higher than in nonstressed cells. The phr mRNA levels were even more increased after prolonged heat shock treatment by a factor of 49 after 60 min and a factor of 50 after 90 min (Fig. 7A).

The transcript levels of both genes were also increased during the stationary growth phase. Compared with early and late exponential growth phase, expression of the aat gene was stimulated by a factor of 1.5 and 3 during early and late stationary phase, that of the phr gene by a factor of 6 and 18 (Fig. 7A). Control experiments using the *P. furiosus* gdh gene showed no increase of mRNA levels after heat shock and during stationary growth phase (data not shown). Taken together, these data provide evidence that the aat gene is activated by heat shock and starvation, and that Phr is a regulator of heat shock and potentially also of general stress response.

**Analysis of the protein levels of Phr by Western blotting** showed that the expression of the regulator was only slightly enhanced after heat shock and remained constant during different growth phases (Fig. 7B). Thus, in contrast to Hsp20 from *P. furiosus* that was only synthesized in significant amounts in heat-shocked cells (26), Phr is both expressed during growth at the optimal temperature and after temperature upshift. This
Several lines of evidence indicate that we have identified an archaeal regulator of heat shock response. A protein conserved among Euryarchaeota (Ref. 27 and Table I) was found to inhibit specifically cell-free transcription of the $aaa^{+}$ atpase gene, of the gene encoding the small archaeal heat shock protein Hsp20, and of its own gene (Fig. 3). The protein bound specifically to the promoter region of the $aaa^{+}$ atpase promoter (Figs. 4 and 5). The data shown in this study indicated that this specific DNA binding site overlapped with the RNAP binding site (Fig. 5) and that promoter-bound Phr inhibited RNAP recruitment at heat shock promoters (Fig. 4). Within the DNA binding site of Phr three DNA signals conserved among the promoters investigated here were identified (Fig. 2B). These three sequences were shown to be essential for binding of Phr to DNA by mutational studies (Fig. 6). Finally, the $aaa^{+}$ atpase, the phr gene, and the small heat shock gene hsp20 (Ref. 26; see below) produced highly elevated mRNA levels after heat shock treatment (Fig. 7). These results demonstrated that the genes regulated in vitro by Phr are clearly affected by heat shock treatment.

The cellular function of the $P$. furiosus AAA$^{+}$ ATPase is not known but members of the AAA$^{+}$ superfamily are often involved in energy-dependent proteolysis, molecular chaperone-like activities (28), and transcriptional activation (29). The small heat shock proteins (sHsps) from Archaea have been studied to some extent. The sHsp from $M$. jannaschii is a 16.5-kDa protein that forms a unique spherical oligomer of 24 subunits and has the ability to protect proteins in $E$. coli extracts from thermal aggregation (30, 31). Hsp20 from $P$. furiosus (that is identical with Pfu-sHSP described by Laksanalamai et al., Ref. 26) the product of the gene investigated in this study, showed a sequence similarity of 33.8% with $M$. jannaschii sHsp and of 43% and 31% with two sHsps of bacterial origin (26). Both $P$. furiosus Hsp20 and its mRNA were shown to be induced by heat shock. More than 90% of cellular proteins from $E$. coli remained soluble after 40 min at 105°C when Hsp20 from $P$. furiosus was overexpressed. In addition, Hsp20 prevented a mesophilic glutamate dehydrogenase from aggregation as a result of heat treatment in vitro. These results (26) demonstrate clearly that the product of the $P$. furiosus hsp20 gene investigated in our study has chaperone activity. mRNA levels of the $aaa^{+}$ atpase and of the gene phr were shown in this work to be elevated during heat shock. These findings support the conclusion that the DNA-binding protein and DNA signals described in this work can be consid-
Archaeal Heat Shock Regulator

Fig. 6. Identification of Phr binding signals at an archaeal heat shock promoter. A, binding of Phr to different sized DNA fragments. The DNA fragments represent the Phr binding site of the aae<sup>+</sup> atpase gene promoter. The three conserved sequences of heat shock promoters are shown in bold letters in the lower part of the figure. BRE and the TATA box are boxed, the vertical arrows indicate the boundaries of the Phr footprint. Binding reactions were performed with radiolabeled DNA for 30 min at 70 °C; complexes were analyzed by native gel electrophoresis (EMSA). B, binding of Phr to DNA fragments carrying certain mutations. Mutations and nucleotides, which are conserved among the binding sites of the aae<sup>+</sup> atpase, the hsp20, and the phr gene are indicated below the figure. The three conserved putative binding signals are shown in bold letters in the wild type sequence. Mutated bases are underlined.
tured at 103 °C in vitro, and dissociation of the denatured protein from its operator sequence may account for the high increase of phr mRNA detected after temperature upshift (Fig. 7). Our finding that heat shock triggered the accumulation of Phr mRNA dramatically but that of the regulator protein only slightly (Fig. 7, A and B) suggests that regulation of archaeal heat shock response is complex, and that more than one regulator might be involved. Regulation on the level of mRNA stability and/or translation might occur, and a protein or cofactor modulating the binding activity of Phr might exist.

Also during the stationary growth phase the levels of the aaa⁺ ATPase and of phr mRNA were significantly increased (Fig. 7A) compared with logarithmic phase suggesting that the transcription of these genes is also affected by starvation and general stress. Since the promoter of the aaa⁺ ATPase gene contains a Phr binding site and binding of Phr prevents RNA polymerase recruitment at this promoter (Fig. 4) it is likely that Phr is also involved in the growth phase-dependent regulation of this gene. Protein levels of the AAA⁺ ATPase were not analyzed here. The finding that Phr protein levels remained constant during various growth phases (Fig. 7B) indicates again that an additional hitherto unknown mechanism modulating its binding activity seems to be required for regulation of stress response in Archaea. The identification of the transcriptional regulator described here offers the opportunity to address exciting questions concerning the crystal structure of Phr and its interplay with cellular factors and proteins. These studies will be important not only

---

2 M. Thomm, A. Engelmann, G. Vierke, and R. Ladenstein, unpublished data.
for studies of the mechanism of heat shock regulation in Archaea but will also contribute a deeper understanding of the evolution of stress response in all three domains of life.

Acknowledgments—We thank Jens Thomsen for the initial help in cloning of Phr from genomic DNA of *P. furiosus*, and the valuable advice of Winfried Hausner in cloning and bioinformatic work is appreciated. We thank Uta Wehmeyer for technical assistance and Holger Preidel for large scale cultivation of *P. furiosus*.

REFERENCES

1. Gross, C. A. (1996) in *Escherichia coli and Salmonella Cellular and Molecular Biology* (Neidhardt, F. C., ed) pp. 1382–1399, ASM Press, Washington, D.C.
2. Hecker, M., Schumann, W., and Volkmer, U. (1996) *Mol. Microbiol.* **19**, 417–428
3. Narberhaus, F. (1999) *Mol. Microbiol.* **31**, 1–8
4. Narberhaus, F. (1998) *Genes Dev.* **12**, 3788–3796
5. Macario, A. J. L., Lange, M., Ahring, B., and Conway de Macario, E. (1999) *Microbiol. Mol. Biol. Rev.* **63**, 923–967
6. Dittrich, L., Lowe, J., Stock, D., Stetter, K. O., Huber, H., Huber, R., and Steinbacher, S. (1998) *Cell* **93**, 125–138
7. Andrae, S., Frey, G., Jaenicke, R., and Stetter, K. O. (1998) *Eur. J. Biochem.* **255**, 93–99
8. Minuth, T., Henn, M., Rutkat, K., Andrae, S., Frey, G., Rachel, R., Stetter, K. O., and Jaenicke, R. (1999) *Biol. Chem.** 380**, 55–62
9. Fiaux, G., and Stetter, K. O. (1998) *Arch. Microbiol.* **165**, 56–61
10. Hethke, C., Geering, Ana C. M., Hausner, W., de Vos, W., and Thomm, M. (1996) *Nucleic Acids Res.* **24**, 2369–2376
11. Thomm, M. (1996) *FEMS Microbiol. Rev.* **18**, 159–171
12. Bell, S. D., and Jackson, S. P. (2001) *Curr. Opin. Microbiol.* **4**, 208–213
13. Hausner, W., Wettach, J., Hethke, C., and Thomm, M. (1996) *J. Biol. Chem.* **271**, 30144–30148
14. Bell, S. D., Kosa, P. L., Sigler, P. B., and Jackson, S. D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 13662–13667
15. Aravind, L., and Koonin, E. V. (1999) *Nucleic Acids Res.* **27**, 4648–4670
16. Bell, S. D., Magill, C. P., and Jackson, S. P. (2001) *Biochem. Soc. Trans.* **29**, 392–395
17. Piisser, P., and Pfeifer, F. (2002) *Mol. Microbiol.* **45**, 511–520
18. Bell, S. D., and Jackson, S. P. (2000) *J. Biol. Chem.* **275**, 31624–31629
19. Dahlke, I., and Thomm, M. (2002) *Nucleic Acids Res.* **30**, 701–710
20. DiRuggiero, J., and Robb, F. T. (1995) *in Archaea: A Laboratory Manual* (Robb, F. T., Place, A. R., Sowers, K. R., Schreier, H. J., DasSarma, S., and Fleischmann, E. H., eds) pp. 97–99, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
22. Frey, G., Thomm, M., Brudigam, B., and Hausner, W. (1990) *Nucleic Acids Res.* **18**, 1361–1367
23. Sambrook, J., Maniatis, T., and Fritsch, E. F. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 6.39–6.43, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. Hausner, W., and Thomm, M. (1995) *J. Biol. Chem.* **270**, 17649–17651
25. Hausner, W., and Thomm, M. (2001) *J. Bacteriol.* **183**, 3025–3031
26. Laksanamalai, P., Maeder, D. L., and Robb, F. T. (2001) *J. Bacteriol.* **183**, 5198–5202
27. Gelfand, M. S., Koonin, E. V., and Mironov, A. A. (2000) *Nucleic Acids Res.* **28**, 695–705
28. Maupin-Furlow, J. A., Wilson, H. L., Kaczowka, S. J., and Ou, M. S. (2000) *Front. Biosci.* **5**, D837–865
29. Zhang, X., Chaney, M., Wigleshwararaj, S. R., Schumacher, J., Bordes, P., Cannon, W., and Back M. (2002) *Mol. Microbiol.* **45**, 895–903
30. Kim, R., Kim, K. K., and Kim, S.-H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9129–9133
31. Kim, K. K., Kim, R., and Kim, S.-H. (1998) *Nature* **394**, 585–589
32. Thompson, D. K., and Daniels C. J. (1998) *Mol. Microbiol.* **27**, 541–551
33. Fernandes, M., O’Brien, T., and Lis, J. T. (1994) *in The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. J., Tissiéres, A., and Georgopoulos, C., eds) pp. 375–393, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
34. Bell, S. D., Cairns, S. S., Robson, R. L., and Jackson, S. P. (1999) *Mol. Cell* **4**, 971–982
