FEBS Letters 542 (2003) 42–46 FEBS 27178

CO_2 response for expression of ribulose-1,5-bisphosphate carboxylase/oxygenase genes is inhibited by AT-rich decoy in the cyanobacterium

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Received 26 November 2002; revised 21 February 2003; accepted 28 March 2003

First published online 11 April 2003

Edited by Marc Van Montagu

Abstract The CO_2-regulatory function of the AT-rich element in the promoter for ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*) genes in the cyanobacterium *Synechococcus* sp. PCC7002 was analyzed using the transcription factor decoy approach. Double-stranded phosphorothioate AT-rich oligonucleotides with high affinity for a sequence-specific DNA-binding protein were successfully introduced into cyanobacterial cells in culture without any transfection reagent. The AT-rich decoy oligonucleotides interfered with CO_2 regulation of *rbc* expression by blocking the binding of the sequence-specific DNA-binding protein, indicating that the AT-rich element plays a critical role in CO_2 regulation for *rbc* genes. The decoy oligonucleotide approach to cyanobacteria provides a simple and excellent tool for investigating transcriptional regulation in vivo.

Key words: CO_2-regulatory element; Transcription factor decoy; Ribulose-1,5-bisphosphate carboxylase/oxygenase; *Synechococcus* sp. PCC7002 (*Agmenellum quadruplicatum* PR-6)

1. Introduction

Gene transcription is regulated by regulatory DNA elements and their related DNA-binding proteins [1]. Recently, a transcription factor decoy approach using synthetic double-stranded phosphorothioate oligonucleotides containing a *cis*-element was developed [2–4]. Synthetic double-stranded phosphorothioate oligonucleotides as decoy elements that can penetrate cells, can block the binding of DNA-binding proteins to promoter regions of targeted genes and interfere with eukaryotic transcription [3–5]. This approach provides a powerful tool for elucidating the role of the regulatory DNA elements and related DNA-binding proteins.

Cyanobacteria are prokaryotes that carry out oxygen-evolving photosynthesis in a manner similar to higher plants. Their prokaryotic organization provides an excellent model system for the study of photosynthetic processes and several other important biological phenomena on a molecular basis. The unicellular cyanobacterium *Synechococcus* sp. PCC7002 (*Agmenellum quadruplicatum* PR-6) is one of the species that have an efficient and naturally occurring mechanism for the uptake of exogenous DNA [6,7]. We noted this characteristic, which might make it possible to introduce decoy oligonucleotides into cyanobacterial cells efficiently.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is the key enzyme in the Calvin–Benson cycle, catalyzes the incorporation of CO_2 into carbohydrate in plants and photosynthetic prokaryotes [8]. The enzyme is a hexadecameric assembly and is composed of eight large subunits (RbcL) of about 55 kDa and eight small subunits (RbcS) of about 13 kDa. In cyanobacteria, the cellular concentration of Rubisco increases at low CO_2 levels [9]. Rubisco is located in the carboxysomes, which are subcellular structures involved in efficient CO_2 utilization, and cells growing at the atmospheric level of CO_2 contain more carboxysomes than those growing at 5% CO_2 in air [10]. We previously reported that the CO_2-responsive *cis*-acting element containing the AT-rich sequence was present upstream of the cyanobacterial *rbc* promoter, and that a sequence-specific protein bound to the AT-rich element [11].

In the present study, we examined the applicability of the transcription factor decoy approach to cyanobacteria, and examined the role of the AT-rich element and corresponding *trans*-acting factor. We found that phosphorothioate oligonucleotides were efficiently introduced into cyanobacterial cells in culture without any transfection reagent. The transcription factor decoy approach revealed that the expression of *rbcLS* is regulated by the AT-rich element and related DNA-binding protein, and that the CO_2 dependence in the production of RbcLS was suppressed by inhibiting the binding of the sequence-specific DNA-binding protein to the AT-rich element.

2. Materials and methods

2.1. Materials and strains

Restriction enzymes were purchased from Takara (Kyoto, Japan). The DNA sequencing kits were from Applied Biosystems (Norwalk, CT, USA). The *Escherichia coli* strain JM109 purchased from Takara was grown in LB medium. The cyanobacterial strain *Synechococcus* sp. PCC7002 (*A. quadruplicatum* PR-6) [6,12], obtained from the American Type Culture Collection (ATCC 27264), was cultured at 30°C in medium A [13] under aeration with 0.03%, 1% or 5% CO_2. Continuous illumination was provided at 50 μE/m^2/s by three FL40SS (37 W) fluorescent lamps. Agar plates were prepared using medium A solidified with 1.5% agar and incubated at 30°C.

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Abbreviations: FITC, fluorescein isothiocyanate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00336-3

2.2. Oligonucleotides

AT-rich decoy and control oligonucleotides used in this study were phosphorothioate oligonucleotides. Their sequences are as follows: 30-mer AT-rich oligonucleotides, 5′-ATT TTA TGG CTT TTT TAG GTA TTT TTG TAA-3′ and 5′-TTA CAA AAA TAC CTA AAA AAG CCA TAA AAT-3′; 30-mer non-specific control oligonucleotides, 5′-TCA TCG GCG CTA ATT ACA AAT CCT ACG AGT-3′ and 5′-ACT CGT AGG ATT TGT AAT TAG CGC CGA TGA-3′. The DNA-binding protein binds to the above AT-rich oligonucleotides [11]. Synthetic oligonucleotides were dissolved in sterile TEN buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA). The single-stranded oligonucleotides were annealed for 16 h while the temperature decreased from 95 to 25°C.

2.3. Treatment of cyanobacterial cells with oligonucleotides

Cyanobacterial cells were grown to the exponential phase under aeration with 1% CO_2, inoculated into fresh medium A and grown for 2 days. The culture was then diluted by medium A (OD_{550} = 0.2) containing the AT-rich decoy or control oligonucleotides at indicated concentrations. The cells were grown for 2 days under aeration with indicated CO_2 concentrations (OD_{550} = 3–6), where cells were in the exponential phase, and collected.

2.4. Cellular uptake of fluorescein isothiocyanate (FITC)-labeled oligonucleotides

Cells were incubated with 0, 100 or 200 nM of FITC-labeled control oligonucleotides (5′-end-labeled) in the growth medium under aeration with 1% CO_2. Twenty-four hours after incubation, the medium was removed, cells were washed three times with phosphate-buffered saline (PBS) and fixed with 100% methanol for 30 min at room temperature. FITC-labeling was analyzed on a Zeiss Axioplan 2 fluorescence microscope equipped with a FITC fluorescence filter set (excitation 450–490 nm, emission filter 590 nm; Carl Zeiss, Jena, Germany). Images were captured with an Axiocam CCD camera (Carl Zeiss). Image analysis and presentation were performed using the AxioVision 2.05 software package (Carl Zeiss).

2.5. DNA constructions and assay for luciferase activity in cyanobacterial transformants

Two fragments in the *rbc* promoter region were prepared using the polymerase chain reaction (PCR) technique. Each fragment was amplified with *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA) during 25 cycles of 94°C denaturation for 15 s, 55°C annealing for 30 s, and 68°C extension for 1 min with a final extension time of 3 min. PCR products, P304 with an AT-rich element and P251 without an AT-rich element, were digested with *Eco*RI and cloned into the *Eco*RI and *Sma*I sites on pAQJ4-Luc, which was constructed by inserting the luciferase gene from cassette vector 2 (Nippon Gene, Tokyo, Japan) into pAQJ4-MCS [14,15] at *Bam*HI and *Xba*I restriction sites. The resulting vectors were confirmed by DNA sequence analysis using a 377 DNA sequencer (Applied Biosystems). pAQJ4-Luc without *rbc* promoter region (P0) was used as a control vector.

Synechococcus sp. PCC7002 was transformed by the method described by Buzby et al. [6], except that the concentration of DNA was modified to 0.4 pmol/ml. Single colonies from cyanobacterial transformants were cultured in 4 ml of medium A containing 4 μg/ml of ampicillin under aeration with 0.03% or 5% CO_2. The culture was then diluted with medium A (OD_{550} = 0.2) and grown for 2 days (OD_{550} = 3–6), in which cells were growing exponentially. Each culture was collected, resuspended in 0.3 ml of PBS buffer. Protein extracts were prepared using a FastPrep system (Qbiogene, Carlsbad, CA, USA) as described by the manufacturer, and centrifuged at 10 000 rpm for 15 min at 4°C. Dilutions of the resulting supernatant were assayed for luciferase activity determined with a photometer AB-2100 (Atto, Tokyo) [16]. Luciferase activity was normalized by the protein content. Protein concentrations were determined by the method of Bradford [17] with bovine serum albumin as a standard.

2.6. Western blot analyses of RbcLS

Two oligopeptides, a 17-mer of RbcL and a 13-mer of RbcS, were chemically synthesized and specific antibodies against them were raised in rabbits. Protein extracts were prepared (see Section 2.5 for luciferase activity). Cellular proteins (1.3 μg) were separated on 4–20% sodium dodecyl sulfate–polyacrylamide gel, and separated proteins were transferred onto a nitrocellulose membrane. Anti-RbcL or anti-RbcS antibodies (1:1500 dilution) and anti-rabbit IgG antibody (1:100 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) conjugated with horseradish peroxidase were used as primary and secondary antibodies, respectively. Immunodetection was performed by an enhanced chemiluminescence method as recommended by the manufacturer (Amersham Biosciences, Piscataway, NJ, USA).

3. Results

3.1. Uptake of FITC-labeled oligonucleotides by cyanobacterial cells

We verified that double-stranded oligonucleotides labeled with FITC could be introduced efficiently into cyanobacterial cells in culture without any transfection reagent. Cells were fixed 24 h after culture with FITC-labeled oligonucleotides and observed by fluorescence microscopy. No fluorescent signal was seen in cells cultured without the FITC oligonucleotide (Fig. 1A,B). When the oligonucleotides (100 nM or 200 nM) were added to the culture, fluorescence was detected in most of the cells observed (Fig. 1C–F). The intensity of the recorded signal showed that the cellular uptake of the oligonucleotide was dose-dependent (Fig. 1C–F). Thus, there was efficient uptake of FITC-labeled oligonucleotides by cyanobacterial cells in culture even without any transfection reagent.

3.2. Functional analysis of the rbc promoter by a luciferase reporter gene assay

To confirm the site of the CO_2-regulatory element, we constructed pAQJ4-Luc, by inserting the luciferase gene from cassette vector 2 into pAQJ4-MCS [14,15]. Two fragments, P304 with an AT-rich element or P251 without an AT-rich element, in the *rbc* promoter obtained using a PCR technique were fused to the luciferase-coding region (Fig. 2A), and their promoter activities in *Synechococcus* sp. PCC7002 were characterized by a luciferase reporter gene assay.

Fig. 2B shows luciferase activities in cell extracts from cyanobacterial transformants carrying each luciferase reporter gene fusion. CO_2-dependent luciferase activities were detected in cyanobacterial transformants containing P304/pAQJ4-Luc. Extracts from these transformants grown under 5% CO_2 showed diminished activities compared to those from 0.03% CO_2-grown cells. However, luciferase activities with loss of CO_2 dependence were seen in extracts from P251/pAQJ4-Luc transformants grown under 5% CO_2. Thus, the region, −304 to −251 upstream of the translation start site of *rbcL*, which contains the AT-rich sequence (−291 to −262), contributes to the response of the *rbc* promoter in *Synechococcus* sp. PCC7002 to CO_2.

3.3. Effect of AT-rich decoy oligonucleotides on CO_2 response of the rbc promoter activity

We examined whether AT-rich decoy oligonucleotides can modulate the CO_2 response of the transcriptional activity in the *rbc* promoter. In cyanobacterial transformants containing P304/pAQJ4-Luc, addition of 200 nM AT-rich decoy oligonucleotides resulted in nearly the same luciferase activity between 0.03% and 5% CO_2 levels, indicating that CO_2 dependence of P304 was lost by AT-rich decoy oligonucleotides (Fig. 3). By contrast, addition of non-specific control oligonucleotides had no effect on the CO_2 dependence of luciferase activity. AT-rich decoy oligonucleotides failed to affect the

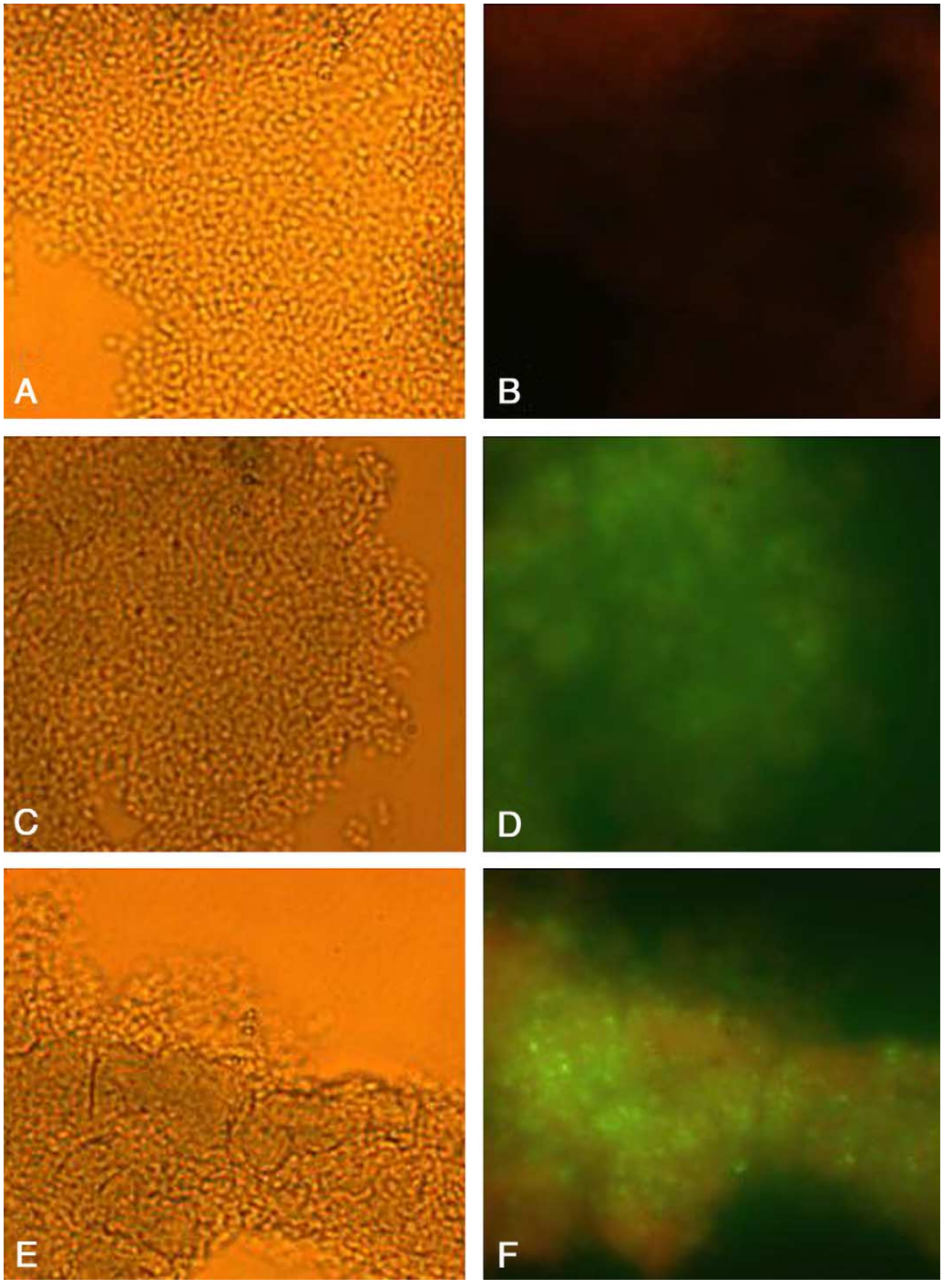

Fig. 1. Cellular uptake of FITC-labeled oligonucleotides in cyanobacterial cells in culture. Cyanobacterial cells were incubated with FITC-labeled oligonucleotides for 24 h under aeration with 1% CO_2 at the indicated concentrations. A,B: No oligonucleotide; C,D: 100 nM; E,F: 200 nM. A,C,E: phase contrast images; B,D,F: fluorescent images corresponding to A, C and E, respectively. Magnification, ×400.

luciferase activity in the transformants with the P251/pAQJ4-Luc reporter gene fusion, which contains no AT-rich element, exhibiting similar results to those in the P251/pAQJ4-Luc transformants described in Fig. 2B.

3.4. *Effect of AT-rich decoy oligonucleotides on CO_2-regulated expression of RbcLS*

We next examined whether blockade at the AT-rich element in the *rbc* promoter by AT-rich decoy oligonucleotides would affect the production of RbcLS regulated by CO_2 levels. We performed Western blotting and densitometric quantification of peak areas in detected signals. Extracts from cells grown under 5% CO_2 showed a decrease in expression levels of RbcLS compared to extracts from cells grown under 0.03% CO_2, indicating that a high CO_2 level inhibits the expression of RbcLS (Fig. 4). However, transfection with 200 nM AT-rich decoy oligonucleotides abolished the CO_2-dependent decreases in protein levels of RbcLS completely, whereas transfection with non-specific control oligonucleotides did not (Fig. 4).

4. Discussion

Analysis of the CO_2-regulatory function of *rbc* promoter by the luciferase reporter gene assay indicated that the expression of *rbc* genes is regulated by CO_2 conditions, and the region, −304 to −251 upstream from the translation start site of *rbcL*, which contains the AT-rich sequence (−291 to −262), contributes to the CO_2 response of the *rbc* promoter in *Synechococcus* sp. PCC7002. These results are compatible with our previous finding that the CO_2-dependent *cis*-acting element, the AT-rich region, was present upstream of the cyanobacterial *rbc* promoter and that the transcription of *rbcLS* may be regulated by this AT-rich element and a DNA-binding protein [11].

To define the role of the AT-rich element and corresponding DNA-binding protein in cyanobacteria, we applied a transcription factor decoy approach that uses synthetic double-stranded phosphorothioate oligonucleotides containing a *cis*-element [2–4]. In this approach, the synthetic double-stranded phosphorothioate oligonucleotides, as decoy elements that can be introduced into the cells with the aid of a transfection reagent, compete with the binding of sequence-specific DNA-binding proteins to promoter regions of targeted genes and interfere with eukaryotic transcription. We examined whether cyanobacterial cells could take up double-stranded oligonucleotides labeled with FITC successfully in culture without any transfection reagent. *Synechococcus* sp. PCC7002 possesses an efficient, naturally occurring mechanism for the uptake of exogenous DNA [6,7]. Cyanobacterial cells were transfected in culture by merely adding double-stranded oligonucleotides into growth medium. Thus, a transcription factor decoy approach in cyanobacterial cells is a convenient and ideal method for investigating *cis*-elements and corresponding transcription factors.

Addition of oligonucleotides to cyanobacteria induced growth inhibition and a decrease in luciferase activities slightly in transformants containing P304/pAQJ4-Luc. These findings may be due to some non-specific toxic effects, as non-

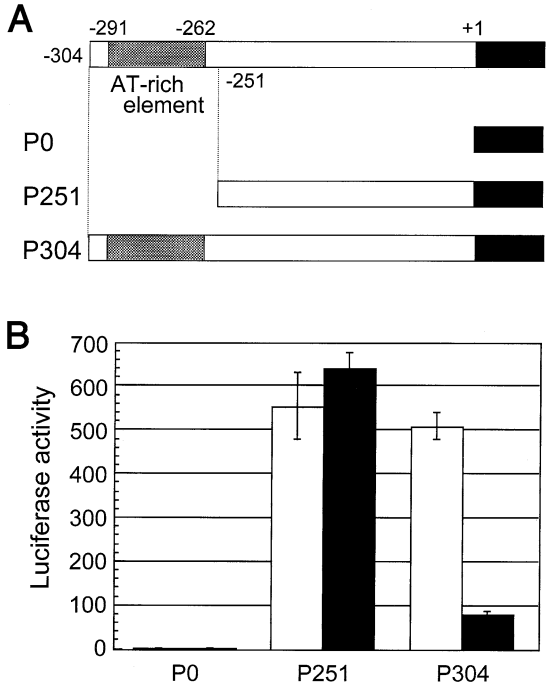

Fig. 2. Expression of luciferase in *Synechococcus* sp. 7002 transformants containing pAQJ4-Luc derivatives grown under aeration with 0.03% or 5% CO_2. A: Promoter regions used in luciferase reporter gene fusions. Numbers refer to the distance from the translation start site of the *rbcL* gene. B: Luciferase activities of luciferase reporter gene fusions in *Synechococcus* sp. 7002 grown under aeration with 0.03% (white bars) or 5% CO_2 (black bars). Luciferase activity is given as photons normalized by the protein content. Data represent mean ± S.D. obtained from three independent measurements.

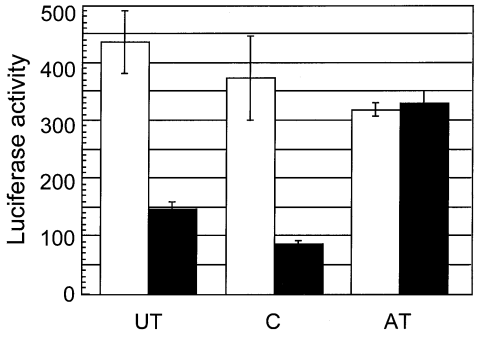

Fig. 3. Effect of transfection with decoy oligonucleotides on the expression of luciferase in response to CO_2 in *Synechococcus* sp. 7002 transformed by the promoter-luciferase gene fusions containing AT-rich regions. Cells were transfected with oligonucleotides as described in Section 2. *Synechococcus* sp. 7002 was grown under aeration with 0.03% (white bars) or 5% CO_2 (black bars) for 2 days. Luciferase activity is given as photons normalized by the protein content. UT, untransfected control cells; C, 200 nM non-specific control oligonucleotide-transfected cells; AT, 200 nM AT-rich decoy oligonucleotide-transfected cells. Data represent mean ± S.D. obtained from three independent measurements.

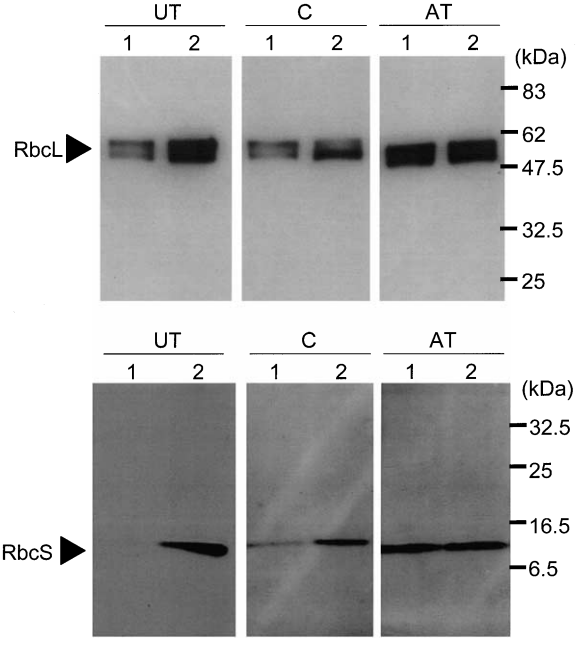

Fig. 4. Effect of transfection with decoy oligonucleotides on RbcLS expression in *Synechococcus* sp. PCC7002. *Synechococcus* sp. 7002 was grown under aeration with 5% (lane 1) or 0.03% (lane 2) CO_2. Protein extracts were prepared from cells treated with TEN buffer (UT), non-specific control oligonucleotides (C), or AT-rich decoy oligonucleotides (AT) at 200 nM for 2 days. The RbcLS contents were determined by Western blot analysis as described in Section 2.

specific binding of oligonucleotides or their degradation products to biological targets has been shown [18]. Cellular uptake of AT-rich oligonucleotides, however, was clearly related to inhibition of the CO_2 response of the *rbc* promoter in cyanobacteria. Addition of 200 nM AT-rich decoy oligonucleotides suppressed the CO_2 dependence of the *rbc* promoter in transformants containing P304/pAQJ4-Luc (Fig. 3), but not in transformants containing P251/pAQJ4-Luc reporter gene fusion (data not shown). Transfection of non-specific control oligonucleotides had no effect on the CO_2 dependence of luciferase activity. These results suggest that the AT-rich element in the *rbc* promoter contributes to the CO_2-dependent repression and that blockade of the sequence-specific DNA-binding protein by AT-rich decoy oligonucleotides results in loss of CO_2 dependence.

This blockade at the AT-rich element in the *rbc* promoter affected the expression of RbcLS regulated by CO_2 levels. Western blot analysis revealed that a high CO_2 level inhibits the expression of RbcLS, but that transfection with 200 nM AT-rich decoy oligonucleotides abolished this inhibitory effect completely. Cells treated with non-specific control oligonucleotides exhibited the CO_2 dependence less clearly than untreated cells did, because non-specific toxic effects of oligonucleotides might influence the results, as discussed above. However, non-specific oligonucleotides did not inhibit the CO_2 response of the *rbc* promoter (Fig. 4). Therefore, it appears that the inhibition of binding of the sequence-specific DNA-binding protein by AT-rich decoy oligonucleotides suppressed the CO_2 dependence in the expression of RbcLS.

In conclusion, using the decoy approach, we elucidated that the AT-rich element and sequence-specific DNA-binding protein play a role in negative CO_2 regulation of *rbcLS* expression and RbcLS production. The decoy oligonucleotides were efficiently introduced into cyanobacterial cells in culture without any transfection reagent, providing a useful approach to investigate *cis*-elements and corresponding transcription factors in photosynthetic organisms.

Acknowledgements: We thank Dr. Akiho Yokota of the Nara Institute of Science and Technology for helpful and useful advice. We are also grateful to Mrs. Mina Usui-Takeshige for DNA sequencing analysis.

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