A Cold Shock-Induced Cyanobacterial RNA Helicase

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Received 14 October 1998/Accepted 13 January 1999

The ability to modify RNA secondary structure is crucial for numerous cellular processes. We have characterized two RNA helicase genes, crhB and crhC, which are differentially expressed in the cyanobacterium Anabaena sp. strain PCC 7120. crhC transcription is limited specifically to cold shock conditions while crhB is expressed under a variety of conditions, including enhanced expression in the cold. This implies that both RNA helicases are involved in the cold acclimation process in cyanobacteria; however, they presumably perform different roles in this adaptation. Although both CrhB and CrhC belong to the DEAD box subfamily of RNA helicases, CrhC encodes a novel RNA helicase, as the highly conserved SAT motif is modified to FAT. This alteration may affect CrhC function and its association with specific RNA targets and/or accessory proteins, interactions required for cold acclimation. Primer extension and analysis of the 5' untranslated region of crhC revealed the transcriptional start site, as well as a number of putative cold shock-responsive elements. The potential role(s) performed by RNA helicases in the acclimation of cyanobacteria to cold shock is discussed.

RNA helicases are single-stranded RNA-dependent ATPases that convert double-stranded RNA into single-stranded RNA through unwinding activity. These enzymes have been implicated in a diverse range of cellular processes including ribosome biogenesis, translation initiation, cell growth and differentiation, oogenesis, and oncogenesis (5, 28). RNA helicases comprise three related families of proteins based on the spatial and sequence conservation of eight amino acid motifs, including DEAD, DEAH, and DEXH, from which the family names are derived (6). Theoretically, RNA helicases may be involved in any process in which modulation of RNA secondary structure is required. In fact, putative RNA helicase-encoding gene sequences are ubiquitous, having been identified in organisms ranging from bacteria to humans, as well as in positive-strand RNA viruses (5, 28).

Although sequences encoding putative RNA helicases have been identified in a number of prokaryotic species, including those of the genus Bacillus (GenBank accession no. P42305), the domain Archaea (30), and the genus Synechocystis (12), they have been studied most extensively for Escherichia coli, whose genome encodes several RNA helicases belonging to all three families. Functions that have been identified for E. coli DEAD box RNA helicases include 23S rRNA function (dbpA [17]), ribosome biogenesis (smrB [18]), RNA turnover (rhlB [23]), and cold shock adaptation (csdA [9]). csdA expression increases upon a temperature shift from 37 to 15°C, with the 70-kDa CsdA protein being ribosome-associated at low temperatures and possessing helix-stabilizing activity (9). A function has not been ascribed to a fifth E. coli DEAD box RNA helicase, rhlE; however, a null mutant grows normally under a variety of conditions, implying that rhlE is not an essential gene (19).

RNA helicases have not been studied to date in the diverse, heterogeneous group of gram-negative photosynthetic prokaryotes, the cyanobacteria (29). Cyanobacteria are an excellent model system in which to study RNA helicase function as they are the ancestors of modern chloroplasts (7) and, in addition, perform a number of complex physiological phenomena involving cellular differentiation, such as aerobic nitrogen fixation and akinete formation (29). Evidence from other systems implicates RNA helicases in similar differentiation processes (5, 28).

Here we report the first molecular characterization of RNA helicase genes from a cyanobacterium, the filamentous, nitrogen-fixing cyanobacterium, Anabaena sp. strain PCC 7120 (referred to hereafter as Anabaena). The results indicate that Anabaena contains a minimum of two divergent RNA helicase genes, crhB and crhC, which exhibit expression under a broad range of conditions, and the novel RNA helicase gene, crhC, which is specifically expressed only under cold shock conditions.

MATERIALS AND METHODS

Organisms and culture conditions. Axenic cultures of Anabaena sp. strain PCC 7120, obtained from the University of Toronto Culture Collection (UTCC 387), were grown photoautotrophically at 30°C in BG-11 medium with a 16-h-light-8-h-dark cycle. Illumination was provided by fluorescent lamps at 150 microeinsteins m⁻² s⁻¹. Aeration was provided by continuous bubbling with air and shaking on a rotary shaker at 150 rpm.

DNA manipulation. Standard methods, as described elsewhere (26), were utilized for DNA manipulations including Southern blotting and in situ colony hybridization with the cloning vector pBluescript KS(+) (Stratagene) and the E. coli host, DH5α. For Southern blot analysis, genomic DNA, isolated from Anabaena (10), was transferred to a Hybond N membrane (Amersham). Hybridization was performed overnight at 60°C with either the crhB or the crhC PCR products labelled with [³²P]dCTP (Amersham) with a random-primer labelling kit (New England Biolabs). DNA fragments were sequenced on both strands with Sequenase version 2.0 (Amersham). DNA sequence analysis was performed with the University of Wisconsin Genetics Computer Group Sequence Analysis Software (GCG) programs, version 8.1.

PCR amplification. To isolate RNA helicase-encoding sequences, three degenerate PCR primers, based on conserved amino acid motifs found in five E. coli DEAD box RNA helicases (11), were synthesized (R as purine, Y as pyrimidine, and N as any of the four nucleotides): 5'-RTNTTYNGAYGARGC NGA-3' from the conserved motif VLDEAD, 5'-CNACN(C/A)NGGARYT NGC-3' from the conserved motif PTREL, and 5'-GNGCNACRCTNCNGT NGC-3' from the conserved motif ATDVAA. PCRs were performed in a reaction volume of 50 μl containing approximately 0.5 U of Taq DNA polymerase and each of the primers at a 1 μM concentration. The PCR program consisted of a 3-min denaturation-incubation at 94°C, followed by 20 cycles of 1 min of denaturation at 92°C, 1 min of primer annealing at 70°C, and 1.5 min of primer

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extension at 72°C and then by 20 cycles of 1 min of denaturation at 92°C, 1 min of primer annealing at 60°C, and 1.5 min of primer extension at 72°C, and terminated with a final incubation of 5 min of primer extension at 72°C. The annealing temperature was initially 70°C and was dropped by 0.5°C per cycle during the first 20 cycles. The correct-sized PCR products were purified from agarose gels and ligated into pcRII (Original TA cloning kit; Invitrogen). Cloned PCR products were subjected to Southern blot hybridization with the tobacco DEAD box RNA helicase gene, NeIF-4A2 (20), as probe.

Cloning *Anabaena* *crh* genes. Libraries containing *XbaI*-digested *Anabaena* genomic DNA ranging from 3 to 5 and 5 to 10 kb were constructed by ligation into *XbaI*-digested and dephosphorylated pBluescript KS (+) (Stratagene). The libraries were first screened by Southern blot hybridization with either the cloned *crhb* or *crhc* PCR fragments, followed by in situ colony hybridization (26).

**Northern analysis.** Total *Anabaena* RNA was isolated from liquid cultures (27) grown under the stated conditions. Denatured RNA (15 μg) was size fractionated on 1.2% formaldehyde-agarose gels and transferred to a Hybond N+ membrane (Amersham) (26). Hybridization was performed overnight at 65°C in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na₂HPO₄, and 1 mM EDTA [pH 7.7])–0.2% sodium dodecyl sulfate (SDS)–5× Denhardt's solution with antisense probes generated from either the *crhb* or the *crhc* PCR-cloned products labelled with [³²P]UTP (Amersham) with a Riboprobe kit (Promega). A 500-bp *EcoRI*-HindIII fragment encoding the *Anabaena* RNA Nase P gene was radiolabelled as described for DNA manipulations and used to probe Northern blots overnight at 65°C in aqueous buffer according to the manufacturer's protocol (Amersham). Blots were washed for 15 min at 65°C once in ×1 SSPE-0.1% SDS and twice in ×0.1× SSPE-0.1% SDS. Blots were stripped and reprobed according to the manufacturer's protocol.

**Primer extension analysis of the *crhc* transcript.** Total RNA was isolated from *Anabaena* cells grown under cold shock-inducing conditions for 3 h, as described above. Primer extension reactions were performed (26), with 50 μg of RNA and ×10× edpm of [³²P]ATP end-labelled primer. The primer was a 19-base oligonucleotide, 5′-CGTCTCTGATAAGACCGAGC-3′, corresponding to the noncoding strand of the *crhc* gene beginning 98 nucleotides downstream of the initiator ATG. Primer extension products were separated on 6% sequencing gels in parallel with sequencing reactions of the *crhc* gene performed with the same primer.

**Nucleotide sequence accession numbers.** The nucleotide sequences reported in this study have been submitted to the EMBL data bank under the accession no. AF040044 (*crhb*) and AF040045 (*crhc*).

## RESULTS

**PCR cloning of RNA helicase gene fragments from *Anabaena*.** Sequencing of cloned PCR products which hybridized with the tobacco DEAD box RNA helicase gene, NeIF-4A2 (20), revealed three clones with significant sequence similarity to DEAD box RNA helicases. Two of the clones contained identical 758-bp inserts, named *crhb* (for cyanobacterial RNA helicase cold), while the third contained a 348-bp insert, named *crhc*. Identical 4.3-kb inserts, containing identical 348-bp inserts, named *crhb*, *crhc* and *crhb* encode distinct RNA helicase-related amino acid sequences. *Crhb* exhibits 32% identity and 51% similarity at the deduced amino acid level to the corresponding region of CrhC. *Crhc* is a novel RNA helicase gene, as it encodes a FAT motif, a unique alteration of the strictly conserved SAT motif, found in all but one other DEAD box RNA helicase.

**Gene copy number determination.** Southern blot analysis indicated that both *crhb* and *crhc* genes are present as single distinct gene copies in the *Anabaena* chromosome (Fig. 1).

**Cloning and sequence analysis of a *crhc* genomic clone.** Two full-length *crhc* clones, containing identical 4.3-kb inserts, were isolated from size-fractionated *XbaI* genomic libraries with the cloned *crhc* PCR fragment as a probe. A physical map of one *crhc* clone, pWM75, indicated that the open reading frame was located on an internal *HindIII*-EcoRV fragment (data not shown). Sequence analysis indicated that this 1,964-bp fragment contained a 1,275-bp open reading frame, encoding a 425-amino-acid polypeptide having a predicted molecular mass of 47,481 Da and a pI of 10.79 (data not shown). Crhc contains seven of the conserved amino acid motifs (Fig. 2, thick bars) diagnostic of all DEAD box RNA helicase proteins in terms of both sequence and spatial orientation. The eighth motif, the SAT box, is present as a FAT motif in Crhc (Fig. 2, arrowhead).

The deduced *Anabaena* Crhc gene product exhibits similarity to a number of established and putative RNA helicases (Fig. 2). Crhc is most closely related to the *E. coli* RNA helicase, RhlE, having 74% similarity (60% identity). The partial Crhb sequence exhibits the highest similarity, 70% (64% identity), with the corresponding region, amino acids 153 to 268, of a putative RNA helicase gene in *Synechocystis* sp. strain PCC 6803 (12) (data not shown). *Crhb* and *Crhc* are as equally related to other DEAD box RNA helicase sequences with which they have 55 to 60% similarity (36 to 39% identity) as they are to each other.

Numerous attempts to isolate a full-length *crhb* genomic clone were not successful, apparently as a result of toxicity of the cloned gene in *E. coli*.

**crhb gene expression.** Northern blot analysis indicates that the *crhc* transcript is specifically expressed in cells which have been temperature shifted from 30 to 20°C, i.e., under cold shock conditions (Fig. 3A, lane 2). The *crhb* transcript was differentially expressed under any condition in which the cells were grown in the light, including stimulation by cold shock (Fig. 3B, lane 2). RNA P hybridization indicates that the presence or absence of hybridizing transcripts for *crhc* or *crhb* is not due to RNA loading (Fig. 3C).

**Identification of the *crhb* transcription start site.** To identify the promoter region responsible for cold shock induction of *crhc* transcription, total RNA isolated from cold shock-induced *Anabaena* cells was subjected to primer extension analysis. Total RNA from noninduced cells was also subjected to primer extension analysis as a control. A primer extension product was detected only in RNA isolated from cold shock-induced cells, as an intense GG doublet (Fig. 4, lane 2). The proximal G residue, located 115 nucleotides upstream of the *crhc* translational start codon, was designated as the transcriptional start site (Fig. 5, asterisk). A minor signal corresponding to a G residue 120 nucleotides from the *crhc* translational start codon was also observed. Although the importance of this second potential transcription initiation site is not known, a variety of cyanobacterial genes have been shown to have multiple start sites (3). A primer extension product was not observed in RNA isolated from non-cold-shock-induced cells (Fig. 4, lane 1), in agreement with the Northern blot analysis (Fig. 3A).

**Sequence analysis of putative *crhc* regulatory elements.** Analysis of the *crhc* promoter for transcriptional elements indicates that a σ⁷₀-dependent –10-like sequence, TAAGT, is present beginning 9 nucleotides upstream of the transcription start site (Fig. 5, boldface). A –35-like region within the
space constraints of typical *E. coli* promoters was not observed, as expected for a gene which is not expressed under normal growth conditions. An AT-rich sequence resembling an upstream enhancer element (16) is located at positions −53 to −65 (Fig. 5, underlining). A putative *E. coli*-like cold shock box involved in transcriptional attenuation is located at positions +87 to +97 (Fig. 5, thickly outlined box). The *Anabaena* cold shock box, 5'−TGCAAGGCCGA−3', matches the *E. coli* cold shock box (5'−TGACGTACAGA−3') (8) at 7 of 11 positions.

Analysis of the sequences flanking the initiator methionine residue (position +116 from the transcription initiation site) for translational activators indicates that the 5' untranslated region (UTR) possesses a putative *E. coli*-like Shine-Dalgarno sequence 3 nucleotides upstream of the translation initiation codon with six of eight residues base pairing (Fig. 5, double underlining) with the *Anabaena* 16S rRNA (14). The 16S rRNA-**crhC** mRNA base-pairing potential continues downstream from this site, through the translation initiation codon, up to a 15-nucleotide sequence beginning at position +137, in which 9 of 15 nucleotides are base paired (Fig. 5, thinly outlined box). This region strongly resembles a downstream box (16) involved in the translational activation of cold shock-induced *E. coli* genes. The 5' region of **crhC** from −115 to +71 is also predicted to fold into a complex, stable secondary structure, with a ΔGo of −35.4 kcal/mol (data not shown). In this structure, the AUG is located in a loop while the proposed Shine-Dalgarno sequence and the downstream box are partially base paired.

The 3' UTR contains a 22-nucleotide stem-loop sequence corresponding to a weak rho-independent transcriptional terminator consisting of an 8-bp GC-rich stem and a 6-base loop structure followed by a string of four T residues. This potential transcriptional terminator is located 26 nucleotides downstream of the translational stop codon (data not shown). Similar sequences have been shown to be involved in transcription termination and/or mRNA stability in prokaryotic organisms (24).

**DISCUSSION**

We report the first molecular characterization of cyanobacterial RNA helicase genes. The results indicate that the *Anabaena* genome possesses two distinct genes, as confirmed by Southern and Northern analyses. At this point, we cannot discount the possibility that additional, more divergent cyanobacterial RNA helicase genes exist in *Anabaena*, since **crhC** and **crhB** do not hybridize. Divergent, multimember DEAD box RNA helicase gene families exist in other organisms, including *E. coli* (11) and tobacco (1, 20, 21).

Deduced amino acid sequence analysis did not provide clues to the function performed by the **crhC** gene product, as the most closely related gene, the *E. coli rhlE* gene, has not been characterized. Amino acid analysis did reveal, however, that CrhC is a novel DEAD box RNA helicase, as it possesses a
FAT box instead of a diagnostic SAT box. The Ser-to-Phe modification can be accounted for by a C-to-T transition, from a TCC Ser codon to a TTC Phe codon. DEXH box RNA helicases also contain a modified SAT box, a TAT motif (5). In this case, however, the replacement of Ser with Thr is conservative, as both amino acids are polar and uncharged. A putative tobacco DEAD box RNA helicase, NeIF-4A12, also contains a modified SAT motif, a YAT box (1). In this case, the Ser-to-Thr modification also results from a transition at the second base of the codon. Interestingly, in both NeIF-4A12 and CrhC, the modified amino acid is aromatic and thus hydrophobic, a significant departure from the polar, uncharged Ser or Thr residues normally present at this position.

The FAT alteration may have significant effects on the mechanism by which CrhC functions. In vitro and in vivo mutational analyses of mammalian and yeast eIF-4A have implicated the SAT box in coupling the ATPase and RNA helicase activities (22, 28). Crystal structure analysis of a viral DEXH RNA helicase supports these results (2) and led to the proposal that the TAT motif acts as a flexible hinge linking the active sites for ATPase and helicase activities. This would involve hydrogen bond switching between the His residue in the DEXH motif, which is involved in the ATPase activity, and either of the two Thr residues present in the TAT motif, involved in RNA helicase activity. This scenario would not be possible in CrhC, as Phe is not capable of hydrogen bond formation. This implies a more rigid CrhC protein which may limit the RNA substrates with which it can interact.

It is interesting that crhC expression is specifically induced upon a downshift in temperature of 10°C but is not induced by a broad range of other stress conditions. This indicates that crhC is not a general stress-induced gene. The cold shock-specific expression of crhC is similar to that of the E. coli RNA helicase gene cshA (9); however, CrhC is not a CshA homologue, as CrhC most closely resembles the E. coli RNA helicase RhIE. This implies that cold acclimation in prokaryotes involves RNA helicase activity; however, the helicase need not be conserved between genera.

The cold shock-induced gene expression of crhC may be regulated by a number of elements in both the promoter and the translation initiation codon regions. On the transcriptional level, crhC contains both an AT-rich upstream element which functions as a transcriptional activator for cspA in E. coli (16) and a cold shock-like box which is involved in transcriptional attenuation of cspA in E. coli (8). On the translational level, crhC may be regulated negatively by its relatively long, highly structured 5' UTR (115 nucleotides), similar to that observed in the extended autoregulatory 5' UTRs found in the E. coli cold shock-induced genes, cspA, cspB, and cshA (4, 8). CrhC also encodes a downstream element complementary to the 3' end of the 16S rRNA. Similar downstream elements are essential for translation of cold shock-induced mRNAs in E. coli (16).

The cold shock induction of RNA helicase gene expression represents a novel gene family which is induced upon cold acclimation in cyanobacteria. Other cyanobacterial gene families that have been shown to be cold shock induced include those encoding RNA-binding proteins in Anabaena (27) and fatty acid desaturases in Synechococcus (25) and Synechocystis (15). In these cold shock-induced cyanobacterial gene families, as with the crh gene family in Anabaena, regulation of expression is gene specific. The presence or absence of crhC

![FIG. 3. Northern analysis of crhC and crhB expression. A Northern blot of total RNA extracted from Anabaena grown under the stated conditions was hybridized with crhC (A). The blot was consecutively stripped and probed with crhB (B) followed by the Anabaena RNase P gene (C). The resulting autoradiograms are shown. Lane 1, 22 h in the absence of fixed nitrogen; lane 2, 3 h at 20°C; lane 3, 30 min at 44°C; lane 4, 3 min of UV illumination, followed by 3 h in the dark; lane 5, 3 h in the presence of 0.02% methanesulfonic acid methyl ester; lane 6, 3 h in the presence of 90 μg of nalidixic acid per ml; lane 7, 3 h in the presence of 5 μg of mitomycin C per ml; lane 8, 3 h in the presence of 0.5 M NaCl; lane 9, 3 h in the dark; lane 10, cells grown continuously at 30°C in the light. Cells for the experiments whose results are shown in lanes 1, 2, and 10 were grown continuously in the light, while cells were grown in the light and transferred to the dark during the course of the experiments whose results are shown in lanes 3 to 9.](http://jb.asm.org/)

![FIG. 4. Primer extension analysis of the transcription start site. An autoradiogram shows primer extension experiments performed with 50 μg of RNA extracted from Anabaena grown continuously at 30°C (lane 1) or for 3 h at 20°C (lane 2). Lanes A, C, G, and T are sequencing reactions performed on crhC with the primer extension oligonucleotide. An arrow indicates the hybridizing extension product corresponding to the G residue taken to be the start of transcription.](http://jb.asm.org/)
scripts is similar to that observed for desB (15, 25), while the constitutive but cold-enhanced expression of crhB is similar to that of desA (15, 25) and rpbB and rpbC (27).

The characteristics of the crhC promoter and crhC expression are consistent with a class I cold shock gene (31), while the cold shock enhancement of crhB expression implies that it is a class II cold shock gene. In this scenario, CrhC and CrhB would perform distinct roles in the acclimation of cyanobacteria to the cold shock state. These roles could involve an interaction of the RNA helicases with cold-induced RNA substrates, the ribosome, and/or accessory proteins required for cold acclimation. Specifically, RNA helicases could remove cold-stabilized secondary structures in cold shock mRNAs, thereby overcoming the cold-induced blockage of translation initiation under cold shock conditions (31).

In conclusion, our results indicate that the RNA helicase genes characterized in this report are differentially expressed in Anabaena. While crhB expression is enhanced in the cold, crhC expression is limited specifically to cold shock conditions. This implies that both RNA helicases are involved in the cold acclimation process in cyanobacteria; however, they appear to perform different roles in this adaptation. Division of labor between RNA helicases in a model prokaryotic organism like a cyanobacterium will provide an ideal system in which to study the factors controlling the expression of and the physiological functions(s) performed by RNA helicases. We are currently investigating the physiological and enzymatic functions performed by the crh RNA helicase genes in Anabaena.

ACKNOWLEDGMENTS

We are grateful to M. Pickard for providing Taq polymerase and A. Voque for providing the Anabaena RNAse P gene.

This work was supported by a Natural Sciences and Engineering Research Council (NSERC) of Canada postgraduate scholarship to W.C.M. and an NSERC operating grant to G.W.O.

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