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The Transcription Termination Factor Rho Is Essential and Autoregulated in *Caulobacter crescentus*

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The impossibility of obtaining a *rho* null mutant and sensitivity to bicyclomycin have indicated that *rho* is essential for the viability of *Caulobacter crescentus*. Transcription gene fusions of sequences with serial deletions of the *rho* 5′ untranslated region (5′-UTR) with a *lacZ* reporter gene indicated that *rho* is autoregulated at the level of attenuation of transcription in the 5′-UTR.

The importance of Rho-dependent termination in bacterial gene expression is well established for genes that are regulated by termination of transcription in the leader sequences, as described for the *tna* operon from *Escherichia coli* (15, 33, 35). Studies with *E. coli*, *Rhodobacter sphaeroides*, and *Micrococcus luteus* showed that *rho* is an essential gene in these bacteria (6, 8, 26) but not in *Bacillus subtilis* and *Staphylococcus aureus* (29, 34). The current model proposes that Rho loads onto nascent mRNAs at a cytosine-rich region that lacks a strong secondary structure, known as the Rho utilization (*rut*) site (1, 23, 30). It has been proposed that once bound, a hexameric Rho complex hydrolyzes ATP, moves directionally 5′-3′ along the RNA to the site of transcription, and with its helicase function, disengages the message from the paused RNA polymerase and the DNA template, separating the transcription complex (4, 17, 22, 27, 31, 32).

The Rho factor was described to be autogenously regulated in *E. coli* and *B. subtilis* via an attenuation mechanism that occurs in Rho-dependent terminators located within the mRNA leader of the *rho* gene (2, 12, 20). The ATPase activity of Rho in several bacteria has been described and characterized (10, 11, 12, 16, 25), but autoregulation by Rho-dependent terminators in bacteria other than *E. coli* and *B. subtilis* is yet to be characterized.

In a previous work from our group, a *rho* mutation generated by a Tn*5* insertion (strain SP3710) caused deficiency in survival to high salt concentration and acidic pH and resulted in an extreme sensitivity to oxidative stress (13). Experiments showed that transcription of the *C. crescentus rho* gene increases in the *rho* mutant strain, which is indicative of a negative autoregulatory circuit (13).

The Rho factor is essential for *C. crescentus*. The Rho protein in SP3710 was interrupted after residue Gly-112, immediately before the first RNA binding motif (13). Immunoblotting assays of cell extracts probed with a polyclonal Rho antiserum showed that SP3710 still produces a 45-kDa truncated polypeptide but does not present the complete form of the Rho protein of the parental strain, NA1000, except when it is complemented in *trans* with the *rho* gene (Fig. 1A). The *C. crescentus* Rho protein has a predicted molecular mass of 52.8 kDa but migrates with an apparent mass of 60 kDa in sodium dodecyl sulfate-polyacrylamide gel. The 45-kDa protein should

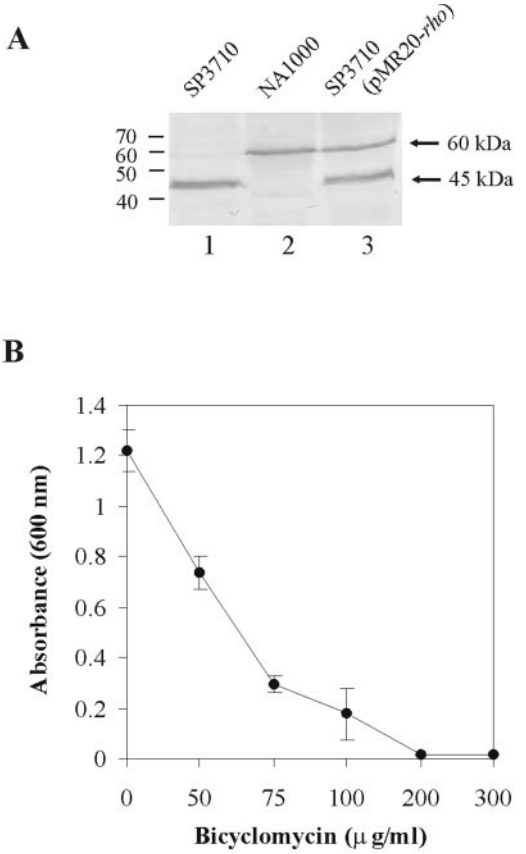

FIG. 1. (A) Immunoblot of *C. crescentus* cell extracts with polyclonal anti-Rho antiserum. Lane 1, extract from strain SP3710; lane 2, extract from strain NA1000; lane 3, extract from SP3710 containing the pMR20-Rho plasmid. (B) Analysis of the sensitivity of *C. crescentus* NA1000 cells to bicyclomycin. Growth was determined by measuring the optical density at 600 nm after 48 h at 30°C of cultures in peptone-yeast extract medium containing increasing concentrations of bicyclomycin: 0, 50, 75, 100, 200, and 300 μg/ml.

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TABLE 1. Primers used in this study

Primer	Sequence (5′-3′)[a]
Rho1	CGGTCATGGCAAGACTCAAG
Rho2	TTGAATTCATGACCGAAGACACCGAAAACC
Rho3	CATAAGCTTTCAGGTGTTCATCGACTGG
Rho4	GCCAAGCTTCGAATGGACCGTCTGTTACC
Rho5	TGGAATTCATCGACACCTATCACCGATC
Rho6	GTCCGCGTGGTTTGCATCCACAG
Rho7	CAGCTCCTGCAGGGACATGGAC
Rho8	GGTTTGGATCCACAGTCGGTTC
Rho9	GCGAATTCTTCCTGCTGCAGCCCTG
Rho10	GGGGATCCCAGGACGGGCGCCAGTC
Rho11	CAGGATCCTCTGTTGACGATGACGC
Rho12	GGGGATCCCGGTCATGGCAAGACTC
Rho14	CAGAATTCGAGCTTGCGGCGGCGGC

[a] Underlined nucleotides indicate restriction sites incorporated into oligonucleotides.

TABLE 2. Antibiotic resistance profile of the colonies after the second recombination event

Strain	No. of colonies		
	Sp^r/Km^s	Sp^r/Km^r	Total
1S	0	73	73
1S(pMR20-Rho)	26	51	77
4S	0	79	79
4S(pMR20-Rho)	15	68	83

have all the residues essential for RNA binding at the high-affinity primary RNA binding site (5, 32), but the missing N-terminal 112 residues may have a role in protein function, probably in the interaction of the subunits to form the active hexamer, as proposed for *E. coli* (32). In order to generate an independent *rho* null mutant, a 3.4-kb fragment containing the *rho* gene was amplified by PCR with primers Rho4 and Rho5 (Table 1) as described in reference 13 and cloned into plasmid pNPTS138. This plasmid carries a *sacB* gene and a kanamycin resistance (Km^r) gene and does not replicate in *Caulobacter*. A 1.0-kb fragment containing the *rho* coding region was replaced by a spectinomycin resistance cassette (ΩSp^r) (28). Two clones (1S and 4S) were selected after the first recombination event (Sp^r and Km^r clones), in plates containing 5 μg/ml kanamycin and 20 μg/ml spectinomycin. These clones were grown in plates containing 3% sucrose and spectinomycin to select for cells in which the plasmid was deleted from the chromosome, leaving the disrupted copy of the gene. The colonies obtained

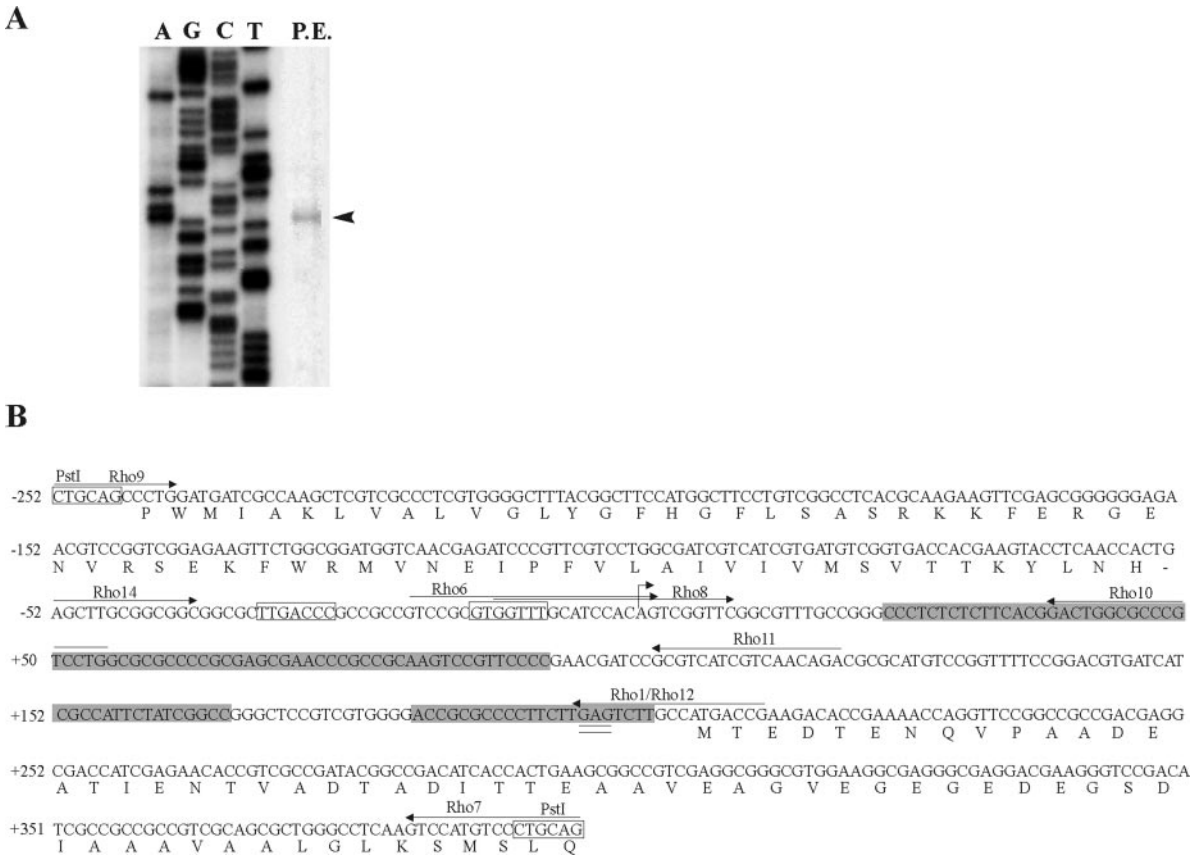

FIG. 2. (A) Determination of the transcription start site of the *rho* gene. Primer extension analysis was carried out with 50 μg total RNA from exponential-phase cells (P.E.). Primer Rho1 was 5′-end labeled with ^{32}P, extended with reverse transcriptase to determine the transcription start site (arrowhead), and also used in the sequencing reaction (shown on the left). (B) Nucleotide sequence of the *rho* regulatory region. The bent arrow indicates the transcription start site and was designated position +1, and the −35/−10 sequences are boxed. C-rich sequences are shaded, the ribosome-binding site is double underlined, and the PstI restriction sites are indicated. The arrows indicate the positions of each oligonucleotide used for primer extension, transcriptional fusions, and RNA gel mobility shift assays.

were then analyzed for their kanamycin resistance pattern, as shown in Table 2. The chromosomal copy of the *rho* gene was lost only in clones 1S and 4S, which carry a copy of the *rho* gene on a plasmid (pMR20-Rho), generating Sp^r/kanamycin-sensitive (Km^s) clones. These results indicate that the *rho* gene is essential for the viability of *C. crescentus* as described for other gram-negative bacteria, such as *E. coli* and *Rhodobacter sphaeroides* and the gram-positive *Micrococcus luteus* (6, 8, 26). These results also indicate that the truncated form of Rho in SP3710 must have some remaining activity.

The antibiotic bicyclomycin is a specific inhibitor of Rho activity in *E. coli* (36) and prevents the growth of several gram-negative bacteria at low dosages (24). In vitro assays have shown that bicyclomycin inhibits the ATPase activity of Rho from *E. coli* (18, 36), *M. luteus* (25), *B. subtilis* (12), and *Streptomyces lividans* (11), but its antimicrobial activity is found only against gram-negative bacteria, with the exception of *M. luteus* (25, 26). The growth of *C. crescentus* NA1000 was tested in the presence of increasing concentrations of bicyclomycin (Fig. 1B), and it was verified that 100 μg/ml was the minimal concentration tested where there was still growth and 200 μg/ml abolished it completely. These results further confirm that the *rho* gene is essential for the viability of *C. crescentus* and that the truncated form of Rho in strain SP3710 is probably functional enough to ensure viability, although the cells are still severely affected (13). The minimal bicyclomycin concentrations that prevented growth were 75 μg/ml for *E. coli* DH5α (11) and 90 μg/ml for *M. luteus* (26). However, higher bicyclomycin concentrations did not affect the growth of bacteria for which Rho is not essential, such as *B. subtilis* (300 μg/ml) (12) and *Streptomyces lividans* ZX7 (600 μg/ml) (11).

Sequences downstream of the *rho* promoter are required for autoregulation. The transcription start site of the *rho* gene was determined by primer extension analysis using 50 μg of total RNA from the NA1000 strain and primer Rho1 (Table 1), labeling with [γ-^{32}P]ATP, and extension with SuperScript III reverse transcriptase (Invitrogen) (Fig. 2A). The *rho* mRNA presented a 206-nt-long 5′ untranslated region (5′-UTR). Such long UTRs were also described for the *rho* genes from *E. coli* (255 nucleotides [nt]) and *B. subtilis* (at least 293 nt), suggesting that a long leader sequence is important for the regulation of this gene (12, 20, 29).

Different fragments containing the *rho* promoter and the 5′-UTR were amplified by PCR with the following primers (Table 1): Rho10 (Rho9/Rho10), Rho11 (Rho9/Rho11), Rho12 (Rho9/Rho12), Rho14 (Rho14/Rho10), Rho15 (Rho14/Rho7), and Rho7 (Rho8/Rho7) (Fig. 3A). The fragments were cloned in plasmid pRK*lacZ*290 (7), generating transcriptional fusions with the *lacZ* gene, and β-galactosidase activity was measured by the method of Miller (21).

The fragment containing the promoter and 5′-UTR regions of *rho* (RhoPstI) yielded about 850 β-galactosidase units in NA1000 and 4,500 units in SP3710, indicating that autoregulation occurs in this construct (Fig. 3B). As a control, the *tacA* gene (19), which is not regulated by Rho, showed similar levels of expression in both strains. On the other hand, constructs containing either the promoter only (Rho14) or the promoter and 54 bp of the 5′-UTR (Rho10) showed much higher β-galactosidase activities in both strains, indicating that the autogenous regulation is lost in these constructs. These results indicate that the autoregulation does not occur at the level of transcription initiation and that the *rho* gene has a strong promoter.

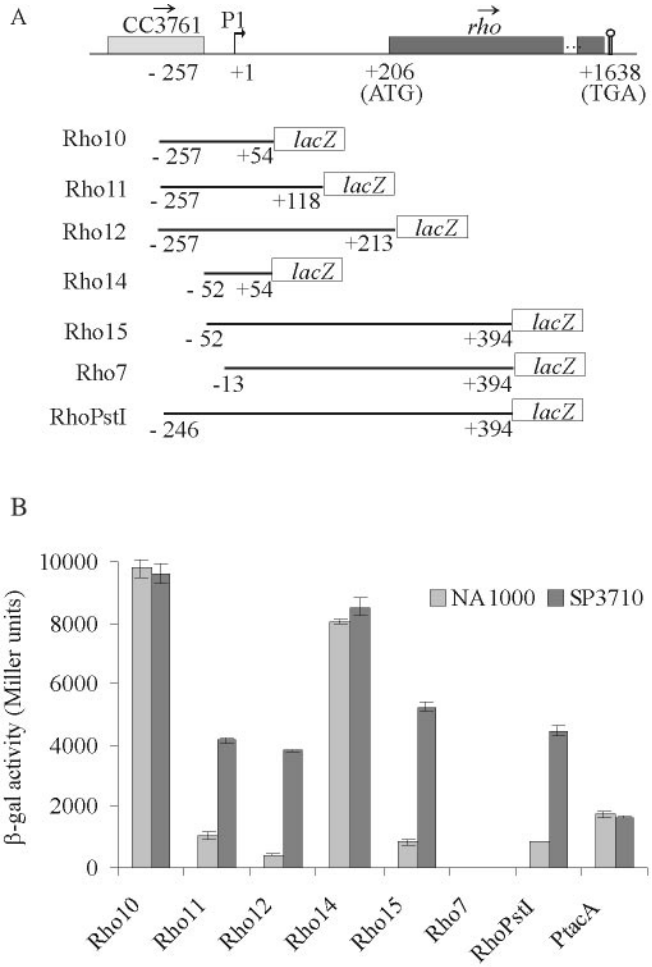

FIG. 3. Analysis of expression driven by *rho* regulatory regions. (A) Map (not to scale) of the region surrounding the *rho* gene in *C. crescentus*. Boxes indicate open reading frames, and the arrows above them show the direction of transcription. Coordinates in nucleotides are relative to the first nucleotide of the *rho* transcription start site (bent arrow), determined by primer extension analysis (+1). The promoter is indicated as P1. The stem and loop indicate a putative Rho-independent transcription terminator. Below the map, each transcriptional fusion construct is indicated, where coordinates indicate the extent of the regulatory *rho* region cloned in front of the reporter *lacZ* gene. (B) β-Galactosidase activity of each construct in the NA1000 and SP3710 strains. The results are in Miller units (21) and are the average of results of at least three independent assays.

Both Rho11 and Rho12 constructs showed β-galactosidase activities similar to that of RhoPstI, restoring the autoregulation. These results suggested that attenuation sites could be present in the region between nt +54 and +213, but there was still a possibility that a second promoter could exist in this region. Rho7, which lacks the −35 region of the *rho* promoter but keeps the complete 5′-UTR and 187 bp of the Rho coding region, showed no β-galactosidase activity, confirming that there is no other promoter downstream of position −13. When the complete promoter was present at the 5′ end (Rho15), it

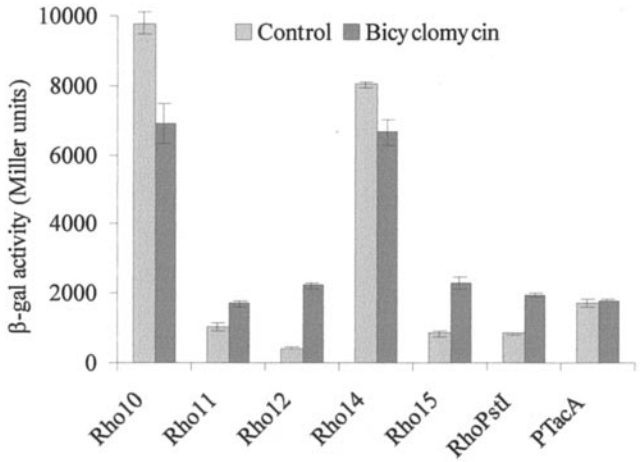

FIG. 4. Analysis of expression driven by *rho* regulatory regions in the presence of bicyclomycin. Expression was determined from NA1000 cells harboring the respective constructs described in Fig. 3 after incubation at 30°C for 3 h in the presence or absence of 100 μg/ml bicyclomycin. The β-galactosidase (β-gal) activities are expressed in Miller units (21) and are the averages of results of at least three independent assays.

showed normal levels of activity. Overall, the results indicated that transcription attenuation sites are present between nucleotides +54 and +213, which is similar to what was described for *rho* genes from *E. coli* and *B. subtilis* (12, 20).

These results were confirmed by measuring *rho* expression in the presence of 100 μg/ml bicyclomycin for 3 h. As shown in Fig. 4, a pattern of *rho* expression was obtained for NA1000 with bicyclomycin that was similar to those presented for SP3710 with all the constructs. These results confirm that the presence of Rho causes a decrease in the transcription of its own gene and that this effect is dependent on the 5′-UTR downstream of position +54.

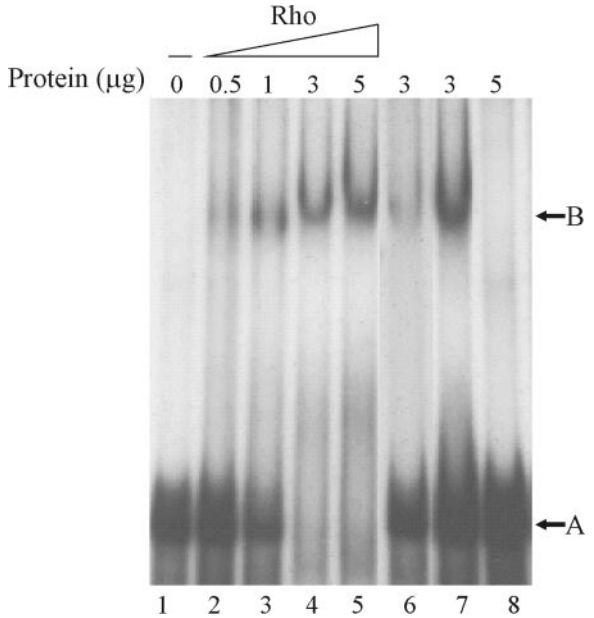

FIG. 5. RNA electrophoretic mobility shift assay of *rho* mRNA. The indicated amount of purified Rho was incubated with 70 ng of ^{32}P-labeled RNA fragment for 20 min, and the complexes were resolved in a 5% polyacrylamide gel. Lane 1, RNA probe; lanes 2 to 5, increasing amounts of Rho (0.5, 1, 3, and 5 μg, respectively) added to the RNA probe; lane 6, the probe incubated with 3 μg of Rho and 0.7 μg of the same nonradioactive RNA competitor; lane 7, the probe incubated with 3 μg of Rho and 1 μg of tRNA; lane 8, the probe incubated with bovine serum albumin (5 μg).

Binding of Rho to the 5′-UTR of *rho* mRNA. The results above suggested that there would be attenuators between position +54 and position +213 (Fig. 2B). This region is notably cytidine/uridine rich and is a strong candidate to contain *rut* sites, similar to those proposed for enteric bacteria (1, 3, 9, 23). The binding of Rho to this region was tested by an RNA electrophoretic mobility shift assay. The *rho* gene was amplified by PCR, using primers Rho2 and Rho3 (Table 1), and the products were cloned into pProEX-HT (Invitrogen). Expression of His-Rho in *E. coli* DH5α was obtained after induction with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h, and the protein was purified in Ni-nitrilotriacetic acid agarose columns (QIAGEN).

A probe extending from primer Rho6 to primer Rho12 was transcribed in vitro by T7 RNA polymerase in the presence of [α-^{32}P]UTP from a pGEM-T Easy plasmid (Promega). An RNA binding assay was carried out essentially as described in reference 14, using an RNA template (5×10^5 cpm) and purified His-Rho protein (dialyzed against the binding buffer). Incubation of the probe with different amounts of purified His-Rho protein (Fig. 5) caused retardation in its migration (lanes 2 to 5). The entire amount of probe was shifted with 3 μg/ml of Rho protein (lane 4). The same unlabeled RNA fragment was able to compete efficiently for binding (compare lanes 4 and 6), but tRNA was not (compare lanes 4 and 7). As a control, the assay was carried out with 5 μg/ml of bovine serum albumin, and no shift in the band was observed (lane 7). The interaction of Rho protein with the *rho* 5′-UTR mRNA confirms the presence of *rut* sites in this region and indicates that expression of the *C. crescentus rho* gene is autoregulated by attenuation of transcription.

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