Expression Profiles and Physiological Roles of Two Types of Molecular Chaperonins from the Hyperthermophilic Archaeon *Thermococcus kodakarensis*[▽][†]

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***Thermococcus kodakarensis* possesses two chaperonins, CpkA and CpkB, and their expression is induced by the downshift and upshift, respectively, of the cell cultivation temperature. The expression levels of the chaperonins were examined by using specific antibodies at various cell growth temperatures in the logarithmic and stationary phases. At 60°C, CpkA was highly expressed in both the logarithmic and stationary phases; however, CpkB was not expressed in either phase. At 85°C, CpkA and CpkB were expressed in both phases; however, the CpkA level was decreased in the stationary phase. At 93°C, CpkA was expressed only in the logarithmic phase and not in the stationary phase. In contrast, CpkB was highly expressed in both phases. The results of reverse transcription-PCR experiments showed the same growth phase- and temperature-dependent profiles as observed in immunoblot analyses, indicating that the expression of *cpkA* and *cpkB* is regulated at the mRNA level. The *cpkA* or *cpkB* gene disruptant was then constructed, and its growth profile was monitored. The *cpkA* disruptant showed poor cell growth at 60°C but no significant defects at 85°C and 93°C. On the other hand, *cpkB* disruption led to growth defects at 93°C but no significant defects at 60°C and 85°C. These data indicate that CpkA and CpkB are necessary for cell growth at lower and higher temperatures, respectively. The logarithmic-phase-dependent expression of CpkA at 93°C suggested that CpkA participates in initial cell growth in addition to lower-temperature adaptation. Promoter mapping and quantitative analyses using the Phr (*Pyrococcus* heat-shock regulator) gene disruptant revealed that temperature-dependent expression was achieved in a Phr-independent manner.**

Living microorganisms face various kinds of stress and protect themselves by inducing several stress-responsive proteins. Under temperature stress, which is a major form of stress in nature, cells produce a group of proteins called heat shock proteins (HSPs) (13). The predominant HSPs are classified by their molecular masses as HSP104, HSP90, HSP70, HSP60, and small HSP (3, 13). The best-studied chaperone is HSP60. HSP60s, called chaperonins, assist in refolding denatured proteins and folding newly synthesized proteins. Chaperonins are classified into two groups, I and II (14). Group I chaperonins consist of two protein components, GroEL and GroES, with molecular masses of approximately 60 and 10 kDa, respectively, and are found in bacteria, mitochondria, and chloroplasts. Group II chaperonins, which have a structure similar to that of the GroEL/GroES complex, are found in archaea and the cytosol of eukaryotes and can facilitate protein folding in the absence of cochaperonin GroES. Cytosolic group II chaperonins are closely related to cell growth and markedly enhanced at the early S phase of the cell cycle in mouse and human cultured cells (29). The cytosolic group II chaperonin that is upregulated around the early S phase is associated with tubulin in vivo. In archaea, it is unclear whether or not chaperonin expression is under cell cycle control; however, some homologues involved in cell cycle regulation have been identified in archaea (15). A cell cycle-dependent variation in Orc1/Cdc6 levels in *Sulfolobus* species has been demonstrated, reminiscent of the variation in cyclin levels during the eukaryotic cell cycle (19). The three Orc1/Orc6 replication initiation proteins of *Sulfolobus acidocaldarius* have been shown to vary in abundance over the cell cycle in a cyclin-like fashion, providing a clear example of cell cycle-specific events among archaea (19). In addition, a CDC48/VCP homologue has also been identified in *Thermococcus kodakarensis*, and growth phase-specific fragmentation was observed only in the stationary phase (11). These findings led us to examine whether the hyperthermophilic archaeal chaperonin is under cell cycle or growth phase regulation.

Thermococcus kodakarensis is a sulfur-reducing hyperthermophilic archaeon isolated from Kodakara Island, Japan (1, 17). It has two kinds of group II chaperonin genes, *cpkA* and *cpkB*, in its genome, and CpkA and CpkB were upregulated by growth temperature down- and upshifts, respectively (9, 28). Based on the phylogenetic similarities between members of the *Archaea* and *Eukarya*, CpkA and CpkB are also expected to have a cell cycle-dependent mechanism in their expression.

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However, the details are not available because a synchronized cultivation has not been established for an anaerobic hyperthermophile such as *T. kodakarensis*. Cell cycle regulation is correlated with growth phase regulation because the gap period is prolonged in the stationary phase due to an extended generation time. Hence, we have investigated in more detail whether or not two chaperonins, CpkA and CpkB, are expressed under growth phase-dependent regulation.

In the present study, the growth phase dependencies of the expression of these chaperonins were examined at various cell cultivation temperatures, 60°C, 85°C, and 93°C, by quantitative reverse transcription-PCR (RT-PCR) and immunoblot analyses. In addition, to obtain precise information on the temperature-dependent expression, the promoter region was analyzed by determining the transcriptional start points. In archaea, only limited information is available regarding the temperature-responsive factor. The heat shock regulation of members of the *Archaea* is predicted to be different from that of prokaryotes and eukaryotes because no sigma-like factor, such as sigma factor σ^H (σ^{32}) in *Escherichia coli* (31), and no homologues of heat shock factor (HSF) in *Drosophila* and mammals have been identified in the genomes (18). Phr (*Pyrococcus* heat shock regulator) is the only known factor that has been identified as a heat shock regulator in *Pyrococcus furiosus* (25). Phr is involved in the heat-dependent expression of several heat shock genes by repressing transcription at low temperatures in *P. furiosus*. It has been suggested that the expression of the thermosome in *P. furiosus* is controlled by Phr (25). Recently, Phr has been shown to be involved in the heat-dependent induction of prefoldins in *T. kodakarensis* (4). In this report, the transcriptional levels of *cpkA* and *cpkB* were analyzed in a *phr* disruptant of *T. kodakarensis*, and the Phr dependency on chaperonin induction was also examined.

MATERIALS AND METHODS

Microorganisms, media, and growth conditions. *Thermococcus kodakarensis* KOD1 (wild type) and its derivatives were cultivated anaerobically in a nutrient-rich medium (MA-YT or ASW-YT) with an addition of 5.0 g/liter of elemental sulfur or pyruvate or a synthetic medium (ASW-AA) containing amino acids and elemental sulfur (22). The construction procedure for the *phr* gene (TK2291) disruptant (*T. kodakarensis* KHR1) was described elsewhere (5). To obtain cells for RT-PCR and immunoblot analyses, growth curves at 60°C, 85°C, and 93°C in MA-YT medium with pyruvate were carefully monitored and cells were harvested at middle logarithmic and stationary phases. The optical densities at 660 nm of *T. kodakarensis* cells at 60°C, 85°C, and 93°C were 0.07, 0.2, and 0.2 in logarithmic phase and 0.3, 0.7, and 0.7 in stationary phase, respectively. *E. coli* TG-1 was used for general DNA manipulation and sequencing. *E. coli* cells were routinely cultivated at 37°C in Luria-Bertani (LB) medium. Ampicillin (50 µg/ml) was added to the medium when needed.

DNA manipulation and sequencing. DNA manipulations were carried out by standard techniques as described previously by Sambrook and Russell (20). Restriction enzymes and other modifying enzymes were purchased from Takara or Toyobo. Plasmid DNA was isolated with a Wizard Plus miniprep DNA purification system (Promega). GFX PCR DNA and a gel band purification kit (GE Healthcare) were used to recover DNA fragments from agarose gels after electrophoresis. DNA sequencing was performed with a BigDye Terminator cycle-sequencing ready reaction kit, version 3.1, and a model 3130 capillary DNA sequencer (Applied Biosystems).

RT-PCR. Total RNAs extracted from cells of *T. kodakarensis* KOD1 and the Δ*phr* mutant (5) that were cultivated at 60°C, 85°C, and 93°C were purified by using an RNeasy midi kit (Qiagen). Each RNA (0.25 µg) in 10 µl of the reaction mixture was reverse transcribed at 55°C for 30 min with Transcriptor reverse transcriptase (Roche) using the primer for each (*cpkA*-IR, 5′-CAT GCC CAT GTC CAT TCC GC-3′, and *cpkB*-IR, 5′-TGA GGA TCA TTA TGG CAG CC-3′). The cDNA obtained was then amplified by PCR using the primer pair

for each (for *cpkA*, *cpkA*-IF, 5′-CGT CAA AGT CAT CAG CGA GC-3′, and *cpkA*-IR; for *cpkB*, *cpkB*-IF, 5′-GAC AAG ATC AAG GAG GTC GG-3′, and *cpkB*-IR). The reactions were carried out by using KOD Plus polymerase (Toyobo) for 20 cycles according to the reported procedure. The RT-PCR products were analyzed by 2% agarose gel electrophoresis. The amplified products for *cpkA* and *cpkB* were 256 bp and 712 bp, respectively. As a control to ensure that the signal intensities reflected the initial levels of each transcript directly, RT-PCRs were performed with the same total RNAs and the 16S rRNA gene-specific primers 16SrRNA-Fw, 5′-CCA TAG GCC TGA GGT ACT GG-3′, and 16SrRNA-Rv, 5′-CGC TCC CCT GGC CTT CGT CCC TCA CCG TCG-3′. The amplified product was 570 bp.

Immunoblot analyses. The preparation of the specific antibodies anti-CpkA and anti-CpkB was described previously (9). Cell extracts were obtained from the *T. kodakarensis* cells cultivated at 60°C, 85°C, and 93°C and were subjected to electrophoresis in 0.1% sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gels and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked using 5% skim milk and incubated with the mouse anti-CpkA and anti-CpkB serum at dilutions of 1:5,000 and 1:10,000, respectively. The detection of immunocomplexes was performed by using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Cappel) as the secondary antibody and a BCIP/NBT stock solution (Roche Diagnostics) according to the manufacturer's instructions.

Primer extension analysis. The nonradioisotope primer extension analysis was carried out according to the reported procedure (8). Total RNA was isolated as described above. Cy5.5-labeled primer (*cpkA*-Cy5.5, 5′-TAA CAA CCG GCT GTC CAC TA-3′, or *cpkB*-Cy5.5, 5′-TAA CAA CTG GCT GGC CTG CG-3′) and total RNA (17 µg) were mixed and preincubated at 90°C for 5 min and then added to the reaction buffer. The reverse transcriptase reaction was carried out with Transcriptor reverse transcriptase (Roche) for 30 min at 50°C. The products were purified by ethanol precipitation and incubated for 3 min at room temperature in the presence of RNase. Then, the reverse transcripts were resuspended in loading buffer containing 95% formamide, 20 mM EDTA, and 0.05% bromophenol blue. After heat treatment at 90°C for 5 min, samples were loaded on 6% polyacrylamide gel containing 7 M urea. In order to prepare sequence ladders, the DNA fragments, including the upstream regions of *cpkA* and *cpkB*, respectively, were cloned into the plasmid pUC19. The fragments were amplified from the chromosomal DNA using primers 5′-ACT TCT CGA ATT CCC TGA CG-3′ and 5′-GTA TCC TGA GGA GTC CCT G-3′ for *cpkA* and 5′-CTT CCT TCC TTC AAA GAT TG-3′ and 5′-TGA GGA TCA TTA TGG CAG CC-3′ for *cpkB* under PCR conditions of 94°C for 2 min for the 1st cycle and 94°C for 15 s, 55°C for 30 s, and 68°C for 30 s for the 2nd to 25th cycles. The amplified products were cloned into pUC19, and the plasmids obtained were designated pUCcpkA and pUCcpkB, respectively. DNA sequence ladders using the same primers and pUCcpkA or pUCcpkB as a template were prepared according to the manufacturer's instructions provided with a Thermo Sequenase cycle sequencing kit (USB). The reverse transcripts were visualized by using an Odyssey infrared imaging system (LI-COR).

Construction of disruption vectors. The vectors to disrupt *cpkA* and *cpkB* through single-crossover homologous recombination were constructed as follows. DNA fragments containing each target gene together with its flanking regions (ca. 800 bp each) were amplified by using *T. kodakarensis* genomic DNA as the template and primer sets (sense for *cpkA* disruption, *cpkA*-OF, 5′-TTC GAA TTC GAG TTC TCG CTT CCC TTA CG −3′; antisense for *cpkA* disruption, *cpkA*-OR, 5′-GAG GAA TTC CTC CCT TGA CCT GCT TAT CG-3′; sense for *cpkB* disruption, *cpkB*-OF, 5′-GCC GAA TTC CTC CCA GAA GTT CTT TCT GG-3′; antisense for *cpkB* disruption, *cpkB*-OR, 5′-AGC GAA TTC TTG AGG AGA ACG GCG TTA GG-3′ [underlined sequences indicate EcoRI site in the sense and antisense primers]). These DNA fragments were subcloned into pUD2 (21), and the constructs were named pUDcpkAfr and pUDcpkBfr. An inverse PCR was performed in order to amplify the entire pUDcpkAfr or pUDcpkBfr excluding the region of the disruption target (the primer sets for *cpkA* disruption were 5′-CAG AAA CGC TTC GTT AGT GCT CT-3′ and 5′-GAA CTT CTG CAT AGG CTG GGG CA-3′, and the primer sets for *cpkB* disruption were 5′-GAG GGT GGA TGA GTT TTA GTT AC-3′ and 5′-GAA TCT GCT CTT CTC TGG TCA GG-3′). After self-ligation, the plasmid containing the 5′- and 3′-flanking regions of the target gene was constructed. The DNA sequences of all vectors were confirmed by sequence determination.

Transformation of *T. kodakarensis*. The theoretical background for specific gene disruption has been described previously (21). *T. kodakarensis* KU216 (*pyrF* disruptant, *cpkA*⁺ *cpkB*⁺) was cultivated in an ASW-YT liquid medium for 12 h, and cells were harvested (3,200 × *g* for 5 min) from 3 ml of culture. The cells were resuspended in 200 µl of 0.8× ASW and kept on ice for 30 min. Plasmid DNA (5 µg) was added to the cell suspension, and the mixtures were kept on ice
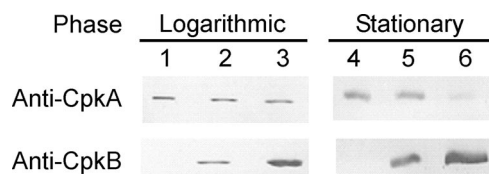
FIG. 1. Protein levels of CpkA and CpkB in the logarithmic and stationary phases. Immunoblot analysis with anti-CpkA and anti-CpkB was performed using cytoplasmic extracts obtained from cells cultivated at 60°C (lanes 1 and 4), 85°C (lanes 2 and 5), and 93°C (lanes 3 and 6) as described in Materials and Methods. Cell extracts containing 1.0 μg of total protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and detected with anti-CpkA and anti-CpkB.

for 1 h. The treated cells were cultured in 20 ml of ASW-AA liquid medium containing 5.0 g/liter of elemental sulfur in the absence of uracil for the period of 2 generations. They were then spread on an ASW-YT plate medium containing 7.5 mg/ml of 5-fluoroorotic acid in order to select transformants that had completed the second, pop-out recombination and incubated at 85°C for isolation of the *cpkA* disruptant or 60°C for isolation of the *cpkB* disruptant. Transformants with a host genotype (*cpkA*⁺ *cpkB*⁺) and those with the intended disruption (Δ*cpkA* or Δ*cpkB*; entire deletion of *cpkA* or *cpkB* gene) were distinguished by confirming the fragment length using PCR methods with specific primers that anneal outside of the target (for Δ*cpkA* confirmation, primers *cpkA*-OF and *cpkA*-OR, and for Δ*cpkB* confirmation, primers *cpkB*-OF and *cpkB*-OR). The 1,365-bp or 1,584-bp DNA was amplified by the above primer combinations from the *cpkA* disruptant or *cpkB* disruptant, respectively, indicating that both disruptants were satisfactorily constructed. (Primers *cpkA*-OF and *cpkA*-OR or primers *cpkB*-OF and *cpkB*-OR amplify 3,094 bp or 3,299 bp of DNA, respectively, from wild-type chromosomal DNA.) Furthermore, the construction of *cpkA* and *cpkB* disruptants was confirmed by no DNA amplification by primers *cpkA*-IF and *cpkA*-IR and primers *cpkB*-IF and *cpkB*-IR, respectively.

RESULTS

Quantitative analyses of protein expression. The expression of CpkA and CpkB has been reported to be upregulated by down- and upshifts, respectively, in growth temperature (9, 28). It has not been clarified whether this temperature-dependent expression occurs in all growth phases. In the present study, the expression levels of CpkA and CpkB were examined by specific antibodies in both the logarithmic and stationary phases at the optimum growth temperature (85°C) and at lower (60°C) or higher (93°C) growth temperatures (Fig. 1). In the stationary phase, CpkA expression was detected only at 60°C and 85°C and not at 93°C. However, in the logarithmic phase, the levels of CpkA were not significantly changed at any of the temperatures examined. The temperature-dependent pattern of CpkB expression in the logarithmic phase was similarly observed in the stationary phases at all temperatures. The expression level increased as the cell growth temperature rose. It is noteworthy that the expression of CpkB is regulated depending on the cell growth temperature; however, that of CpkA is regulated depending on the growth phase in addition to the growth temperature. The growth phase-dependent manner of CpkA expression is a unique phenomenon that has not been observed in other chaperonins.

Quantitative analyses of *cpkA* and *cpkB* transcripts. It has been unclear whether CpkA and CpkB expression is regulated at the mRNA or the protein level. Most genes are regulated at the transcriptional level (mRNA level); however, some cold-inducible proteins are regulated at the posttranscriptional level
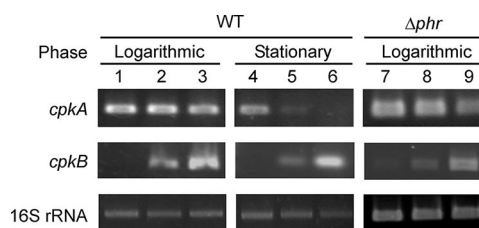

FIG. 2. Comparison of mRNA levels of *cpkA* and *cpkB* in the logarithmic and stationary phases. RT-PCRs were performed with total RNAs isolated from the wild type (WT) and KHR1 cultivated at 60°C (lanes 1, 4, and 7), 85°C (lanes 2, 5, and 8), and 93°C (lanes 3, 6, and 9) as described in Materials and Methods. The levels of the *cpkA* and *cpkB* transcripts were evaluated as the signal intensities of fragments amplified with the respective gene-specific primers. As a control, to ensure that the intensities directly reflected the initial levels of each transcript, the levels of 16S rRNA were examined.

(7). To investigate the amounts of mRNA of *cpkA* and *cpkB*, we performed quantitative analysis by RT-PCR using mRNAs isolated from *T. kodakarensis* grown in the logarithmic and stationary phases at 60°C, 85°C, and 93°C (Fig. 2). In *cpkA* during the logarithmic phase, no significant differences were observed in the patterns at all temperatures examined. However, at 85°C and 93°C, the signal intensity of the *cpkA* transcript decreased in the stationary phase. In the stationary phase in particular, the *cpkA* signal decreased as the cell cultivation temperature rose, and it almost disappeared at 93°C. As for *cpkB* in the logarithmic phase, its transcript was hardly detected at 60°C, but the level of the *cpkB*-specific transcript increased with upshifted growth temperatures, as shown in Fig. 2. A similar pattern was observed in the stationary phase. Growth phase-dependent expression was not observed in the transcriptional pattern of *cpkB*, but growth temperature-dependent expression was observed. We examined the *cpkB*-specific amount in the stationary phase by quantitative real-time PCR and observed that the transcriptional level at 93°C was 78-fold higher than that at 60°C (see Fig. S1 in the supplemental material). In the previous report, the expression levels were examined by using cells obtained from the stationary-phase condition (9). Focusing on both the logarithmic and stationary growth phases, the unique growth-dependent profiles of *cpkA* were observed, and regulation was shown to occur at the mRNA level.

Promoter analyses of *cpkA* and *cpkB*. As reported above, it has become clear that the expression of CpkA and CpkB is regulated at the transcriptional level, as for other known HSPs (31). We then compared the promoter regions of two chaperonin genes. The *cpkA* and *cpkB* genes show high sequence identity in their coding regions. However, the sequences of both genes vary in the region upstream from the initiation codon. Therefore, we expected that the difference between the upstream regions of *cpkA* and *cpkB* would contribute to their different manners of expression. To compare the promoter regions of *cpkA* and *cpkB*, their transcriptional start points were determined using RNA extracted from logarithmic-phase cells by the nonradioisotope-labeled primer extension method (8). As shown by the results in Fig. 3, the transcriptional start sites of *cpkA* and *cpkB* were identified at 32 and 67 nucleotides upstream of the initiation codon, respectively, indicating that
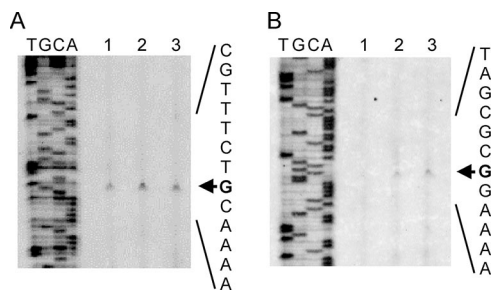
FIG. 3. Results of primer extension analyses for *cpkA* (A) and *cpkB* (B). Primer extension reactions were performed with total RNAs isolated from *T. kodakarensis* bacteria cultivated at 60°C (lane 1), 85°C (lane 2), and 93°C (lane 3) as described in Materials and Methods. The extended products were analyzed with sequencing ladders generated using the same primer and template (lanes A, T, G, and C). The transcriptional start sites are indicated by arrows.

cpkA and *cpkB* are transcribed from a single start point. The same start points were identified at all temperatures examined, and no additional points were identified. This indicates that multiple-promoter regulation was not involved in the temperature-dependent expression. The transcriptional machinery of archaea is generally similar to that of a eukaryote. The putative promoter region of *cpkA* and *cpkB* includes a transcription factor B (TFB)-binding element (CNANANC) and a TATA box (TTTAT/AA) (Fig. 4). In the heat shock response of a eukaryote, an HSF is known as a positive regulator of *hsp* genes that binds to the inverted repeat of heat shock element sequence NGAA (18). However, archaea do not have homologues of HSF- or heat shock element-like sequences. The regulation mechanisms of the heat shock responses of bacteria and eukarya are not applicable to archaea. The Phr-binding region is the only known archaeal heat-shock element that possesses three conserved regions, including the transcriptional start site (Fig. 4, conserved regions indicated by underlines). The Phr orthologue is also identified in *T. kodakarensis*

as TK2291 (5). The effect of Phr on *cpkB* induction was examined by using a *phr* disruptant named KHR1.

Effect of Phr on chaperonin gene expression. To investigate the effect of the *phr* gene disruption on the transcription of *cpkA* and *cpkB*, RT-PCR experiments were performed using a logarithmic-phase mRNA from KHR1. There was no significant difference between the mRNA amounts of *cpkB* in the wild type and KHR1 (Fig. 2). Phr is a heat-sensitive repressor and inhibits transcription by binding to DNA at lower temperatures (25). If the expression of *cpkB* were repressed by Phr binding, the level of *cpkB* would increase at 60°C in KHR1. The result obtained indicated that Phr is not involved in the heat-dependent expression of *cpkB*. The results of sequence analysis also indicate that Phr does not bind to the promoter region because the Phr-binding consensus was not identified (Fig. 4). The results of the experiments using the KHR strain also indicated that *cpkA* expression is not under Phr regulation.

Growth characteristics of *cpkA* or *cpkB* disruptants. To examine the physiological roles of *cpkA* and *cpkB* in *T. kodakarensis* cells, we constructed *cpkA* or *cpkB* disruptants (harboring Δ*cpkA* or Δ*cpkB*, respectively) using the strategy described in Materials and Methods. The genotypes of the disruptants were first confirmed by PCR analysis with primer sets that anneal inside or outside of the disrupted region. The expected shortening in the length of the amplified DNA was confirmed as mentioned in Materials and Methods. An internal primer that anneals within the coding region was also used to confirm the deletion, indicating the absence of the target on the disruptant's chromosome. It was estimated that the gene disruptant obtained would show growth defects in response to the growth temperature. Therefore, the *cpkA* disruptant (Δ*cpkA* *cpkB*[+]) and *cpkB* disruptant (*cpkA*[+] Δ*cpkB*) were precultivated at 93°C and 60°C, respectively, at which temperatures no growth defects were expected. These disruptants were then cultivated at 60°C, 85°C, and 93°C, and the growth profiles

FIG. 4. Sequence comparison of promoter regions of various heat shock elements. The transcription start sites that were determined are indicated by arrows. The positions of the TATA elements and TFB recognition element are indicated by boxes and dashed underlines, respectively. Phr recognition elements are underlined. Regions conserved between *cpkB* and *hsp60*s of *Pyrococcus* spp. are shaded. The transcriptional start sites are indicated by arrows. aaa[+] ATPase, ATPase associated with diverse cellular activities; sHSP, small HSP; *P. horikoshi*, *Pyrococcus horikoshii*; *P. abyssi*, *Pyrococcus abyssi*.
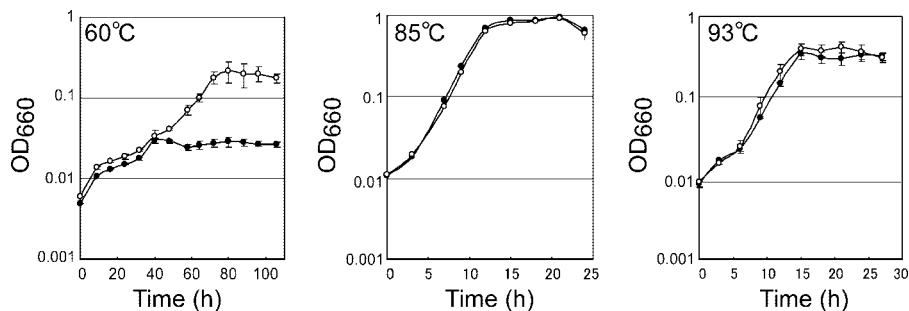
FIG. 5. Growth characteristics of the *cpkA* disruptant and the parental strain. Representative growth curves of the *cpkA* disruptant (Δ*cpkA* *cpkB*⁺) and KU216 (*cpkA*⁺ *cpkB*⁺). Cells were cultivated at 60°C (left panel), 85°C (center panel), and 93°C (right panel), respectively. Error bars show standard deviations. ○, KU216; ●, *cpkA* disruptant; OD$_{660}$, optical density at 660 nm.

were compared with those of wild-type strain KU216 (*cpkA*⁺ *cpkB*⁺) (Fig. 5 and 6). As for the effect of *cpkA* disruption, no obvious difference was observed between the growth of strain KU216 and that of the *cpkA* disruptant at 85°C and 93°C (Fig. 5, center and right panels). It was expected that CpkA would be required for initial cell growth at all temperatures because it was expressed in the logarithmic growth phase at all temperatures. However, a clear growth defect was observed only at 60°C (Fig. 5, left panel), and no growth lag was observed at 85°C and 93°C for the *cpkA* disruptant. In the *cpkA* disruptant, CpkB could probably be substituted for the CpkA. In contrast, the *cpkB* disruptant showed a growth defect only at 93°C, and no defect was observed at 85°C or 60°C (Fig. 6). It was concluded that CpkB participates in cell survival in a higher-temperature environment. The results obtained indicate that CpkA and CpkB are important for cell growth at lower and higher growth temperatures, respectively, than the optimum one.

DISCUSSION

The results of a previous immunoprecipitation study revealed that the subunit composition of the chaperonin in *T. kodakarensis* changes depending on the growth temperature (9). The results of the present study revealed that the subunit composition also changes depending on the growth phase because the expression level of CpkA is controlled by not only the growth temperature but also the growth phase. The increased ratio of CpkB to CpkA would enable the complex to be ther-

mostable, because the biochemical analysis of the chaperonin of another *Thermococcus* sp., *Thermococcus* sp. strain KS-1, which possesses identical α and β chaperonin subunits and has amino acid sequences identical to those of CpkA and CpkB, respectively, has revealed that the homooligomer consisting of only β subunits was more thermostable than that consisting of only α subunits (30). A mildly hydrophobic Gly-Gly-Met (GGM) repeat sequence of GroEL exists in the C-terminal amino acid sequence of CpkA but not in that of CpkB (10, 30). It has been reported that the protrusion of the flexible and mildly hydrophobic C-terminal GGM repeat sequence and repulsion effect from the negatively charged wall inside the chaperonin molecule were required for the rapid folding of substrate proteins (23). Therefore, the change in the composition of the chaperonin may cause a change in the substrate preference. However, no growth defect was observed in *cpkA* and *cpkB* disruptants at the optimal temperature, at which both CpkA and CpkB were expressed in the wild type. These results suggested another possibility, namely, that CpkA and CpkB are alternatives for each other at 85°C and that specific substrates for CpkA or CpkB seem to be produced at 60°C or 93°C, respectively.

We speculated that the expression level of an archaeal chaperonin is related to the cell growth conditions. In the present study, it has been shown that the level of CpkA expression changed depending on the growth phases at 85°C and 93°C. The expression may be correlated with the cell cycle rather than the growth phase. A synchronized culture is required to examine cell cycle dependency; however, this has not been
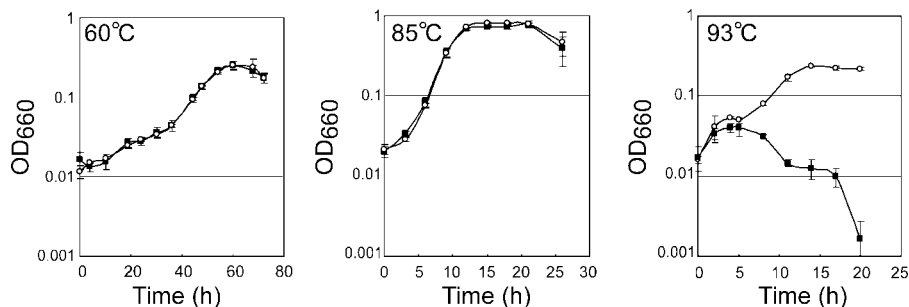

FIG. 6. Growth characteristics of the *cpkB* disruptant and the parental strain. Representative growth curves of the *cpkB* disruptant (*cpkA*⁺ Δ*cpkB*) and KU216 (*cpkA*⁺ *cpkB*⁺). Cells were cultivated at 60°C (left panel), 85°C (center panel), and 93°C (right panel), respectively. Error bars show standard deviations. ○, KU216; ●, *cpkB* disruptant; OD$_{660}$, optical density at 660 nm.

achieved for the hyperthermophilic anaerobic archaeon. Among archaea, cell cycle-specific gene expression has been analyzed in two microorganisms, the aerobic thermophilic archaeon *S. acidocaldarius* (16) and the halophilic archaeon *Halobacterium salinarum* (2). In *H. salinarum*, 87 genes out of 2,547 genes were found to be cell cycle regulated, and seven clusters were discovered in regulated genes (2). Among them, cluster five contained 34 genes with their highest transcriptional levels about 1.5 h after cell division. Several of them had a very low transcriptional level until the cell division was completed and were rapidly induced thereafter, similar to the M/G_1-induced genes found in eukaryotes (2). In the cluster, the chaperonin homologues named thermosomes (OE3925R and OE4122R), members of the HSP60 family, were identified. *H. salinarum* OE3925R shows higher sequence similarity to CpkA than to CpkB, suggesting that CpkA is also under cell cycle regulation.

As for *cpkB*, growth phase-dependent expression was not observed at any of the temperatures examined. Instead, the typical heat dependency was observed. By transcription start site mapping, a single start site was identified at 67 nucleotides upstream of the initiation codon (Fig. 3B), indicating that multiple-promoter regulation was not involved in the temperature-dependent expression of *cpkA* and *cpkB*. In *E. coli*, *rpoH*, encoding the heat shock regulator σ^H (σ^{32}), is transcribed in a complicated manner. *rpoH* transcription is regulated by multiple promoters and modulated by several factors, including two σ factors (σ^{70} [6] and σ^{24} [27]), the global regulator cyclic AMP receptor protein (12), and the DnaA protein (26). Such regulators are not involved in the archaeal system, because no homologues are found in the genomes. The transcriptional machinery of archaea is generally similar to that of a eukaryote. Both the TFB-binding element and a TATA box were identified in the promoter region (Fig. 4). The results of the RT-PCR experiment revealed that enhanced expression of *cpkB* at 93°C is not under Phr control (Fig. 1). When the promoter regions of *cpkB* and *Pyrococcus* HSP60 were compared, a typical conserved area was observed downstream of the TATA box, showing CAAANGAAC-N_8-GGAA (Fig. 4, conserved regions indicated by shading). It has been reported that the nucleotide alignment of the 5′-flanking regions of archaeal chaperonin genes in *Haloferax volcanii* showed a high degree of sequence conservation between positions −37 and +1, especially in and immediately surrounding the TATA element of the putative core promoter (24). The results of mutation and deletion studies of the promoter region of *H. volcanii* chaperonin indicated that the regulatory sequences involved in heat-induced transcription lie within the promoter region (24). We speculate that the specific region containing CAAANGAAC-N_8-GGAA participates in the heat-responsive regulation. The sequence of this element in *cpkA* is C<u>T</u>AAN GAA<u>G</u>-N_8-G<u>C</u>AA (different bases are indicated with underlining) (Fig. 4). The differences and the gap would be involved in heat inducibility. The unknown factor might bind this region to control the *cpkB* transcription.

The promoter region of *cpkB* shares sequence similarity with that of *Pyrococcus* HSP60, suggesting that they are all phylogenetically related and evolved from the same origin. However, the upstream region of *cpkA* shows less similarity with *Pyrococcus* elements. *cpkA* is considered to be a typical paralogue

of *cpkB* acquired in the course of evolution to adapt to a lower-temperature environment. In fact, the *cpkA* disruptant shows a growth defect at lower growth temperatures. CpkA might participate in the maintenance of protein structures of stationary-phase cells at lower cell growth temperatures. However, the manner in which CpkA is involved in cell cycle regulation or growth phase regulation is still unclear. To investigate this issue in detail, a trial to develop a synchronized culture for *T. kodakarensis* is needed.

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