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ORIGINAL PAPER

Differential regulation of *groESL* operon expression in response to heat and light in *Anabaena*

Hema Rajaram · Shree Kumar Apte

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Abstract The HrcA protein is known to bind the *cis*-element CIRCE and repress expression of *hsp60* in certain bacteria. However, recent data from cyanobacteria have seriously questioned the HrcA/CIRCE interaction paradigm. A *hrcA* null mutant showed constitutive expression of Hsp60 proteins [GroEL/Cpn60(GroEL2)], and an unexpected further increase in GroEL during temperature upshift, suggesting involvement of regulatory mechanisms other than HrcA in *groESL* expression in *Anabaena*. The negative regulation of both *hsp60* genes [*groEL* and *cpn60* (*groEL2*)] at CIRCE element was established by: (1) constitutive expression of Green Fluorescent Protein gene, tagged to *Anabaena hsp60* promoters, in *E. coli*, and its repression upon co-expression of *Anabaena* HrcA and (2) specific binding of *Anabaena* HrcA to the CIRCE element. Deletion analysis of other *cis*-elements further distinguished (a) a photo-regulation by the K-box and (b) thermoregulation from a novel H-box, over and above the negative regulation by HrcA at CIRCE.

Keywords Heat-regulation · HrcA · CIRCE · *groESL* · *cpn60* · *Anabaena*

Introduction

The heat shock response (HSR) entails rapid and increased expression of heat shock proteins (Hsps). The major heat-shock regulons in bacteria are (i) positively regulated by σ^{32} (RpoH) or (ii) negatively regulated by HrcA (Narberhaus 1999; Yura and Nakahigashi 1999). The RpoH protein in association with RNA Polymerase binds to σ^{32} promoters upstream of heat shock genes, thereby enhancing their expression during heat shock. Low RpoH level at ambient temperature is controlled at the transcriptional and translational level and by manipulation of its stability (Yura et al. 1993). The negative regulation by a repressor protein HrcA involves a consensus 9 bp inverted repeat sequence [TTAGCACTC-N_9-GAGTGCTAA] termed Controlling Inverted Repeat of Chaperone Expression (CIRCE) in close proximity to the promoter (Zuber and Schumann 1994). The HrcA binds to the CIRCE element preventing transcription of the downstream genes. During heat stress, the inability of denatured HrcA to bind to the CIRCE element derepresses transcription of the downstream genes (Roberts et al. 1996; Mogk et al. 1997). X-ray crystallographic studies have revealed that the native HrcA protein is a dimer (Liu et al. 2005). Synthesis of GroEL during heat stress regenerates HrcA dimers and shuts off HSR (Schumann et al. 1998). The presence of CIRCE element and the corresponding *hrcA* gene has been observed in over 40 bacteria, including the HrcA-based negative regulation at CIRCE has been demonstrated only in few bacteria (Narberhaus 1999).

Cyanobacteria neither possess a *rpoH*-like gene nor the *cis* elements similar to σ^{32}-like promoter and do not show the typical σ^{32}-based positive regulation of HSR. Deletion of three alternate sigma factors SigB, SigC and SigD in *Synechocystis* PCC6803 hampered growth at higher temperatures. Of these, SigB has been shown to be essential for *hspA* expression, while the downstream target genes of SigC and SigD are unknown (Tuominen et al. 2006, 2008). Recent studies on heat-shock regulation in cyanobacteria

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H. Rajaram · S. K. Apte (✉)
Molecular Biology Division,
Bhabha Atomic Research Centre, Trombay,
Mumbai 400 085, India
e-mail: aptesk@barc.gov.in

have identified three putative negative regulatory mechanisms: (i) the *hspA* gene of *Synechococcus vulcans* is negatively regulated by an upstream AT-rich imperfect inverted repeat sequence (ACAAgcAAA-N_x-TTTagTTGt), with the aid of a DNA-binding protein (Kojima and Nakamoto 2002), (ii) the mutation in *hik34*, a gene encoding a putative histidine kinase of *Synechocystis* PCC6803, constitutively expresses certain heat shock genes, suggesting their possible negative regulation by Hik34 protein (Suzuki et al. 2005, Slabas et al. 2006), and (iii) the *groESL* operon and the *cpn60* (*groEL2*) gene of *Synechocystis* PCC6803 (Chitnis and Nelson 1991; Lehel et al. 1993) harbour the CIRCE element and are negatively regulated by HrcA (Nakamoto et al. 2003) in this unicellular cyanobacteria. Additionally, two *cis*-elements termed, the N-box upstream of the *groESL1* gene of *Synechocystis* PCC6803 and the K-box found in the *hsp60* promoters and *dnaK*2 of *Synechocystis* PCC6803 and *Synechocuccus elongatus* PCC7942, appear to positively regulate *hsp60* expression in response to heat and light (Kojima and Nakamoto 2007; Sato et al. 2007). Occurrence of such elements upstream of the *hsp60* genes seems to differ in different cyanobacteria.

All cyanobacteria possess at least two or rarely three *hsp60* genes (Lund 2009). One gene, *groEL*, is found with its co-chaperonin-encoding gene, *groES*, in a bicistronic *groESL* operon; the other, *cpn60* (*groEL2*), is present as a single gene. Occurrence of *hrcA* gene and CIRCE in *hsp60* genes/operons of several cyanobacteria suggests that HrcA-based negative regulation of *hsp60* genes may occur in these microbes. However, the following recent observations have seriously questioned the paradigm of CIRCE/HrcA interaction in the regulation of cyanobacterial *hsp60* genes: (i) absence of CIRCE element upstream of some *groE* genes e.g. the *groEL2* in *Synechococcus vulcanus* that possess *hrcA* (Furuki et al. 1996), (ii) presence of CIRCE-like elements but absence of *hrcA* gene in certain marine cyanobacteria, e.g. *Prochlorococcus marinus* (http://genome.kazusa.or.jp/cyanobase), (iii) up-regulation of genes which do not possess CIRCE element in Δ*hrcA* mutants of *Synechocystis* PCC6803, e.g.. the *clpB2* gene (Nakamoto et al. 2003) or *pilA1*, *pilA2* and *dnaK2* genes (Singh et al. 2006), and (iv) the presence of additional *cis*-elements (N-box and K-box), which positively regulate *hsp60* expression in response to light and heat, irrespective of CIRCE and HrcA (Kojima and Nakamoto 2007). The CIRCE/HrcA-negative interaction thus appears to be neither exclusive nor universal among cyanobacteria.

The heat regulation of *hsp60* genes has not been investigated in filamentous heterocystous nitrogen-fixing *Anabaena* strains. The HrcA proteins from different *Anabaena* strains show 93–95% similarity of amino acid residues among themselves, but only 45–50% similarity with the HrcA of *Synechocystis* PCC6803 and about 18–25% with that of heterotrophic bacteria, wherein it is restricted to specific regions (http://blast.ncbi.nlm.nih.gov). Binding of a protein from cell-free extract of the thermophilic cyanobacterium *Thermosynechococcus elongatus* to CIRCE was revealed recently (Sato et al. 2008), though the identity of the binding protein was not established. Direct physical interaction of HrcA with CIRCE has never been demonstrated in cyanobacteria and deserves attention in view of the above facts. Additional *cis*-elements are also present upstream of the *groESL* operon in *Anabaena* strains. The K-box element is found upstream of the *groESL* operon, but not upstream of the *cpn60* (*groEL2*) gene, in *Anabaena* sp. strain PCC7120, hereafter referred to as *Anabaena* 7120, and *Anabaena* sp. strain L-31, hereafter referred to as *Anabaena* L-31. The N-box which contributes to both light- and heat-induction in *Synechocystis* is not detected upstream of the *groESL* operon of both *Anabaena* 7120 and *Anabaena* L-31 but is present upstream of the *cpn60* (*groEL2*) gene of *Anabaena* 7120 (Kojima and Nakamoto 2007). *Anabaena* L-31 harbours several unique additional direct/inverted repeats upstream of the *groESL* operon. The role(s) of these various elements in *hsp60* regulation remains to be explored in *Anabaena* strains. In this study, we demonstrate negative regulation of *hsp60* genes in *Anabaena* L-31, by direct binding of the HrcA dimer at the CIRCE element. A new negative heat-regulatory element designated as H-box has been identified along with light-specific positive regulatory K-box upstream of the *groESL* operon in *Anabaena* L-31.

Materials and methods

Organism and growth conditions

Escherichia coli cells were grown in Luria–Bertani (LB) medium at 37°C. Axenic cultures of *Anabaena* L-31 and *Anabaena* 7120 were grown in BG-11 liquid medium, pH 7.0 (Castenholz 1988) with (BG-11, N^+) or without (BG-11, N^-) combined nitrogen (17 mM $NaNO_3$) under continuous illumination (30 Me m^{-2} s^{-1}) and aeration (3 L min^{-1}) at 27°C. Heat-shock treatment involved exposure to 42°C. The different antibiotics used were 100 μg carbenicillin mL^{-1} (Cb_{100}), 34 μg chloramphenicol mL^{-1} (Cm_{34}) and 50 μg kanamycin mL^{-1} (Kan_{50}) for *E. coli* and 25 μg neomycin mL^{-1} (Neo_{25}) in BG-11 agar plates or 10 μg neomycin mL^{-1} (Neo_{10}) in BG-11 liquid medium for recombinant *Anabaena* 7120 strains.

PCR amplification and DNA electrophoresis

PCR amplification of DNA fragments involved genomic DNA of *Anabaena* L-31 (100 ng) and 1 μmole each of

Table 1 Primers and PCR products used in this study

Primer	Sequence*	RE
P_{gro}CFwd	5′ GCGAGCTCACGGGGCTGTTAAATCAG 3′	*Sac*I
P_{gro}DR10Fwd	5′ GCGAGCTCCACTGATAACTGTTAACT 3′	*Sac*I
P_{gro}IR11Fwd	5′ GCGAGCTCTGTCAGGTTATTTCCACT 3′	*Sac*I
P_{gro}DR12Fwd	5′ GCGAGCTCCAGTTATCAGTTGTCAGG 3′	*Sac*I
P_{gro}Fwd1	5′ GCGAGCTCCCGTACAACCAAAACA 3′	*Sac*I
P_{gro}Rev1	5′ GCGGTACCATTTCTCCAGAGTTAGCACTC 3′	*Kpn*I
P_{gro}Fwd2	5′ GCGAGCTCCACTGATAACTGTTAACT 3′	*Sac*I
P_{gro}Rev2	5′GCGGTACCGCTAATTTACCGAAAAAC3′	*Kpn*I
P_{cpn}Fwd	5′ GCGAGCTCCCTACATGAGCATCAAGTG 3′	*Sac*I
P_{cpn}Rev	5′ GCGGTACCCCTTTCTCTTGCGCTTCC 3′	*Kpn*I
*hrcA*Fwd	5′ GCCATATGCAAGTTCAGTTGACTAATC 3′	*Nde*I
*hrcA*Rev	5′ GCCTCGAGACTGAACGCTTCTGAGAG 3′	*Xho*I

* The underlined sequence denotes the restriction sites of the different restriction enzymes (RE) as indicated. *P* signifies that the PCR product represents a promoter fragment

Table 2 Plasmids used in this study

Plasmid	Description	Source/reference
pBluescriptSKII (pBS)	Cb^r, cloning vector	Stratagene
pUC19	Cb^r, cloning vector	Lab collection
pBS*npt*II	Cb^r, Kan^r, 1.1-kb *npt*II gene from pAM1956 cloned at *EcoR*V site of pBS	This study
pUC*hrcA*	End filled 1.1-kb *hrcA* PCR product cloned at *EcoR*V site of pUC19	This study
pUC*hrcA*$^-$	*npt*II gene inserted at *Xba*I site of pU*ChrcA*	This study
pET29a	Cm^r, expression vector	Novagen
pET*hrcA*	1.1-kb *hrcA* PCR product cloned between *Nde*I and *Xho*I sites of pET29b	This study
pAM1956	Kan^r, promoterless vector with *gfpmut2* reporter gene	(Yoon and Golden 1998)
P_{gro}C:*gfp*	P_{gro}C PCR product cloned at *Sac*I and *Kpn*I sites of pAM1956	This study
P_{gro}DR10:*gfp*	P_{gro}DR10 PCR product cloned at *Sac*I and *Kpn*I sites of pAM1956	This study
P_{gro}IR11:*gfp*	P_{gro}IR11 PCR product cloned at *Sac*I and *Kpn*I sites of pAM1956	This study
P_{gro}DR12:*gfp*	P_{gro}DR12 PCR product cloned at *Sac*I and *Kpn*I sites of pAM1956	This study
P_{gro}:*gfp*	P_{gro} PCR product cloned at *Sac*I and *Kpn*Iv sites of pAM1956	This study
P_{cpn}:*gfp*	P_{cpn} PCR product cloned at *Sac*I and *Kpn*I sites of pAM1956	This study

specified forward and reverse primers (Table 1), 100 μM dNTPs, 1U Taq DNA Polymerase in the Taq Buffer provided (Roche Diagnostics, Germany). DNA fragments were electrophoretically resolved on 0.8–1.2% agarose gels in TBE at 80 V for 2 h.

Cloning, overexpression and purification of *Anabaena* L-31 HrcA protein from *E. coli*

Amplification of *Anabaena* L-31 chromosomal DNA with *hrcA*Fwd and *hrcA*Rev primers (Table 1) yielded a 1.1-kb fragment. The ends of the PCR product were filled using dNTPs and Klenow enzyme and ligated to pUC19 vector at *EcoR*V restriction site. Restriction analysis ensured that the *hrcA* gene was cloned in the same direction as the *lacZ* promoter to achieve HrcA expression in *E. coli DH5α* (data not shown). The resulting construct was designated as pU*ChrcA* (Table 2). The gene sequence of *hrcA* was submitted to Gen Bank (Accession No. AY897588).

The 1.1-kb *Nde*I-*Xho*I fragment from pU*ChrcA* was ligated to pET29b at identical sites to obtain pET*hrcA* (Table 2) and transformed into *E. coli* BL-21(pLysS) cells. The cells were induced with 1 mM IPTG at 37°C for 1 h, then resuspended in lysis buffer containing 8 M urea and lysed using One Shot model Cell Disruptor (Constant Systems, UK). The HrcA protein was purified from the cell lysate using Ni^{2+}-nitrilotriacetic acid (NiNTA) column as per the manufacturer's protocol (Qiagen, Germany). HrcA protein was obtained in native form by gradual removal of urea by dialysis against decreasing concentration of urea at

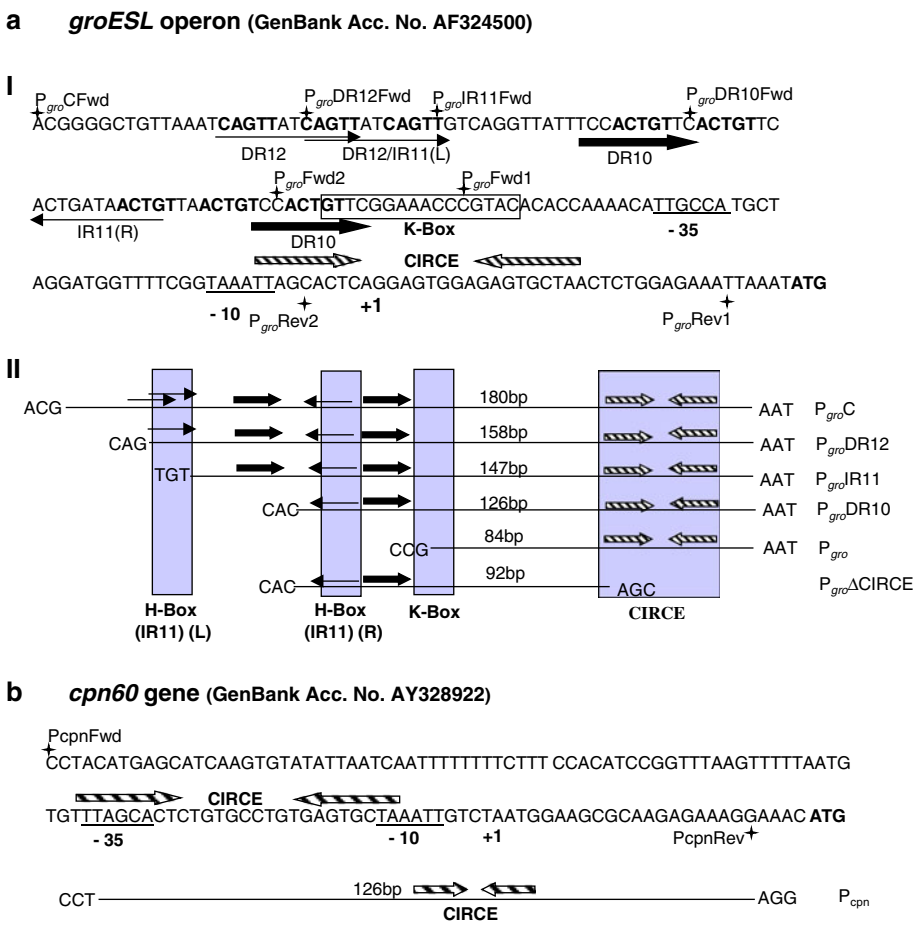

Fig. 1 Upstream DNA sequence of (**a**) *groESL* operon and (**b**) *cpn60* gene of *Anabaena* L-31. The CIRCE element [TTAGCACTC-N_x-GAGTGCTAA] is marked with *striped arrows*. The −35 and −10 regions of the putative σ^{70}-dependent promoter are *underlined*. The +1 indicates the putative transcription start sites. The first nucleotide of different forward and reverse primers is indicated by a *plus sign*. **a** (I) Nucleotide sequence of the upstream putative regulatory sequence of the *groESL* operon. The 12-bp direct repeats (DR12) [CAGTTATCAGTTATCAGTT] are marked with *arrows* pointing in the same direction (→). The 11-bp inverted repeat (IR11) [CAGTTAT-CAGT-N_x-ACTGATAACTG] is marked by *arrows* facing each other (→ and ←). The 10-bp direct repeat (DR10) [TCCACTGTTC-N_x-TC-CACTGTTC] is marked by *block arrows*. The *boldface* nucleotides represent the 5-bp direct repeats (CAGTT and ACTGT). The K-box sequence [TGTTCGGAAACCCGTA] has been *boxed*. (II) Different PCR products with their corresponding sizes and relative positions upstream of the *groESL* operon. The *left* (L) and *right* (R) arms of IR11 constituting the proposed H-box are marked in I and II. **b** Nucleotide sequence of the upstream region of *cpn60* gene

4°C. The purified HrcA protein was used to raise specific anti-*Anabaena* HrcA antibodies in rabbit.

Cloning of the putative regulatory regions of the *hsp60* genes

The upstream regulatory region of the *groESL* operon (Fig. 1a) was amplified from the cloned *groESL* operon of *Anabaena* L-31 (Rajaram et al. 2001) using P_{gro}Rev1 (Table 1) as a reverse primer in combination with P_{gro}CFwd, P_{gro}DR12Fwd, P_{gro}IR11Fwd, P_{gro}DR10Fwd and P_{gro}Fwd1 (Table 1) as the five forward primers. Positions of these primers are indicated in Fig. 1a, I. The different PCR products were designated P_{gro}C, P_{gro}DR12, P_{gro}IR11, P_{gro}DR10 and P_{gro}, respectively (Fig. 1a, II; Table 1). The *groESL* promoter element lacking the CIRCE element was generated by amplification with P_{gro}Fwd2 and P_{gro}Rev2 primers (Table 1; Fig. 1a), which resulted in a 92 bp PCR product, designated as P_{gro}ΔCIRCE. The upstream regulatory region of the *cpn60* (*groEL2*) gene was obtained by amplification of the cloned *cpn60* gene of *Anabaena* L-31 (Rajaram and Apte 2008) using P_{cpn}Fwd and P_{cpn}Rev primers (Table 1), and the resulting 125-bp PCR product was designated P_{cpn} (Fig. 1b). All the PCR products were cloned in pBluescript SKII vector and sequenced to confirm their identity with the original sequence.

The different promoter fragments were ligated to the vector pAM1956, at *Sac*I-*Kpn*I restriction sites. The vector

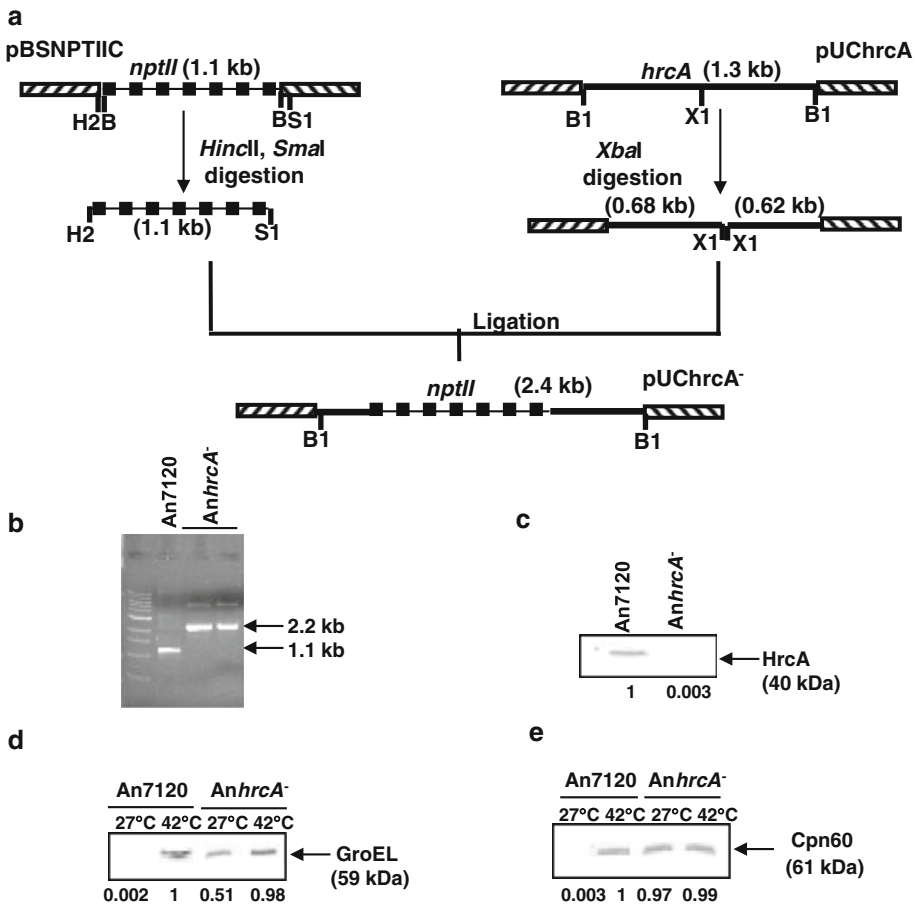

Fig. 2 Hsp60 expression in the *hrcA* mutant of *Anabaena* 7120. **a** Schematic representation of the construction of the plasmid pUC*hrcA*$^-$. The different restriction sites indicated are B: B*am*HI, S: *Sma*I and X: *Xba*I **b** PCR amplification of *Anabaena* 7120 and An*hrcA*$^-$ cells with *hrcA*Fwd and *hrcA*Rev primers. The PCR products obtained are indicated by *arrows*. **c** Immunodetection of HrcA. Protein extracts from three-day-old nitrogen-fixing cultures of *Anabaena* 7120 and An*hrcA*$^-$ were resolved by 10% SDS–PAGE and electroblotted. HrcA protein was immunodetected with an anti-AnHrcA antibody. **d** and **e** Immunodetection of Hsp60 proteins. Three-day-old nitrogen-fixing *Anabaena* 7120 (An7120) and An*hrcA*$^-$ cells were incubated either at 27°C or at 42°C for 16 h. Immunodetection was carried out with (**d**) anti-AnGroEL or (**e**) anti-AnCpn60 antibodies. Values below the *lanes* depict protein levels relative to wild-type *Anabaena* 7120 under control (27°C) conditions (**c**) and during heat stress (**d**, **e**)

pAM1956 has a promoterless *gfpmut2* gene coding for Green Fluorescent Protein (GFP) (Yoon and Golden 1998). The corresponding promoter constructs were designated P_{gro}C:*gfp*, P_{gro}DR12:*gfp*, P_{gro}IR11:*gfp*, P_{gro}DR10:*gfp*, P_{gro}:*gfp* and P_{cpn}:*gfp* (Table 2) and were maintained in *E. coli* strain DH5α, hereafter referred to as 'Ec'. For some experiments, *E. coli* clones carrying different promoter:reporter constructs were co-transformed with plasmids pUC*hrcA* or pUC19 and selected on $LBCb_{100}Kan_{50}$ plates. Such co-transformants were confirmed by cell-based PCR using appropriate corresponding primers (data not shown).

Mutagenesis of *hrcA* gene in *Anabaena* 7120

The pUC*hrcA* plasmid DNA was linearised with *Xba*I, ends filled and ligated with the 1.1-kb *Hinc*II-*Sma*I fragment of pBS*npt*II comprising the *npt*II gene (Fig. 2a). The resulting construct pUC*hrcA*$^-$ (Table 2) was electroporated into *Anabaena* 7120 as described earlier (Chaurasia et al. 2008), and the electrotransformants were selected on BG-11, N^+, Neo_{25} plates for several generations to allow complete segregation. The *hrcA* mutant of *Anabaena* 7120 thus obtained was designated An*hrcA*$^-$ (Table 3).

Transfer and expression of the promoter constructs in *Anabaena* 7120

The different promoter constructs and the empty vector, pAM1956, were conjugated into *Anabaena* 7120 using a conjugal *E. coli* donor [HB101(pRL623 + pRL443)] (Table 3) as described earlier (Elhai and Wolk 1988) and exconjugants selected on BG-11, N^+, Neo_{25} plates. The different transgenic *Anabaena* 7120 strains obtained were designated as AnP_{gro}C:*gfp*, AnP_{gro}DR12:*gfp*, AnP_{gro}IR11:*gfp*, AnP_{gro}DR10:*gfp*, AnP_{gro}:*gfp*, AnP_{cpn}:*gfp* and AnpAM (Table 3).

Fluorimetric analysis of GFP expression

Three-day-old *Anabaena* 7120 cultures were directly subjected to heat stress (42°C). The stressed and control cells were centrifuged, the pellet washed three times and resuspended in BG-11, N^- medium. Fluorimetric analysis was carried out using a Perkin Elmer Fluorimeter (Model LS50B) with excitation at 480 nm and emission measured at 510 nm. Turbidity (OD) of the cells was estimated at 750 nm. Promoter activity was measured as 'GFP fluorescence (arbitrary units) $(OD)^{-1}$.

Table 3 Strains used in this study

Strain	Description	Source/reference
E. coli strains		
DH5α	F$^-$ *recA*41 *endA*1 *gyrA*96 *thi*-1 *hsd*R17 (rk$^-$ mk$^-$) *supE*44 *relA*λ Δ*lacU*169	Lab collection
BL21(pLysS)	Cm^r F– *ompT* h_S dS_B (r_B^- m_B^-) *gal dcm* Novagen (DE3) pLysS	
Ec(pUC*hrcA*)	Cb^r, DH5α harbouring pUC*hrcA* plasmid	This study
Ec(P_{gro}:*gfp*)	Kan^r, DH5α harbouring P_{gro}:*gfp* plasmid	This study
Ec(P_{cpn}:*gfp*)	Kan^r, DH5α harbouring P_{cpn}:*gfp* plasmid	This study
Ec(pUC*hrcA* + P_{gro}:*gfp*)	Cb^r, Kan^r, DH5α harbouring pUC*hrcA* and P_{gro}:*gfp* plasmids	This study
Ec(pUC*hrcA* + P_{cpn}:*gfp*)	Cb^r, Kan^r, DH5α harbouring pUC*hrcA* and P_{cpn}:*gfp* plasmids	This study
Ec(pUC19 + P_{gro}:*gfp*)	Cb^r, Kan^r, DH5α harbouring pUC19 and P_{gro}:*gfp* plasmids	This study
Ec(pUC19 + P_{cpn}:*gfp*)	Cb^r, Kan^r, DH5α harbouring pUC19 and P_{cpn}:*gfp* plasmids	This study
HB101(pRL623 + pRL443)	Donor strain expressing cargo plasmid pRL623 (encoding methylase) and conjugal plasmid pRL443	(Wolk, C.P.)
Anabaena strains		
Anabaena L-31	Wild type	(Thomas 1970)
Anabaena 7120	Wild type	Lab Collection
An *hrcA*$^-$	Neo^r, *hrcA* mutant of *Anabaena* 7120	This study
AnP_{gro}C:*gfp*	Neo^r, *Anabaena* 7120 harbouring P_{gro}C:*gfp*	This study
AnP_{gro}DR10:*gfp*	Neo^r, *Anabaena* 7120 harbouring P_{gro}DR10:*gfp*	This study
AnP_{gro}IR11:*gfp*	Neo^r, *Anabaena* 7120 harbouring P_{gro}IR11:*gfp*	This study
AnP_{gro}DR12:*gfp*	Neo^r, *Anabaena* 7120 harbouring P_{gro}DR12:*gfp*	This study
AnP_{gro}:*gfp*	Neo^r, *Anabaena* 7120 harbouring P_{gro}:*gfp*	This study
AnP_{cpn}:*gfp*	Neo^r, *Anabaena* 7120 harbouring P_{cpn}:*gfp*	This study

Protein electrophoresis, western blotting and immunodetection

Proteins were resolved by 10% SDS–PAGE and electroblotted on positively charged nylon membranes (Roche Diagnostics, Germany), as described previously (Alahari and Apte 1998). Immunodetection was carried out with anti-AnGroEL, anti-AnCpn60 (Rajaram and Apte 2008) or anti-AnHrcA antibodies raised against the corresponding purified proteins of *Anabaena* L-31, hence the prefix 'An'.

Electrophoreticmobility shift assays

The 84-bp P_{gro} and 92-bp P_{gro}ΔCIRCE DNA fragments used for electrophoreticmobility shift assays (EMSA) (Fig. 1a) were 3′-end labelled using DIG-11-ddUTP and terminal transferase (Roche Diagnostics, Germany). Labelled DNA was incubated with purified HrcA protein from *Anabaena* L-31 for 20 min in binding buffer [25 mM HEPES, pH 7.9; 5 mM $MgCl_2$, 25 mM NaCl, 5 µg BSA and 0.5 µg poly (dI-dC)] (Koksharova and Wolk 2002). For supershift assay, anti-AnHrcA antibody was added to the mixture after 20 min of binding at 1:100 dilution and incubated for another 30 min. The DNA/HrcA or DNA/HrcA/anti-AnHrcA antibody mixtures were electrophoretically separated on 12% polyacrylamide gel in Tris–Acetate buffer, pH 7.4 at 4°C, blotted onto nylon membrane and detected by chemiluminescence as per the manufacturer's protocol (Roche diagnostics, Germany).

Statistical analyses

All experiments were repeated at least three times, and typical results from one experiment are shown. Variations between experiments were less than 10%. Each treatment comprised of three replicates, and mean ± SE values are shown**.**

Results and discussion

De-repression of Hsp60 proteins in *Anabaena* 7120 *hrcA* mutant

The conserved CIRCE element upstream of both the *groESL* operon (Fig. 1a) and *cpn60* (*groEL2*) gene (Fig. 1b) and the presence of *hrcA* gene are common occurrence in the genomes of different *Anabaena* strains including *Anabaena* L-31. In order to assess the involvement of *hrcA* in regulating *hsp60* expression, a *hrcA* mutant was

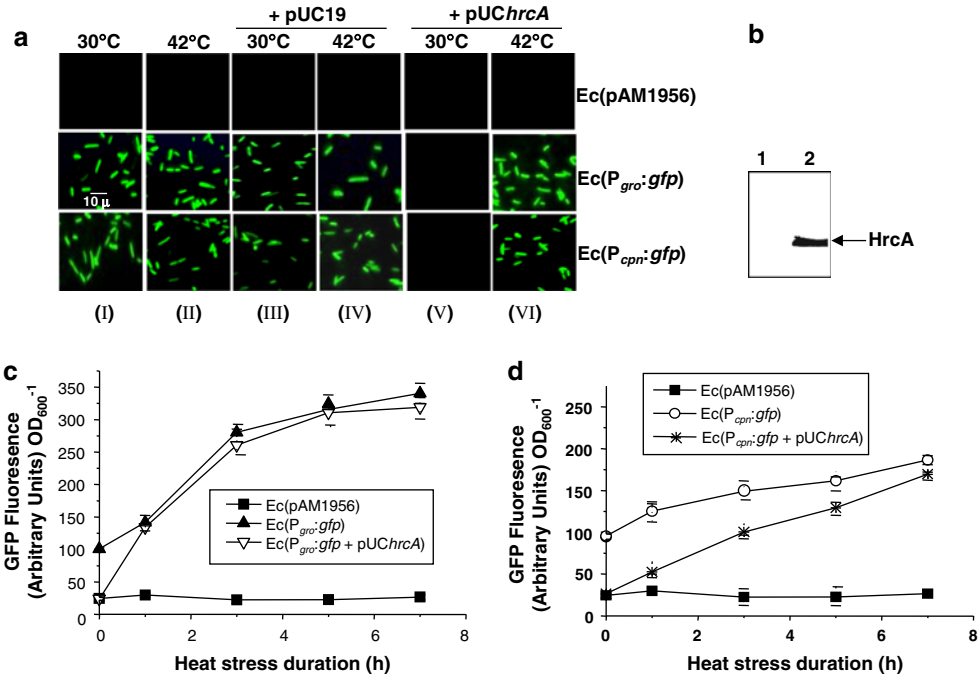

Fig. 3 Analysis of *Anabaena* L-31 *hsp60* promoters in *E. coli*. **a** Fluoromicrographs (×1,000 magnification) of *E. coli* DH5α (Ec) cells transformed with pAM1956, P_{gro}:*gfp* or P_{cpn}:*gfp*, and of cells co-transformed with pUC19 or pUC*hrcA*, at 30°C and 42°C. **b** Proteins extracted from Ec (pUC19) (*lane 1*) and Ec (pUC*hrcA*) (*lane 2*) were separated by 10% linear SDS–PAGE, followed by western blotting and immunodetection using anti-AnHrcA antibody. The 40-kDa HrcA protein is indicated by an *arrow*. **c** and **d** Fluorimetric analysis of GFP expression in *E. coli* DH5α (Ec) cultures transformed with *Anabaena* L-31 (**c**) *groESL* and (**d**) *cpn60* promoters and exposed to heat stress

constructed in *Anabaena* 7120 as shown in Fig. 2a. The mutant, *AnhrcA*$^-$, yielded a single DNA fragment of 2.2 kb upon PCR amplification with *hrcA*Fwd and *hrcA*Rev primers and lacked the 1.1-kb fragment corresponding to the native *hrcA* gene (Fig. 2b), indicating gene replacement. Immunodetection studies revealed that while the 40-kDa HrcA protein could be detected in wild-type *Anabaena* 7120, the corresponding protein was absent in the mutant, An*hrcA*$^-$ strain (Fig. 2c). The mutant cells expressed the 59-kDa GroEL (Fig. 2d) and the 61-kDa Cpn60 (GroEL2) (Fig. 2e) proteins at elevated levels under control (27°C) growth conditions, unlike *Anabaena* 7120 (Fig. 2d, e), suggesting that negative regulation of *hsp60* expression by HrcA occurs in *Anabaena* strains. An unexpected additional increase in the levels of the GroEL protein, but not of Cpn60 (GroEL2) protein, was observed upon heat stress in the An*hrcA*$^-$ cells (Fig. 2d, e). This suggested that, even the HrcA protein, may not be the sole regulator of the *groESL* operon and a second level of thermal regulation may operate. This was ascertained by: (a) assessment of negative regulation by HrcA at CIRCE and (b) deletion analysis of DNA elements from *groESL* promoter region.

Constitutive expression of *Anabaena* L-31 *hsp60* promoters in *E. coli* and their repression by *Anabaena* L-31 HrcA

To ascertain if HrcA of *Anabaena* L-31 can *per se* regulate *Anabaena* L-31 *hsp60* promoters, GFP expression driven from the *Anabaena* L-31 *groESL* (P_{gro}) and *cpn60* (P_{cpn}) promoters (Fig. 1b) was monitored in *E. coli*. *E. coli* does not have a *hrcA*-like gene, while the *Anabaena* L-31 *hsp60* genes have typical *E. coli* σ^{70}-like promoters. Thus, it provides a clean background, wherein the possible interaction between the *cis*-elements and *trans*-acting proteins of *Anabaena* L-31 can be unambiguously assessed. *E. coli* cells harbouring P_{cpn}:*gfp* and P_{gro}:*gfp* plasmids strongly expressed GFP both at 30°C (Fig. 3a, I) and during heat shock (Fig. 3a, II). Co-transformation of such cells with pUC*hrcA* resulted in (a) strong expression of *Anabaena* L-31 HrcA in *E. coli* as confirmed by immunodetection with anti-AnHrcA antisera (Fig. 3b) and (b) loss of GFP expression from both P_{gro}:*gfp* and P_{cpn}:*gfp* plasmids at ambient temperature (Fig. 3a, V). Co-transformation with empty pUC19 vector did not affect GFP expression (Fig. 3a, III and IV), indicating that the observed result is not due to low copy number of the pUC clones at 30°C. The HrcA repression of P_{gro} and P_{cpn} promoters was alleviated upon shift to higher growth temperatures (42°C) (Fig. 3a, VI).

The above results were also confirmed quantitatively by fluorimetric analysis (Fig. 3c, d), which clearly showed: (i) constitutive expression of GFP from both *hsp60* promoters in *E. coli* at 30°C (control), (ii) loss of *hsp60* promoter driven GFP expression at 30°C in *E. coli* co-expressing *Anabaena* L-31 HrcA, and (iii) restoration of *hsp60* promoter-driven GFP expression in *E. coli* cells expressing *Anabaena* L-31 HrcA, at 42°C (Fig. 3c, d). It may be argued that such results obtained in an unnatural host (*E. coli*) may not truly reflect the physiological regulation native to *Anabaena* strains. But the data shown in Fig. 3 strongly suggested that *Anabaena* HrcA can directly represses *Anabaena* L-31 *hsp60* genes at ambient conditions, and its inactivation during heat shock can result in

expression from both the *hsp60* promoters. Interestingly, in the absence of *Anabaena* L-31 HrcA, even recombinant *E. coli* showed over threefold induction of *Anabaena* L-31 P_{groESL} promoter by heat stress (Fig. 3c), confirming the data shown in Fig. 2d and suggesting the possibility of a second level of regulation independent of HrcA.

Binding of HrcA dimer to CIRCE element

The physical binding of HrcA to the *Anabaena* L-31 CIRCE element was also ascertained. A recombinant His_6-tagged *Anabaena* L-31 HrcA protein was purified from *E. coli*, its purity and identity ascertained by MALDI-ToF analysis–based peptide mass fingerprinting (UniProtKB/TrEMBL Acc. No. Q5EF74) and used in electrophoretic mobility shift assays (EMSA) (Fig. 4a, b). An 84-bp DNA fragment (P_{gro}, Table 1; Fig. 1a) from *Anabaena* L-31 containing the CIRCE element showed a concentration-dependent mobility shift with purified HrcA protein (Fig. 4a), but not with heat-denatured (65°C, 15 min) HrcA protein. *Anabaena* L-31 HrcA did not bind to a 92-bp (P_{gro}ΔCIRCE, Table 1) DNA fragment (Fig. 4a), which lacked the CIRCE element. A supershift with several slow migrating products was observed when anti-AnHrcA antibody was used along with HrcA protein in such assays (Fig. 4b), confirming that the observed mobility shift was indeed due to HrcA and not because of any co-eluting *E. coli* protein. The native HrcA protein displayed molecular mass of 81.2 kDa on Sephacryl S-400 gel filtration column suggesting that it was a dimer (Fig. 4c), unlike the heat-denatured HrcA (Fig. 4a), which corresponded to the monomeric molecular mass of 40.3 kDa (Fig. 4c), clearly indicating that HrcA dimer, but not its monomer, binds to the CICRE element.

Additional cis-elements regulate *groESL* expression of *Anabaena* L-31

In silico analysis of the nucleotide sequence upstream of *Anabaena* L-31 *groESL* operon revealed the presence of several direct and inverted repeats (Fig. 1a, I) including the heat/light-regulated K-box reported in *Synehcocystis* PCC6803 (Kojima and Nakamoto 2007). Some of these were unique to *Anabaena* L-31 and were not found in *Anabaena* 7120. Their possible role in regulation of the *groESL* operon was analysed by sequentially deleting one or more of the elements from the 5′ end (Fig. 1a, II) and evaluating their effect on GFP expression in *Anabaena* 7120 (Fig. 5).

Deletion of the first 7 bases of the left arm of DR12 as in P_{gro}DR12 had no significant effect on GFP expression in *Anabaena* 7120 at 27°C or 42°C (data not shown). Deletion of the left arm of IR11, as in P_{gro}IR11 (Fig. 1a), resulted in twofold increase in GFP expression under control conditions and a further five- to sixfold in heat due to HrcA denaturation (Fig. 5a, b). No further increase in GFP expression, either under control or during heat stress, was observed in light upon deletion of the left arm of DR10, as in P_{gro}DR10 (data not shown), or partial deletion of the K-box as in P_{gro} (Fig. 5a). These data suggested that the IR11 element acts as a negative regulatory element at ambient conditions, while the observed heat induction from all the constructs (Fig. 5a, b) could be attributed to alleviation of negative regulation by HrcA at CIRCE.

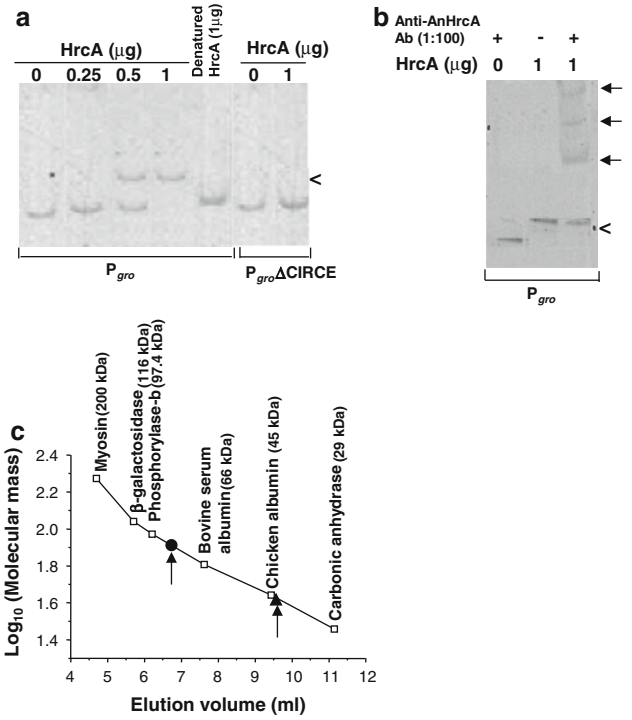

Fig. 4 Binding of *Anabaena* L-31 HrcA to the CIRCE element. **a** and **b** DIG-labelled P_{gro} or P_{gro}ΔCIRCE PCR products (8 ng per lane) were incubated with specified amounts of purified native or heat-denatured HrcA protein (**a**) or native HrcA protein with or without anti-AnHrcA antibody (**b**). The observed shifts (*arrowheads*) and supershifts (*arrows*) are shown. **c** Molecular sieve Sephacryl S-400 chromatography of native or heat-denatured HrcA with standard proteins. Elution volumes of eluted native HrcA (*circle*) and heat-denatured HrcA (*triangle*) are indicated by *arrows*

The K-box (described as MARS "multi-stress associated regulatory sequence" in *Synechococcus elongatus* PCC7942) has been implicated in heat/light regulation of *groESL* operon in *Synechocystis* 6803 (Kojima and Nakamoto 2007) and of *dnaK* gene in *Synechococcus elongatus* PCC7942 (Sato et al. 2007). K-box is found in *groESL* promoters of all *Anabaena* strains sequenced so far. The *groESL* promoters having the K-box (as in P_{gro}C and P_{gro}IR11) were differentially regulated in light and dark in *Anabaena* 7120, the expression being always higher in the presence of light (Fig. 5a, b). However, their expression became insensitive to light upon partial deletion of the K-box (as in P_{gro}) (Fig. 5a, b). This clearly showed that in

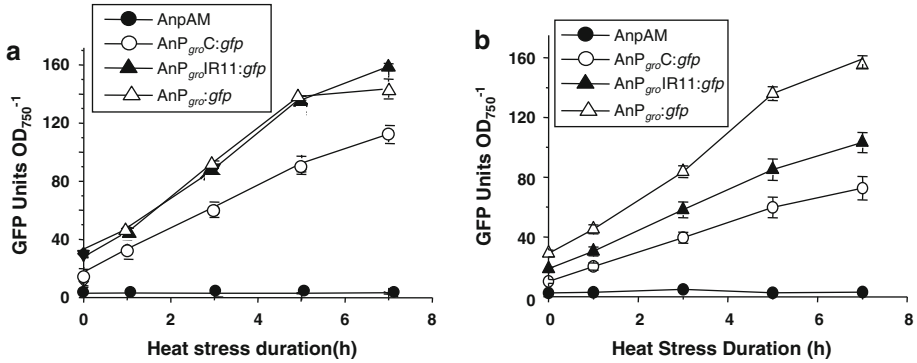

Fig. 5 Functional analysis of *groESL* promoter by GFP expression from different regulatory elements. Recombinant *Anabaena* 7120 (An) strains grown at 27°C carrying different promoter constructs were subjected to different duration of heat stress at 42°C, either in light (**a**) or in dark (**b**). GFP expression in various strains was estimated by fluorimetric analysis and expressed as arbitrary units per unit turbidity

Anabaena, the K-box acts solely as a positive light-responsive element. Repeated attempts to detect an H-box-binding protein from cell-free extracts or from a heparin-Sepharose purified preparation of DNA-binding proteins of *Anabaena* L-31 were not successful (data not shown). Based on the fact that in the absence of HrcA, the *Anabaena* L-31 *groESL* promoter showed heat-induction both in An*hrcA*$^-$ (Fig. 2d) and in *E. coli* (Fig. 3c), it is tempting to speculate that H-box may possibly function as a *cis*-element *per se* without the aid of a regulatory protein.

The present work has revealed complex regulation of the *groESL* operon in *Anabaena* involving: (a) negative regulation by (i) binding of dimeric HrcA repressor to CIRCE and by (ii) the novel H-box element which forms an inverted repeat, and (b) positive regulation by a light-responsive K-box element. In *Synechocystis* PCC6803, which lacks H-box, the K-box is implicated in both light and heat regulation of *groESL* operon (Kojima and Nakamoto 2007). In contrast in *Anabaena*, the responsibility appears to be shared between exclusively light-regulated K-box and heat-regulated H-box and CIRCE/HrcA system.

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