Redox Control of ntcA Gene Expression in *Synechocystis* sp. PCC 6803. Nitrogen Availability and Electron Transport Regulate the Levels of the NtcA Protein

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In this work we have studied the influence of the cellular redox status in the expression of the *Synechocystis* sp. PCC 6803 ntcA gene. Two different ntcA transcripts with different 5’ ends were detected, depending on the different dark/light or nitrogen availability conditions. Accumulation of a 0.8-kb ntcA message was light and nitrogen dependent, whereas a longer 1.2-kb ntcA transcript was neither light nor nitrogen regulated. NtcA protein levels increased concomitantly with the accumulation of the 0.8-kb ntcA transcript. The light-dependent accumulation of the ntcA gene and the NtcA protein was sensitive to electron transport inhibitors. In addition, Glc-grown *Synechocystis* sp. cells showed a similar ntcA expression pattern in darkness to that observed under illumination. These data suggested that electron transport, and not light per se, may regulate ntcA gene expression. Primer extension analysis, together with gel mobility-shift assays, demonstrated that in vitro, the *Synechocystis* sp. NtcA protein specifically bound to the putative promoter region from the light/nitrogen-dependent ntcA transcript but not to that from the constitutive 1.2-kb ntcA mRNA. Band-shift experiments carried out in the presence of thiol oxidizing/modifying agents and different reducing/oxidizing conditions suggested that NtcA binding to its own promoter was under a thiol-dependent redox mechanism. Our results suggest that the cellular redox status plays a central role in the autoregulatory mechanism of the NtcA protein.

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis like higher plants and algae. Most cyanobacteria are able to use nitrate or ammonium as a nitrogen source and some strains are capable of dinitrogen fixation (for review, see Flores and Herrero, 1994). The nitrogen assimilation process is linked to light and photosynthesis. Reduced ferredoxin acts as electron donor to the nitrate and nitrite reductases and reducing power is necessary for the action of glutamine synthetase (GS) and NADH glutamate synthase (GOGAT). This strong coordination also occurs at the molecular level. Both light and nitrogen regulate the expression of genes involved in the nitrogen assimilation metabolism like *glnA* and *glnB* at the transcriptional level (Reyes and Florencio, 1995; García-Dominguez and Florencio, 1997; Reyes et al., 1997). Our understanding of the molecular mechanism of nitrogen control in cyanobacteria has significantly increased during the last years. Several pleiotropic mutants from *Synechococcus* sp. PCC 7942, impaired in the expression of genes involved in nitrate assimilation, allowed the identification of one gene, *ntcA*, that encodes a polypeptide, which acts as a positive transcriptional activator of genes subjected to nitrogen control (Vega-Palas et al., 1990). The *ntcA* gene is widespread in cyanobacteria (Frias et al., 1993) and seems to be highly conserved in all nitrogen-fixing or non-fixing, unicellular or filamentous strains (Frias et al., 1993; Wei et al., 1994; Bradley and Reddy, 1997). NtcA is a DNA-binding protein belonging to the cAMP receptor protein family of transcriptional activators (Vega-Palas et al., 1992). It is required for the full expression of genes subjected to ammonium repression in *Synechococcus* sp. PCC 7942 (Luque et al., 1994). In a similar way, a ntcA mutant from *Anabaena* sp. PCC 7120 requires ammonium for growth and is defective for heterocyst formation (Ramasubramaniam et al., 1994; Wei et al., 1994). In these two strains, NtcA binds to its own promoter and autoregulates its expression in response to nitrogen availability (Luque et al., 1994; Ramasubramaniam et al., 1996). In *Synechocystis* sp. PCC 6803, it has been shown that NtcA controls the expression of the *glnA* and *glnB* genes depending on nitrogen availability conditions (García-Dominguez and Florencio, 1997; Reyes et al., 1997). However, the regulation of the *ntcA* gene in this strain remains still unknown.

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Besides a role in nitrogen assimilation, the NtcA protein has been reported to interact with the promoter fragments of several genes not directly related with nitrogen assimilation or metabolism: the *Synechocystis* sp. PCC 6803 *icd* gene, coding for the isocitrate dehydrogenase (Muro-Pastor et al., 1996); the *Anabaena* sp. PCC 7120 *rbcL* gene encoding for the large subunit of Rubisco (Ramasubramaniam et al., 1994); and the *gor* gene, coding the antioxidant defense enzyme glutathione reductase (Jiang et al., 1997) or the *petH* gene coding for the ferredoxin NADP+-reductase (Valladares et al., 1999). Some of the NtcA up-regulated genes (like *glnA* or *glnB*) have been shown to be under electron transport control in *Synechocystis* sp. PCC 6803 (Reyes et al., 1995; García-Dominguez and Florencio, 1997). In parallel, studies carried out in *Anabaena* sp. PCC 7120 showed that the mechanism by which NtcA binds to the *gor* promoter was regulated in vitro by a redox-dependent mechanism involving Cys residues of the NtcA protein (Jiang et al., 1997). These data open the question whether NtcA is involved exclusively in nitrogen control or may be involved in other regulatory processes depending on other regulatory signals. However, no environmental factors other than nitrogen have been reported to modulate *ntcA* gene expression in cyanobacteria up to date.

The aim of this work was to study how changes in the cellular redox status of the cell induces changes in the expression of the *ntcA* gene in *Synechocystis* sp. PCC 6803. The redox state of both photosynthetic and respiratory electron transport chains was varied by changing light and nutrient regimes and by addition of different electron transport inhibitors. We describe here the influence of the availability of nitrogen and light in the expression of the *ntcA* gene. Our results indicated that the cellular redox state, and not light per se, influences the levels of the regulated 0.8-kb *ntcA* mRNA and the concomitant accumulation of the NtcA protein. The binding of the NtcA protein to its own promoter seems to be also influenced by the redox state in vitro. The role of the cellular redox status in the mechanism by which NtcA autoregulates its own expression and initiates its regulatory cascade will be discussed.

**RESULTS**

**Effect of Nitrogen Source and Nitrogen Availability on ntcA Transcript and NtcA Protein Levels**

Nitrogen control of *ntcA* gene expression has been described in *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7120 (Luque et al., 1994; Ramasubramaniam et al., 1996) and more recently in the unicellular nitrogen-fixing cyanobacterium *Cyanothece* sp. BH68K (Bradley and Reddy, 1997). However, no data are available about the nitrogen control mechanisms of *ntcA* expression in *Synechocystis* sp. PCC 6803. As a first step to study the redox regulation of the *ntcA*

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**Figure 1.** Northern-blot analysis of the *ntcA* gene expression in response to nitrogen source and availability. Total RNA was isolated from mid-log phase *Synechocystis* sp. PCC 6803 cells (A) that were grown during 12 h in medium containing NO$_3^-$, NH$_4^+$, or no nitrogen source. B. Nitrate-grown cells were harvested, washed, and transferred to nitrogen-free medium. Samples for RNA isolation were taken at the indicated times. Ten micrograms of total RNA was loaded per lane. Hybridization was performed with the *ntcA* gene probe. C. Cell extracts were obtained from cultures grown under the same conditions as in A, separated by SDS-PAGE, and western blot was carried out using polyclonal NtcA antiserum raised toward the recombinant *Synechocystis* sp. NtcA protein. Fifty micrograms of protein was loaded per lane.
gen (Fig. 1A). A mRNA with a size of 0.8 kb corresponds well to the ntcA coding region, which is 673-bp long in Synechocystis sp. PCC 6803. The level of the 0.8-kb ntcA mRNA increased after 12 h of nitrogen starvation (approximately 3-fold) when compared with cells grown in the presence of nitrate or ammonium (Fig. 1A). In contrast, the 1.2-kb ntcA transcript level remained almost unchanged. The levels of the different ntcA transcripts were similar in the presence of nitrate or ammonium, suggesting that the nitrogen control system of Synechocystis sp. does not distinguish between these different nitrogen sources. This result is similar to that reported for Anabaena sp. PCC 7120 (Ramasubramaniam et al., 1996) but different to that reported for Synechococcus sp. PCC 7942, where ntcA mRNA levels were negatively regulated by ammonium (Luque et al., 1994).

We examined the time course of the accumulation of the 0.8-kb ntcA transcript during nitrogen deprivation. When Synechocystis sp. cells were transferred from nitrate-containing to nitrogen-free medium, the maximum level of the 0.8-kb ntcA transcript was reached after 2 to 4 h of nitrogen deprivation (3- to 4-fold) (Fig. 1B). Then a slight decrease of this level was observed (data not shown). The level of the 1.2-kb ntcA mRNA remained unchanged.

We also analyzed whether the increase in the 0.8-kb ntcA mRNA level under nitrogen starvation led to a subsequent increase in NtcA protein levels. Synechocystis sp. PCC 6803 crude extracts from cells grown with nitrate, ammonium, or nitrogen deprived for 12 h were subjected to western-blot analysis with polyclonal antibodies raised against the Synechocystis sp. PCC 6803 NtcA protein expressed in Escherichia coli. Similarly to what happened with both ntcA transcripts, the NtcA protein was detected under all the studied conditions. Levels of NtcA were very similar in Synechocystis sp. cells grown with nitrate or ammonium as nitrogen source. Densitometric analysis revealed that only in nitrogen deprived cells a small but consistent increase (less than 2-fold) of the level of the NtcA protein could be detected (Fig. 1C).

The calculated molecular mass for the band obtained in the western-blot experiment was approximately 25 kD. This molecular mass corresponds well with a NtcA monomer as expected after SDS-PAGE denaturing electrophoresis. Gel filtration analysis of NtcA from Anabaena sp. PCC 7120 recently revealed that in vivo, NtcA is in the state of a dimer (Wisén et al., 1999). Most of the cAMP receptor protein family members form dimers when they interact with DNA, each monomer binding a single half-site of the DNA target sequence (Harrison and Aggarwal, 1990).

The 1.2-kb ntcA Transcript Contains a Small Upstream Open Reading Frame

As already described, two different transcripts were detected by the 0.7-kb XhoI ntcA gene probe. Only the 0.8-kb ntcA mRNA was regulated by nitrogen availability. Question arises whether the non-nitrogen regulated 1.2-kb ntcA transcript was an RNA cross-reacting species. Analysis of the genome sequence around the ntcA gene revealed the presence of a small open reading frame (ORF) of 82 residues located upstream the ntcA gene. However, this sequence showed no homologies with known proteins in the protein data bank. Reverse transcriptase-PCR (RT-PCR) was used to determine if the 1.2-kb ntcA transcript detected under all the experimental conditions contained this small ORF and was not an RNA cross-reacting species. cDNA was synthesized by reverse transcription using a specific primer complementary to a sequence in the middle of the ntcA coding region (ntcA-rev). A second primer, specific of the 5' end of the ORF82 (ORF1), was used for a second strand synthesis followed by PCR amplification. The predicted PCR product of 0.5 kb was obtained with total RNA isolated from Synechocystis sp. PCC 6803 grown in a nitrate containing medium (Fig. 2). This band was absent when the RT was omitted in the reaction. These results indicated that the 1.2-kb band was not an RNA cross-reacting species.

![Figure 2](image-url)
Effect of Illumination on \( ntca \) Transcript Levels

Expression of several NtcA up-regulated genes, like \( glnA \) or \( glnB \), has been shown to be light-dependent in \textit{Synechocystis} sp. (Reyes et al., 1995; Garcia-Dominguez and Florencio, 1997). We determined whether different illumination conditions affected \( ntca \) transcript accumulation in this strain. \textit{Synechocystis} sp. PCC 6803 cultures (15 \( \mu \)g chlorophyll mL\(^{-1}\)), grown with nitrate as nitrogen source, were kept in the dark during 1 h and then were illuminated with white light (90 \( \mu \)E m\(^{-2}\) s\(^{-1}\)). Total RNA extracted from cells exposed to dark or light conditions was hybridized using the \( ntca \) gene probe as well as the \( trpA \) and the \( rnpB \) housekeeping gene probes. The 1.2-kb \( ntca \) transcript was detected under both dark and light conditions (Fig. 3A, top). Its level was not modified regardless the different illumination conditions. In contrast, levels of the 0.8-kb \( ntca \) mRNA increased rapidly upon illumination, whereas it was undetectable in darkness (Fig. 3A). After 30 min of light incubation, the level of the 0.8-kb \( ntca \) transcript reached its maximum level. The fact that levels of the 0.8-kb \( ntca \) transcript increased very rapidly, whereas levels of the 1.2-kb \( ntca \) mRNA remained constant, suggested that this longer \( ntca \) transcript did not serve as a precursor of the 0.8-kb \( ntca \) transcript. In contrast to \( ntca \), dark to light transitions did not affect \( trpA \) or \( rnpB \) mRNA transcript levels (Fig. 3A, bottom). These results suggested that the light-dependent accumulation of the 0.8-kb \( ntca \) transcript was specific and was not a consequence of a general response of the cell in \textit{Synechocystis} sp.

We also tested whether the accumulation of the 0.8-kb \( ntca \) transcript was originated from an increase in \( ntca \) gene transcription or \( ntca \) mRNA stability. We determined the half-life of the \( ntca \) gene transcripts under both dark and light conditions. The decay of the 0.8-kb \( ntca \) transcript was similar (approximately 5 min) in darkness or under illumination, suggesting that the light-dependent accumulation of this transcript was due to an increase in the transcription activity (data not shown). In contrast, the 1.2-kb \( ntca \) transcript was very stable (>90 min) under both conditions (data not shown).

NtcA protein levels were determined under dark and light conditions. The cells were incubated in darkness during 1 h and then illuminated during 60 min. Very reduced amounts of NtcA protein (approximately 20% of the initial control values) were detected in extracts from \textit{Synechocystis} sp. cells incubated for 1 h in darkness (Fig. 3B). The 1.2-kb \( ntca \) mRNA was the only \( ntca \) mRNA species detected by northern analysis under these experimental conditions (Fig. 3A). Consistent with the increase of the 0.8-kb \( ntca \) transcript, NtcA protein levels increased between 4- and 6-fold under illumination (Fig. 3B).

**Figure 3.** Time course of \( ntca \) transcript levels in the dark and effect of re-illumination. A, \textit{Synechocystis} sp. PCC 6803 cells were transferred to darkness and after 1 h the culture was re-illuminated. Samples for RNA isolation (10 \( \mu \)g) were taken at the indicated times and hybridized with the \( ntca \) gene probe. The same RNA samples were hybridized with the \( trpA \) (Trp synthetase A subunit) and the \( rnpB \) (ribonuclease B subunit) housekeeping genes. B, Cell extracts (50 \( \mu \)g of protein) were obtained from \textit{Synechocystis} sp. cultures incubated for 1 h in darkness or under illumination, separated by SDS-PAGE, and western blot was carried out using the polyclonal NtcA antiserum.

Effect of Photosynthetic Electron Transport on Multiple \( ntca \) Transcript Accumulation

We investigated whether the light dependence of \( ntca \) expression was susceptible to changes in the redox status of the cell in \textit{Synechocystis} sp. PCC 6803. Cells were incubated for 1 h in darkness and then illuminated in the presence of two photosynthetic electron transport inhibitors, dichloro-methyl urea (DCMU; 20 \( \mu \)m) and di-bromo methyl p-benzoquinone (DBMB; 5 \( \mu \)m). Dark incubation produced an oxidation of the redox state of the cell (Vernotte et al., 1990; Mi et al., 1994; Alfonso et al., 2000). We have already demonstrated that under our dark conditions the plastoquinone pool became more oxidized due to a low reducing power level (NADPH+H\(^+\)), inducing a decrease in the rate of respiration (Alfonso et al., 2000). Under these conditions, the level of ATP only slightly decreased (Vernotte et al., 1990). Upon illumination, DCMU blocks electron transfer at the acceptor side of photosystem II, inhibiting plastoqui-
none reduction via the PS II (Trebst, 1980). DBMIB prevents oxidation of plastoquinone by binding to the quinone oxidation site of the cyt b6f complex (Rich et al., 1991). The effect of both electron transport inhibitors on ntcA transcript accumulation was tested. Transfer of Synechocystis sp. cells from darkness to illumination in the presence of DCMU (20 μM) completely inhibited the 0.8-kb ntcA transcript accumulation observed during the dark to light shift (Fig. 4A, top). Similar results were obtained when DBMIB was used as electron transport inhibitor (data not shown). The levels of the 1.2-kb ntcA transcript remained unchanged.

We further tested the effect of DCMU or DBMIB under illumination without changing the light inten-

tensity. DCMU (20 μM) or DBMIB (5 μM) were added to Synechocystis sp. cells incubated under white light illumination (90 μE m⁻² s⁻¹). Upon addition of DCMU or DBMIB, the 0.8-kb ntcA mRNA quickly disappeared (Fig. 4B); less than 50% of the initial ntcA mRNA levels were detectable after 5 min of DCMU or DBMIB addition. The 1.2-kb ntcA mRNA was not affected. These results suggested that the light-dependent accumulation of the 0.8-kb ntcA transcript was sensitive to the inhibition of photosynthetic electron transport. The level of the rnpB mRNA did not decreased upon addition of DCMU or DBMIB (Fig. 4B).

We decided to further analyze whether the dark decrease of the 0.8-kb ntcA transcript was also related to changes in the redox state of the cell. To that end, we used Glc-grown cells in which a high concentration of NADPH is maintained for a longer time under dark conditions by Glc metabolization. As a consequence, in darkness, the plastoquinone pool and the cyt b6f complex are more reduced than in cells that have been grown in the absence of Glc (Mi et al., 1994). Control (without Glc) and Glc-grown cells were incubated in the dark or under normal illumination for 1 h. Total RNA was isolated from these cells and hybridization was performed with the ntcA gene probe. Both 0.8- and 1.2-kb ntcA transcripts were detected in Glc-grown cells incubated either in darkness or in light (Fig. 4C, right). In contrast, in cells grown without Glc, the 0.8-kb ntcA transcript was detected only in light (Fig. 4C, left). These results suggested that the expression of the 0.8-kb ntcA transcript depends on the redox status of the cells generated by photosynthetic electron transport rather than by light per se.

The changes in NtcA protein levels induced by the addition of photosynthetic electron transport inhibitors were also examined. Upon addition of both photosynthesis inhibitors, the level of the NtcA protein decreased with time; less than 50% of the initial NtcA protein levels were detectable after 30 min of DCMU or DBMIB addition (Fig. 5, top). After 1 h of inhibitor addition, only 20% of NtcA protein remained present in the cell extracts (Fig. 5, top). The levels were similar to those detected in Synechocystis sp. cells incubated for 1 h in darkness (Fig. 3B). These results were consistent with the decrease of the 0.8-kb ntcA transcript observed under the same experimental conditions (Fig. 4B). To further analyze the effect of the redox status on NtcA protein levels, the amount of NtcA was compared between cells grown in the presence or in the absence of Glc. As shown in Figure 5 (bottom), under illumination both cells presented similar levels of the NtcA protein. It is interesting that in darkness and in the presence of Glc, the level of the NtcA protein was only slightly lower than under illumination, whereas in the absence of Glc, only 20% of the light level was detected (Fig. 5, bottom).
Transcriptional Start-Site Mapping of the ntcA Gene

Since northern-blot analysis revealed the presence of two ntcA transcripts, we carried out primer extension analysis to determine the different putative transcription start points (tsp) of the Synechocystis sp. ntcA gene. When the oligonucleotide MAP0.8, complementary to the ntcA coding sequence, was used, an extension product was detected using total RNA from light- and nitrate-grown Synechocystis sp. cells (Fig. 6A). The tsp was mapped to nucleotide 267 with respect to the first translated nucleotide. This tsp, detected by primer extension, corresponded well with a ntcA transcript of 0.8 kb long as detected by northern analysis. The product of the extension reaction was much more abundant with RNA isolated from nitrogen-deprived cells (Fig. 6A). It was undetectable when total RNA was obtained from dark-grown Synechocystis sp. cells (Fig. 6A). A sequence with five out of six matching nucleotides of the −10 σ70 dependent E. coli-like promoter consensus sequence occurred four nucleotides upstream of the ntcA tsp (TGTAAT, Fig. 7). An apparent −35 sequence with three of six nucleotides matching the E. coli-like −35 box was found at 18 nucleotides upstream the −10 box (T777TCTT, Fig. 7). The ntcA gene has been demonstrated to be autoregulated by the ntcA gene product in Synechococcus sp. PCC 7942 (Luque et al., 1994) or Anabaena sp. PCC 7120 (Ramasubramanian et al., 1996). A sequence GTA-N8-TGC, exhibiting near perfect identity with the consensus-binding site of the transcription factor NtcA (GTA-N8-TAC) was detected 60 nt upstream of the −10 sequence (Fig. 7). A similar putative NtcA target site was found in the sequence of the icd gene promoter region from Synechocystis sp. PCC 6803 (Muro-Pastor et al., 1996). The position of the putative NtcA target site deduced from our sequence analysis (centered at −77.5 with respect to the tsp) is striking since in most of the NtcA up-regulated promoters the NtcA binding site was centered at −39.5/−41.5 from the tsp (Luque et al., 1994; Reyes et al., 1995). Only in the

Figure 5. Effect of electron transport inhibitors and reducing power on NtcA protein levels. DCMU (20 μM, top) or DBMIB (5 μM, middle) were added to Synechocystis sp. cells growing under illumination. Samples were taken immediately upon the addition of the inhibitors at the indicated times. Cell extracts were electrophoresed on SDS-PAGE gels, and proteins were blotted onto nitrocellulose membranes. NtcA was detected with an anti-NtcA antibody. The effect of Glc was also analyzed. Synechocystis sp. PCC 6803 cells were grown in the absence (bottom left) or presence of Glc (bottom right) for at least three generations. Cultures were kept in darkness or under illumination for 1 h and cell extracts were obtained. Proteins (50 μg of protein) were blotted onto nitrocellulose membranes. NtcA protein was detected with specific NtcA antibodies.

Figure 6. Mapping of the 5’ ends of the different ntcA transcripts. Total RNA (25 μg) from Synechocystis sp. cultures was extracted from cells after 1 h of darkness, 1 h of illumination, or 12 h of nitrogen deprivation, and used for primer extension analysis with primer MAP 0.8 (A) or MAP 1.2 (B). The position of the fragment with respect to the ATG translation initiation codon is indicated. Lanes A, C, G, and T are dideoxy sequencing ladders of pNtcA1 (A) or pNtcA2 (B) produced by primers MAP0.8 or MAP1.2, respectively.
case of the ntcA gene from *Anabaena* sp. PCC 7120, the NtcA binding site, identified by DNAse footprinting and gene fusion analysis, was centered at 295 from the tsp (Ramasubramaniam et al., 1996).

When the oligonucleotide MAP0.8 was used, no longer transcripts were detected, regardless of the illumination conditions or the source of combined nitrogen. The distance between MAP0.8 and the theoretical tsp of the 1.2-kb ntcA mRNA could be critical (approximately 500 nucleotides). To determine the 5' end of the 1.2-kb ntcA transcript, we designed a second primer, MAP1.2 (Fig. 6B), located closer to the expected position of this tsp. The extension of primer MAP1.2 showed two contiguous extension products that were detected with RNA from cells incubated under light or dark conditions (Fig. 6B). The 5' end of the 1.2-kb mRNA was mapped to nucleotides 2408 and 2409, respectively, to the first ATG of the ntcA gene. These tsp detected by primer extension corresponded well with a ntcA transcript of 1.2-kb length as obtained by northern analysis. The amount of the extension product was similar regardless the dark or illumination conditions. A sequence (TAAAAT) with five of six matching the E. coli-like promoter consensus sequence occurred 6 and 7 nucleotide upstream of the ntcA1.2 transcript tsp (Fig. 7). An apparent 235 sequence with three of six matching the E. coli consensus 235 box (TTTTCT) was located 7 nucleotide upstream of the tsp.

Figure 7. Nucleotide sequence of the ntcA gene and its upstream region cloned in pNtcA2. Position of the putative −10 and −35 boxes is marked in bold letters. The putative NtcA target sequence is underlined. Positions of the tsp are indicated by +. The putative Shine-Dalgarno sequences and position of the primers used for primer extension and for RT-PCR is also indicated. Nucleotide sequences are available on Cyanobase (ntcA, sll1423).
found at 17 nt upstream the −10 box (Fig. 7). No sequence with similarities to a NtcA binding site was found upstream of the 1.2-kb ntcA transcript tsp.

NtcA Binds Upstream of the ntcA Gene in Synechocystis sp. PCC 6803

In Synechococcus sp. PCC 7942 and Anabaena sp. PCC 7120 strains, NtcA has been shown to bind upstream of the ntcA gene in mobility shift assays (Luque et al., 1994; Ramasubramaniam et al., 1996), suggesting that NtcA autoregulates its own expression. Examination of the DNA sequence upstream of the Synechocystis sp. PCC 6803 ntcA gene showed one putative NtcA target sequence near the light- and nitrogen-dependent putative promoter. We performed band-shift experiments with the E. coli expressed, purified Synechocystis sp. NtcA protein to test the NtcA binding to its own promoter. The PCC 6803 NtcA protein retarded an EcoRII-TaqI fragment containing the ntcA gene promoter from −127 to +111 with respect to the previously determined tsp (Fig. 8A, lanes 2 and 4). This DNA fragment contained the putative NtcA target sequence. A 30-fold excess of unlabeled probe reduced the amount of retarded labeled fragment (Fig. 8A, lane 5). Glutathione S-transferase (GST) alone (lane 3) or an unrelated pBluescript fragment (not shown) did not generate any retarded fragments (Fig. 8A). A second EcoRI-BspHII 256-bp fragment, containing the sequence from −510 to −254 with respect to the ntcA translation start site, was also used. This DNA fragment contained the sequence of the σ70 dependent E. coli-like promoter found upstream of the ntcA 1.2 transcript tsp. NtcA did not bind to this fragment in the mobility shift assay (Fig. 8A, lane 6). These data suggest that the NtcA protein specifically binds to the putative promoter region giving rise to the light/nitrogen-dependent transcript but not to that responsible for the constitutive 1.2-kb ntcA mRNA.

Involvement of Reducing Conditions and Thiol Groups in the NtcA-Binding Mechanism to the ntcA Gene

We also studied the influence of reducing and oxidizing conditions in the interaction between NtcA and the ntcA light/nitrogen-dependent promoter. Binding capacity of NtcA to the ntcA light/nitrogen-dependent promoter fragment in the presence of increasing concentrations of the reducing agent dithiothreitol (DTT) was first analyzed. The affinity of NtcA for the ntcA light/nitrogen-dependent promoter was markedly enhanced by increasing the concentration of DTT up to 5 to 10 mM (data not shown). The fact that NtcA binding was sensitive to DTT suggested that reducing conditions are required for NtcA binding.

Free thiol groups of Cys residues have been proposed to be required for NtcA binding to the gor promoter in Anabaena sp. PCC 7120 (Jiang et al., 1997). The effect of a thiol-oxidizing agent and a thiol-modifying agent on NtcA binding to the light/nitrogen-dependent ntcA promoter was then studied. Incubation of the Synechocystis sp. NtcA protein with diamide, a thiol-oxidizing agent resulted in inhibition of NtcA binding to the light/nitrogen-dependent ntcA gene promoter fragment (Fig. 8B, left). Further evidence of the role of thiol groups in NtcA binding to the ntcA promoter fragment was obtained with the thiol-modifying agent NEM (N-ethyl maleimide). Incubation of the NtcA protein with NEM prior to the binding reaction resulted in the inhibition of NtcA binding to the light/nitrogen-dependent ntcA promoter fragment (Fig. 8B, right). On the contrary, incubation of the NtcA protein with the labeled ntcA promoter fragment prior to the incubation with NEM did not modify the NtcA binding activity to ntcA and the ntcA promoter fragment was retarded (Fig. 8B, right). This suggested that in vitro the NtcA binding to the light/nitrogen-dependent ntcA promoter from Synechocystis sp. PCC 6803 is thiol-group dependent.

Figure 8. Gel retardation analysis of the binding of NtcA to the ntcA gene upstream regions. A, Binding of NtcA to the two different Synechocystis sp. PCC 6803 promoter fragments. Lane 1, No protein; lane 2, 5 μM NtcA incubated with a 238-bp EcoRII-TaqI fragment containing the light and nitrogen dependent promoter region (~193 to 45 from the ntcA translation start site); lane 3, same fragment incubated with 5 μM GST; lane 4, same fragment incubated with 1.5 μM NtcA; lane 5, same fragment incubated with 5 μM NtcA and 30-fold excess of unlabeled EcoRII-TaqI fragment; lane 6, 5 μM NtcA incubated with a 256-bp EcoRI-BspHII fragment, containing the constitutive promoter (from −510 to −254 from the ntcA translation start site). B, The 238-bp EcoRII-TaqI fragment containing the light and nitrogen induced promoter region was assayed for NtcA binding in the presence or absence of 1 mM diamide (a thiol oxidizing agent; left) or NEM (a thiol modifying agent; right). NEM was added before or after incubation of the ntcA light- and nitrogen-dependent promoter fragment with the NtcA protein.
Nitrogen control of ntcA expression has been described in several strains of cyanobacteria (Wei et al., 1993; Luque et al., 1994; Bradley and Reddy, 1997). However, the mechanism by which NtcA autoregulates its own expression and initiates its regulatory cascade remains still poorly understood. Information about this regulation in Synechocystis sp. PCC 6803, where the genomic sequence is already known, is not available to our knowledge. In Synechocystis sp., redox controlled expression of several NtcA-up regulated genes has been described (Reyes et al., 1995; García-Dominguez and Florencio, 1997). However, the influence of the redox status on the availability of the NtcA protein or in the affinity of the NtcA protein to its binding site is still unknown. Furthermore, no environmental factors other than nitrogen have been reported to modulate ntcA gene expression in cyanobacteria. The aim of this work was to study the role of the cellular redox status in the regulation of ntcA expression. To that end, the expression of the ntcA gene from Synechocystis sp. under different environmental conditions able to modify the cellular redox status like illumination (darkness/light), nutrient regimes (presence or absence of Glc), or different nitrogen source or availability conditions was studied.

The results presented here indicate that the ntcA gene shows a complex pattern of expression with two different transcripts: a constitutively transcribed 1.2-kb mRNA and a regulated 0.8-kb ntcA transcript. Our data indicate that in Synechocystis sp. PCC 6803, light and nitrogen controlled the accumulation of the 0.8-kb ntcA transcript, whereas the 1.2-kb ntcA transcript was neither light nor nitrogen-dependent as its level was not modified under all the experimental conditions tested. Upon illumination, the accumulation of the NtcA protein followed the accumulation of the 0.8-kb ntcA transcript. Under dark conditions, when the 0.8-kb ntcA transcript decreased or disappeared, levels of the NtcA protein also largely decreased.

Light per se seems not to be the signal that triggers the accumulation of the 0.8-kb ntcA transcript. In Glc-adapted cells, high levels of the 0.8-kb ntcA transcript as well as the NtcA protein were present even in darkness. We have already demonstrated that transcription of the psbA gene, encoding the D1 protein of the Photosystem II and that of other photosynthetic genes (psae and cpcBA), was maintained in darkness in the presence of Glc (Alfonso et al., 2000). The results of that work suggested that the effect of Glc might be related to the redox state of the electron transport chain. The electron transport chain including the plastoquinone pool became more oxidized during dark incubation due to a decrease of the NADPH+H+ concentration (Vernotte et al., 1990; Mi et al., 1994). The Glc metabolism via the pentose phosphate pathway leads to the production of NADPH+H+, and a large level of reducing power is conserved even in darkness. This reducing power can be used for oxidative phosphorylation as well as for reduction of the PQ pool and the thioredoxin, maintaining the electron transport chain more reduced. Under illumination, addition of photosynthetic electron transport inhibitors, such as DCMU or DBMIB, induced the decrease of the 0.8-kb ntcA mRNA. Under these conditions, the level of the NtcA protein largely decreased. Because DCMU and DBMIB have opposite effects on the redox state of the plastoquinone pool we conclude that ntcA gene expression is not directly correlated with the redox state of the plastoquinone pool.

The redox state of the electron transport chain and/or of the cells is also involved in the regulation of the expression of other genes in Synechocystis sp. PCC 6803 different from ntcA or photosynthetic genes like psbA (Alfonso et al., 2000). Non-photosynthetic genes like the secA gene (Mazouni et al., 1998) or the dnaA-like gene (Richer et al., 1998) seem to be also regulated by the redox state of the cells. The expression of genes encoding for enzymes involved in nitrogen metabolism, like glnA and glnB, seems to be similarly redox controlled in Synechocystis sp. PCC 6803 (Reyes and Florencio, 1995; García-Dominguez and Florencio, 1997). However, in cyanobacteria, there are other genes that are not dependent of the redox state of the cell or the presence of light. Here, we showed that the rnpB and trpA genes do not respond to light or to chemical treatments. The ORF sll015s also does not increase under light conditions (Mazouni et al., 1998). Moreover, in Synechococcus sp. PCC 7002, the transcription of the lrtA gene is inhibited by light (Tan et al., 1994). The specific dark expression of other proteins was reported in Synechococcus sp. PCC 6301 and Anacystis nidulans (Singer and Doolitle, 1975; Suranyi et al., 1987). All these data suggest that light and redox control mechanisms are specific even if they control a large number of genes in the genome.

Our data show that both ntcA transcripts had different mRNA stability. The 1.2-kb ntcA mRNA was very stable (t1/2 > 90 min) in opposition with the 0.8-kb ntcA transcript that had a shorter half-life (t1/2 5 min). The high stability of the 1.2-kb ntcA mRNA is striking. Even if there is no general rule in prokaryotes concerning the relationship between translation and mRNA stability, many messages that are actively translated are rapidly degraded. In a similar way, many specific transcripts are stabilized by antibiotics that inhibit translation or under conditions of energy source shift-down or deprivation inducing a slow down of the rate of translation (Petersen, 1993; López et al., 1998). As an example in Synechocystis sp. PCC 6803, the stability of the redox controlled light-regulated psbA2 transcript increased in the dark, whereas there was almost no synthesis of the D1 protein (Mohamed and Jansson, 1991; Alfonso et al., 2000).
Our data indicate that accumulation of the NtcA protein followed the accumulation of the 0.8-kb ntcA transcript. On the other hand, under any oxidizing conditions (darkness, light plus inhibitors), in which the 0.8-kb ntcA transcript decreased or disappeared, the levels of the NtcA protein also decreased. These results suggested that the 0.8-kb ntcA transcript was the major species responsible for NtcA synthesis. However, basal amounts of the NtcA protein were detected even in darkness. Under these conditions, only the 1.2-kb ntcA mRNA was detected, suggesting that the basal levels of the NtcA protein could originate from the constitutive transcript. This may constitute a reasonable stand-by mechanism to activate NtcA when it becomes necessary.

Primer extension experiments presented in this work suggested that similarly to other cyanobacterial strains (Luque et al., 1994; Ramasubramaniam et al., 1996; Bradley and Reddy, 1997), the Synechocystis sp. PCC 6803 gene was expressed from at least two different promoters with different use. A putative tsp, initiated from position −67 (with respect to the ATG), giving rise to the 0.8-kb ntcA transcript, seemed to originate from a proximal promoter. This promoter was induced under illumination or under nitrogen deprivation conditions, whereas it seemed not to be operative under dark oxidizing conditions. A second ntcA putative tsp, initiated from position −408/−409 (with respect to the ATG) giving rise to the 1.2-kb ntcA mRNA, seemed to initiate from a distal promoter. This promoter appeared to represent a constitutive promoter that was functional independently of the illumination conditions or the nitrogen regime of the cells. Constitutive expression of ntcA seemed to be exerted from a σ70-dependent E. coli-like promoter. No NtcA target sequence was found upstream of this tsp, suggesting that this longer ntcA transcript was not subjected to NtcA regulation. Indeed, NtcA retarded a DNA fragment containing the sequence of the light- and nitrogen-dependent promoter where a putative NtcA target sequence was found. However, E. coli-expressed NtcA protein failed to retard a DNA fragment containing the constitutive ntcA gene promoter sequence. These results suggest that similarly to Anabaena sp. PCC 7120 or Synechococcus sp. PCC 7942 (Luque et al., 1994; Ramasubramaniam et al., 1996), NtcA may regulate its own expression in Synechocystis sp. PCC 6803.

It has been reported previously that in Anabaena sp. PCC 7120, NtcA binding to the gor promoter was redox dependent (Jiang et al., 1997). Our band-shift experiments carried out under different reducing or oxidizing conditions indicated that in Synechocystis sp., NtcA binding to the light/nitrogen dependent ntcA promoter was modulated by a similar redox mechanism. Treatment with thiol oxidizing agents like diamide or thiol modifiers like NEM indicated that, similarly to the Anabaena sp. PCC 7120 gor promoter, NtcA binding to ntcA was regulated by a thiol-group dependent redox control mechanism.

In this article, we provide new information about the regulatory mechanism involved in ntcA gene expression. Although our results do not exclude the existence of other control mechanisms, they allow us to propose a working hypothesis about the influence of the redox state of the cell on ntcA gene expression. Reducing power is generated during illumination by photosynthesis. During nitrogen-deprivation, carbon skeletons that are not used for ammonium incorporation are incorporated into the oxidative pentose phosphate pathway also generating reducing power (Reyes et al., 1995). Under these reducing conditions, thiol groups of the NtcA protein could be reduced. Affinity of NtcA for its recognition site in the ntcA gene proximal promoter may increase and transcription of the 0.8-kb ntcA transcript should be induced. Concomitantly, levels of the NtcA protein may increase. Basal levels of NtcA necessary for this initial step could be provided by the 1.2-kb ntcA transcript. On the contrary, under oxidizing conditions, like darkness or light plus inhibitors, thiol groups from the NtcA protein would become oxidized. Affinity of NtcA for its target sequence would decrease and transcription from the light/nitrogen dependent promoter would cease. Concomitantly, levels of the NtcA protein would decrease to basal levels and remain inactive while oxidizing conditions (oxidized thiol groups) are maintained. This mechanism may allow a rapid response of the ntcA gene to changes in environmental conditions, adjusting the different expression programs in which the action of the NtcA protein is required.

MATERIALS AND METHODS

Strain Culture Conditions

Wild-type Synechocystis sp. PCC 6803 was grown as previously described (Herdmann et al., 1978). Dark conditions were obtained by wrapping the flasks with aluminum foil. When indicated DCMU (20 μM) or DBMIB (5 μM) were added. When ammonium was used as the nitrogen source, nitrate was removed from the medium and 10 mM NH4Cl was added. For nitrogen starvation conditions, cells were harvested, washed, and transferred to medium in which nitrate was absent. For all the experiments the medium was buffered with 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.2. When necessary, cells were grown in a 0.1% (w/v) Glc containing medium for at least five generations before used. Rifampicin was used as an inhibitor of transcription to determine the stability of transcripts. Rifampicin was added in excess at a final concentration of 300 μg mL−1 to avoid problems of antibiotic degradation. Samples were collected at different times during the different dark/light, nitrogen, and inhibitory treatments. For RNA isolation, cells were immediately pelleted and frozen in liquid nitrogen. All samples were stored at −80°C until used.
PCR Amplification and Cloning of the ntcA and trpA Genes from Synechocystis sp. PCC 6803

The complete ntcA gene was cloned from Synechocystis sp. PCC 6803 genomic DNA after PCR amplification using specific oligonucleotides. These oligonucleotides were designed from the Synechocystis sp. PCC 6803 genomic map available at Cyanobase (www.kazusa.or.jp/cyano/cyaono.html). The complete ntcA gene was amplified using primers NtcA1 (5'-ATACTCGAGATGGATGACCTCCATTACC-18) and NtcA2 (5'-TCAGGAGGCACTGTGTCATAAGGG-18), from nucleotide 1 to 18 of the ntcA coding region, and the RI site used was present in the pGEM-T-Easy vector (plasmid pNtcA2). Finally, a 792-bp fragment, containing the complete coding sequence of the ntcA gene, was digested with XhoI and the resulting 715-bp fragment was cloned into pGEM-T-Easy vector (plasmid pNtcA1). A second PCR product containing the same sequence from pNtcA1 and 510 nucleotides upstream of the NtcA ORF was also amplified by PCR using the oligonucleotides NtcA2 and NtcA3 (5'-AGTGGCCAGAAACCTGAGCTAA-18) and cloned into the pGEM-T-Easy vector (plasmid pNtcA2). Finally, a 792-bp fragment, containing the complete coding sequence of the trpA (tryptophane synthase gene), was amplified with primers trpA1 (5'-ATGAAAGCCTGGCGCCCTTG-18) and trpA2 (5'-ACTGATGGCGCTTTCGG-18).

RT-PCR of the ntcA Transcripts

Total RNA isolated from Synechocystis sp. cells grown in a nitrate containing medium was treated with RNase-free DNase I (Life Technologies/Gibco-BRL, Cleveland). RT-PCR was carried out using the Superscript Rnase H- Reverse Transcriptase (Life Technologies/Gibco-BRL) and the primer ntcA-rev (5'-ACGGCGTCGACCTAGATACTTACAGG-18) was used to synthesize the cDNA. After reverse transcription, the primer ORF1 (5'-ATGCAAAAAGCAGACGGCCTT-18) was added to run the following PCR.

DNA Manipulation and Gene Sequence

Total DNA from cyanobacteria was isolated as previously described (Alfonso et al., 1999). Plasmid isolation from Escherichia coli, transformation, restriction, and ligation with T4 ligase were performed by standard procedures (Maniatis et al., 1989). Sequencing of plasmids was carried out by the dyeoxy-chain termination method using T7 polymerase (Pharmacia Biotech, Piscataway, NJ).

Expression of the Synechocystis sp. NtcA Protein in E. coli and Purification

Plasmid pNtcA1, containing the complete coding sequence of the Synechocystis sp. ntcA gene, was digested with XhoI and the resulting 715-bp fragment was cloned into the XhoI site of pGEX 4T-1 (Pharmacia Biotech) in phase with the GST gene. GST-NtcA fusion protein and GST were expressed in E. coli DH5α cells. Cells were grown in Luria-Bertani medium to an optical density at 600 nm of 0.6 and then induced with 1 mM isopropyl-β-d-thiogalacto- pyranoside for 3 h. Cells were harvested by centrifugation and resuspended in a 1/20 volume of phosphate-buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 8 mM KH2PO4, pH 7.3) with 4 mM phenylmethyl sulfonyl fluoride and 7 mM β-mercaptoethanol supplemented with 1% (v/v) Triton X-100. Cells were lysed by mild sonication on ice (15–30 s). The extract was centrifuged at 10,000 g for 5 min at 4°C. The supernatant was mixed with 1 mL of glutathione agarose beads (Pharmacia Biotech) and incubated for 2 h at 4°C with gentle agitation. Beads were then transferred to a column and washed extensively with PBS buffer until no more protein was eluted from the column. GST or GST-NtcA fusion protein were eluted with 3 mL of 50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione. Purity of the extract was checked by SDS-PAGE.

Preparation of Synechocystis sp. PCC 6803 NtcA Antibodies and Immunological Detection of the NtcA Protein

GST-NtcA fusion protein was digested with thrombin and the thrombin-cleaved NtcA protein was purified with a glutathione-agarose affinity column. The NtcA fraction was separated by SDS-PAGE and the strong band of approximately 25 kD was excised, homogenized in the presence of 50 mM NH4HCO3, and boiled for 5 min in the presence of 5% (v/v) 2-mercaptoethanol. Protein was electro-eluted overnight at 37°C with stirring and then lyophilized. Rabbits were injected intradermally with the antigen emulsified with complete Freund’s adjuvant according to Berzborn (1980). Antisera were tested in ring tests (Berzborn, 1980). To detect the amount of the NtcA protein, crude extracts from Synechocystis sp. PCC 6803 grown under different conditions were subjected to SDS-PAGE electrophoresis on 12% (w/v) acrylamide gels. Fifty micrograms of total protein was loaded per lane. Western-blot procedures were carried out as described elsewhere (Towbin et al., 1979). Antiserum was used at a 1:1,000 dilution. Incubation with the primary antibody was carried out overnight at 4°C in 1% (w/v) non-fat dry milk, 25 mM Tris, 0.9% (w/v) NaCl, pH 7.5. Detection was carried out using the amplified Alkaline Phosphatase GAR kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were loaded per lane. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard.

Band-Shift Mobility Assays

A 238-bp EcoRI-TaqI fragment containing the sequence from −193 to +45 (with respect to the ntcA translation start site), was generated by digestion of pNtcA2. This fragment contained the −10, −35, and the putative NtcA target sequence. A second EcoRI-BspHI fragment of 256 bp, containing the sequence from −510 to −254 (with respect to the ntcA translation start site), was obtained by digestion of pNtcA2. The EcoRI site used was present in the pGEM-T-Easy polylinker but not in the cyanobacterial sequence. All
the fragments were end labeled with (α-32P) dCTP with sequenease version 2.0 enzyme. One microgram of GST-NtcA fusion protein was cleaved by incubation in PBS buffer supplemented with 1 unit of thrombin and 2.5 mM CaCl2 for 20 min at 31°C. Labeled DNA fragments (4 ng) were incubated at 30°C for 20 min with different concentrations of purified NtcA-thrombin-cleaved protein in binding buffer (25 mM Tris-HCl, pH 8.0, 12% [v/v] glycerol, 60 mM KCl, and 4 mM spermidine) containing 2 μg of poly (dI-dC) in a final volume of 25 μL. Binding reaction with 5 mM of GST protein was also performed. For competition experiments, a 30-fold excess of unlabeled probe was added to the binding reaction mixture. When necessary, different concentrations of DTT were added to the incubation mixture. Treatment with thiol modifying agents was carried out essentially as described in Jiang et al. (1997). Treatment with the thiol-oxidizing agent azodicarboxylic acid bis-dimethylamide (diamide, Sigma, St. Louis) was performed by incubating the protein extract in binding buffer containing 5 mM DTT for 1 h at 37°C in the presence or absence of 1 mM diamide before adding DNA fragments. For thiol modification, the protein extract was incubated in the binding buffer containing 5 mM DTT with the thiol modifying agent N-ethylmaleimide (1 mM NEM, Sigma), before or after adding DNA fragments. The reaction mixtures were separated by electrophoresis on a non-denaturing 6% (v/v) polyacrilamide gel. Electrophoresis was carried out at 4°C and 250 V and the gels were dried and autoradiographed.

RNA Isolation, Northern-Blot Analysis, and Hybridization Probes

Total RNA was isolated from midexponential phase cultures of Synechocystis sp. PCC 6803 as previously described (Alfonso et al., 1999). RNA was separated on formaldehyde gels, transferred to nylon membranes, and hybridized with different probes. Ten micrograms of total RNA was loaded per lane. The ntcA probe was generated by labeling of a 0.7-kb Xhol fragment generated by digestion of pNtcA1, containing the entire ntcA gene sequence from Synechocystis sp. PCC 6803. A trpA (Trp synthase) gene probe obtained by PCR, as described above, was used to detect the levels of the expression of a housekeeping gene under the different treatments used in this study. The rnpB probe contained the coding sequence of the rnpB gene (encoding the constitutive component of RNaseP), from Anacystis nidulans and was a kind gift from A. Vioque (Vioque, 1992). All the probes were radiolabeled by the random priming method, using the multiprime labeling system (Amersham, Buckinghamshire, UK).

Primer Extension Analysis

The oligonucleotides used for primer extension analysis were: 5′-CAGTGCGCTAGGGGACGACTT-3′ (MAP 0.8) for the ntcA 0.8-kb transcript and 5′-GAATGGTCATGAA-ACGGACTT-3′ (MAP 1.2) for the ntcA 1.2-kb transcript. The oligonucleotide (10 pmol) was annealed to 25 μg of total RNA from Synechocystis sp. PCC 6803, grown under different dark, light, or nitrogen conditions in 20 μL of hybridization buffer (40 mM PIPES [1,4-piperazinediethanesulfonic acid], 1 mM EDTA, 0.4 mM NaCl, and 80% [v/v] formamide). Mixtures were incubated at 85°C for 10 min and then at 30°C overnight. The extension reactions were carried out at 37°C for 1 h with 10 units of Superscript Reverse transcriptase (Pharmacia Biotech). Reaction mixtures were then treated with RNAse A (DNase free, Boehringer Mannheim/Roche, Basel) and extracted with phenol. DNA was precipitated with ethanol, resuspended in formamide-loading dye, and then analyzed on a sequencing gel (7 μ urea, and 6% [v/v] acrylamide). To determine the size of the extension product, nucleotide sequencing of an appropriate plasmid was carried out with the same oligonucleotide as a primer.

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LITERATURE CITED


