Characterization of the *Escherichia coli* RNA 3'-Terminal Phosphate Cyclase and Its σ^{54}-Regulated Operon*

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The RNA 3'-terminal phosphate cyclase catalyzes the ATP-dependent conversion of the 3'-phosphate to the 2',3'-cyclic phosphodiester at the end of various RNA substrates. Recent cloning of a cDNA encoding the human cyclase indicated that genes encoding cyclase-like proteins are conserved among Eucarya, Bacteria, and Archaea. The protein encoded by the *Escherichia coli* gene was overexpressed and shown to have the RNA 3'-phosphate cyclase activity (Genschik, P., Billy, E., Swianiewicz, M., and Filipowicz, W. (1997) *EMBO J.* 16, 2955–2967). Analysis of the requirements and substrate specificity of the *E. coli* protein, presented in this work, demonstrates that properties of the bacterial and eukaryotic enzymes are similar. ATP is the best cofactor (K_m = 20 μM), whereas GTP (K_m = 100 μM) and other nucleoside triphosphates (NTPs) act less efficiently. The enzyme undergoes nucleotideylation in the presence of [α-^{32}P]ATP and, to a lesser extent, also in the presence of other NTPs. Comparison of 3'-phosphorylated oligoribonucleotides and oligodeoxyribonucleotides of identical sequence demonstrated that the latter are at least 500-fold poorer substrates for the enzyme. The *E. coli* cyclase gene, named rtcA, forms part of an uncharacterized operon containing two additional open reading frames (ORFs). The ORF positioned immediately upstream, named rtcB, encodes a protein that is also highly conserved among Eucarya, Bacteria, and Archaea. Another ORF, called rtcR, is positioned upstream of the rtcA/rtcB unit and is transcribed in the opposite direction. It encodes a protein having features of σ^{54}-dependent regulators. By overexpressing the N-terminally truncated form of RtcR, we demonstrate that this regulator indeed controls expression of rtcA and rtcB in a σ^{54}-dependent manner. Also consistent with the involvement of σ^{54}, the region upstream of the transcription start site of the rtcA/rtcB mRNA contains the −12 and −24 elements, TTGCA and TGGCA, respectively, characteristic of σ^{54}-dependent promoters. The cyclase gene is nonessential as demonstrated by knockout experiments. Possible functions of the cyclase in RNA metabolism are discussed.

The 2',3'-cyclic phosphate termini are produced during RNA cleavage by many different endoribonucleases. For most of the known enzymes, among them many secretory degradative nucleases such as RNases A or T1, cyclic phosphates are formed as intermediates that are subsequently opened into 3'-phosphomonoesters (1, 2). For some enzymes, such as tRNA-splicing endonucleases from Eucarya and Archaea, the cyclic phosphate is a final product of the cleavage reaction (4–6). In addition, the type I topoisomerase has recently been shown to have endoribonuclease activity yielding 2',3'-cyclic phosphate termini (7). Like some protein enzymes, ribozymes such as hammerheads, hairpins, or the hepatitis delta ribozyme generate 2',3'-cyclic phosphate and 5'-hydroxyl ends during the RNA cleavage reaction (8).

That the 2',3'-cyclic phosphate has an anabolic function in RNA metabolism emerged when it was found that eukaryotic RNA ligases require 2',3'-cyclic ends for RNA ligation (9–17). This requirement applies to both of the known non-organellar RNA ligases, one ligating RNA ends by the 3',5'-phosphodiester, 2'-phosphomonoester linkage and the other joining the ends by the ordinary 3',5'-phosphodiester (reviewed in Refs. 18–20). The two ligases were shown to be involved in nuclear pre-tRNA splicing (12, 14, 21–26). In addition, the ligase generating the 3',5'-phosphodiester, 2'-phosphomonoester linkage functions in splicing the unusual intron present in *HAC1* pre-mRNA in yeast (27, 28) and may also be involved in ligation of virusoid and viroid RNAs in plants (29–31). Interestingly, the only known cellular RNA ligase in eubacteria, which joins RNA ends via the 2',5'-phosphodiester, also requires 2',3'-cyclic ends for ligation (32, 33). Another finding uncovering a potential role for the 2',3'-cyclic phosphate in RNA metabolism was the demonstration that the spliceosomal U6 snRNA in many organisms has a cyclic 2',3'-phosphodiester at the terminus. The mechanism and enzymes responsible for this modification and its biological function are not known (34–36).

In light of the importance of cyclic termini in RNA metabolism, it was interesting to discover that endonucleolytic cleavage is not the only way to generate RNA molecules bearing 2',3'-cyclic phosphates. Such molecules can also be produced by the action of the RNA 3'-terminal phosphate cyclase, an enzyme that catalyzes ATP-dependent conversion of a 3'-phosphate at the end of RNA to the 2',3'-cyclic phosphodiester (11). The cyclase has been purified from HeLa cells and its mechanism of action established (37–40). The cyclization occurs in three steps: (a) Enzyme + ATP → Enzyme-AMP + PP_i, (b) RNA-N^{3'}p + Enzyme-AMP → RNA-N^{3'}pp^{3'}A + Enzyme, and (c) RNA-N^{3'}pp^{3'}A → RNA-N^{3'}p + AMP.

Evidence for the initial two steps were the identification of the covalent cyclase-AMP intermediate complex (37–39, 41) and the demonstration of the RNA-N^{3'}pp^{3'}A molecule accumulation when the ribose at the RNA 3' terminus is replaced with the 2'-deoxy- or 2'-O-methylribose (37). Reaction (c) probably occurs non-enzymatically as the result of nucleophilic attack by the adjacent 2'-OH on the phosphorus in the phosphodiester linkage (40).
To investigate the biological role of the cyclase, we have recently cloned a cDNA encoding the human enzyme (41). The cyclase mRNA was shown to be expressed in all tissues and cell lines analyzed. The protein is localized to the nucleus, consistent with its postulated role in RNA processing. The sequence of the human cyclase has no apparent motifs in common with any protein of known function. However, genes encoding proteins having strong similarity to the cyclase were identified in organisms belonging to all three kingdoms, Eucarya, Bacteria, and Archaea. The protein encoded by the Escherichia coli gene was overexpressed and purified and was shown to have RNA 3′-terminal phosphate cyclase activity (41). Conservation of the cyclase among eukaryotic and prokaryotic organisms suggests that the enzyme performs an important function in RNA metabolism.

In this work, we describe the characterization of the E. coli cyclase and demonstrate that its gene forms part of a so far uncharacterized operon that belongs to the class of operons controlled by the alternative σ^74 factor.

**MATERIALS AND METHODS**

**General Procedures**—Unless stated otherwise, all techniques for manipulation of DNA and RNA, commonly used buffers, and media were as described (42, 43).

**Bacterial Strains and Plasmids**—E. coli strains YMC10 (thi-1 end1I hsdR17 supE44 galU galK strA thi-1 end1I lacU169 hutC5977 lacY1) and YMC22 (like YMC10, but rpoD::Tn10) (44) and plasmid pTH7 were obtained from Drs. M. Carmon-Perez and B. Magasanik (MIT, Cambridge, MA). pTH7 contains the E. coli σ^74 (rpoD::Tn10) gene cloned downstream of the lac promoter on a pBR322 derivative (45). The strain used for gene replacement, MC1061 (F araD139 stra-leu7697/lacY1 galU galK strA bisslacA strA bisslacX74) (46) and plasmid pMAK705 (46) were obtained from Drs. S. Kushner (University of Georgia, Athens, GA) and J. Offengand (University of Miami, FL).

**Bluescript** II KS+ and pRE4 were from Stratagene and Qiagen, respectively.

Plasmid pRtch, containing the rtcB gene and its flanking sequences, was obtained by cloning of the PCR-amplified E. coli DNA fragment into the EcoRV site of pBluescript II KS+; oligonucleotides GCCAC-GACCGTTGGAATTCATCATCG and CAGCGCAATCATCCTTTTTCATC into the SmaI site of pHSG765 vector containing the E. coli ara-leu promoter on a pHSG765 vector containing the E. coli ara-leu promoter. Plasmids pRtch and pRtchΔN, expressing the RtcR activator and its N-terminally truncated version, respectively, were constructed as follows. The rtcR gene with flanking regions was amplified by PCR using as a template a phage DD765 DNA (kindly provided by G. R. Plankett and F. Blutnetter, University of Wisconsin, Madison, WI) and cloned into pBluescript II KS+, yielding plasmid pH1. HindIII and BamHI sites were introduced by oligonucleotide-directed PCR mutagenesis at the 5′ and 3′ ends of the rtcR gene, respectively, using pKD4 as a template. The PCR product was cloned into HindIII and BamHI sites of the pHSG765 vector containing a chloramphenicol resistance marker (47), yielding plasmid pRtch. In this plasmid, the rtcR gene is expressed under the control of the lacZ promoter as a translational fusion with a second DNA fragment of the cyclase gene (obtained by PCR using the E. coli genomic DNA as a template and oligonucleotides TGCGCCATTGCAATCATCGCCTTTCATC and CTGACTACTGTGCGACTGGCACAAGAGATG as the 5′-region-specific primers and oligonucleotides ATTTCTGAGTAACTTTCTGTACCTGTCGTAATC and GGTCGACGGATCTCTTCTATC as the 3′-region-specific primers). The amplified gene was cloned stepwise into pBSKm, yielding the CtaI-SalI and XbaI-BamHI sites, respectively, from plasmid pMAK507 (46). The cyclase was overexpressed as His-tagged protein in the E. coli strain BL21(DE3) and purified on an Ni-nitrilotriacetic acid column as described (47).

**Preparation of the E. coli Cyclase and Its Substrates**—The cyclase was purified by the method of Bradford using the reagent obtained from Bio-Rad and bovine serum albumin as a standard.

**The oligonucleotide substrates** CCCCCACCCGGp^3 and AAAUUAAAGGp^3, were prepared as described previously (41). For use in some experiments, the substrates were additionally purified on a 10% polyacrylamide, 8 m urea gel. Aliquots of radioactive substrates were analyzed by digestion with RNase T2, nuclease P1, or calf intestine phosphatase (9), followed by TLC on cellulose plates in solvent A (see legend) in which over 90% of the incorporated radioactivity was always found at the position of Gp^3. The E. coli S RNA (Boehringer Mannheim) and the in situ transcribed human U14 snoRNA (kindly provided by P. Dragon of this laboratory) were 3′-terminally labeled using [5^32P]pCP and T4 RNA ligase as described (41). Preparation of the unlabeled competitors, AAAUUAAAGG^p (referred to as RNA^p) and AAAAAAAGAG^p (referred to as DNA^p), and their 3′-OH-terminated counterparts (referred to as RNA^OH and DNA^OH, respectively) as well as preparation of CCCCCCGCCGp^3 and (dN)p^3p (requiring a mixture of 3′-phosphorylated oligodeoxyribonucleotides (n = 8–14); referred to as DNA^p (m.n.)) was described (41). TLC was in solvent A (isobutyric acid:concentrated NH_4OH:H_2O (66:33)) or solvent B (saturated NH_4SO_4:3 m sodium acetate:isopropyl alcohol (80:62:6)).

**Cyclase Assays**—Cyclase activity was assayed by the Norit method as described before (40, 41). Unless indicated otherwise, 10-μl assays contained 30 mM Hepes-KOH, pH 7.6, 180 mM NaCl, 2 mM MgCl_2, 0.15 mM EDTA, 0.1 mM spermidine, 1.25 mM dithiothreitol, 0.005% Triton X-100, 5% glycerol, 0.2 mM ATP, 30–90 fmol (10,000–25,000 cpm) of the substrate (either AAAAAAAGAP^3 or CCCCCCGCCGp^3; both substrates yielded similar results), and 20–200 pg of the recombinant E. coli cyclase. Incubations were for 20 min at 25 °C. For pH optimum determination, a three-buffer system containing 0.1 mM MES, 0.05 mM Tris, 0.05 mM ethanolamine (48) was used. The buffer was adjusted to the desired pH at 25 °C. Other details are indicated in the figure legends. K_m values for ATP and GTP were calculated from Lineweaver-Burk plots. Assays were performed under standard conditions in the presence of five different concentrations of ATP (2.5–200 μM) or GTP (7.4 μM–1 mM). Activities were calculated from the initial rates.

Labeling of the cyclase with different [α^32P]NTPs was performed under cyclase assay conditions except that the AAAAAAAGAP^3 substrate was omitted. Reactions (10 μl) contained 1.2 μM [α^32P]NTPs (specific activity 800 Ci/mmol) or 0.33 μM [α^32P]dATP (specific activity 3,000 Ci/mmol) and indicated amounts of the cyclase and were incubated at 25 °C. The reactions were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Immediately before gel electrophoresis, samples were supplemented with respective unlabeled NTPs or dATP to a final concentration of 10 μM.

For AMP release assays, 1.8 ng of the cyclase was first adenylylated under standard conditions in the presence of 1.7 μM [α^32P]ATP (specific activity 800 Ci/mmol), with no substrate added, for 2 h at 25 °C. The reactions were then diluted 7-fold with cyclase assay buffer, different amounts of competitors were added, and incubations were continued for 15 min at 25 °C. The reactions were analyzed as described above.

**RNA Isolation from E. coli Cells**—Bacterial cultures were grown in LB medium containing, when appropriate, 100 mg/liter ampicillin, 20 mg/liter kanamycin, or 10 mg/liter chloramphenicol, to an A_600 of 1.0 (49). When required, isosopt-l-thio-β-D-galactopyranoside was added to an final concentration of 0.1 mM. For RNA isolation, 30 ml of cultures were harvested by centrifugation. Total RNA was isolated with RNasey spin columns (Qiagen) according to the manufacturer’s recommendations, followed by DNase treatment (20 min at 37 °C, 0.1 unit/μl RNase free DNase (Promega) in 50 mM Tris-HCl, pH 7.5, 1 mM MgCl_2). The column fractionation and DNase treatment were repeated twice. After the second DNase treatment, the enzyme was inactivated by heating for 5
min at 72 °C, and RNA was directly used for cDNA synthesis.

RT-PCR—cDNA synthesis was performed in a 40-μl volume. Reactions contained the first-strand synthesis buffer for Superscript II reverse transcriptase (Life Technologies, Inc.), 10 mM dithiothreitol, 1 mM each of dNTP, 2.5 μM random hexamers (Promega), 50 ng/ml RNA, and 100 units of RNase H - SuperScript II reverse transcriptase (Life Technologies, Inc.). Incubations were for 12 min at 21 °C, followed by 45 min at 42 °C. Reverse transcriptase was inactivated by heating for 5 min at 95 °C.

PCR reactions were performed in a 50-μl volume. They contained the native T7 polymerase buffer (Stratagene), 0.5 μM primers, 4 μM of the cDNA synthesis reaction, and 1.5 units of T7 polymerase (Stratagene). In addition, reactions contained 2 mM (~50,000 cpm) of one of the primers that had been 5′-end labeled using [γ-32P]ATP (3,000 Ci/mmole; Amersham Pharmacia Biotech) and T4 polynucleotide kinase and purified on a 20% acrylamide, 8 M urea gel. The concentration of NTPs in B was 0.2 mM.

FIG. 1. pH optimum (A) and effect of different nucleoside triphosphates on cyclization of AAAUAAGAp* (B). Assays were performed as described under "Materials and Methods" except that the three-buffer system containing 0.1 mM MES, 0.05 mM Tris, 0.05 mM ethanola- mine was used in A. A similar pH dependence curve was observed when this buffer was replaced by 30 mM MES-NaOH (pH 5.0–6.5), MOPS-NaOH (pH 6.0–7.5), and Tris-HCl (pH 6.8–8.8). The concentration of NTPs in B was 0.2 mM.

Characterization of the Human cDNA Encoding an RtcB-like Protein—A human cDNA clone (GenBank R61436) encoding RtcB-like protein was obtained from the I.M.A.G.E. Consortium (Livermore, CA). The clone was sequenced on both strands, using appropriate oligonucleotide primers. The sequence encoding 29 N-terminal amino acids missing in this clone was obtained by RT-PCR using sequence information derived from the highly conserved mouse cDNA clone (GenBank W42119) to design the forward PCR primer. Poly(A)+ RNA from HeLa cells, kindly provided by P. Pelczar of this laboratory, was used to generate single-stranded cDNA as described above. The PCR reaction was performed on 200 ng of cDNA using as a forward primer the oligonucleotide ATGACTGTAACTACAGAGATG. This sequence encompasses the ATG initiation codon (shown in italics) and the adjacent 5′- untranslated region nucleotides of the mouse cDNA. The backward primer, ATCATTCACATAGAAAACACC, was complementary to the human cDNA sequence, 120 nucleotides downstream of the ATG codon. The PCR-amplified fragment was cloned into the EcoRI site in plasmid pBluescript II KS+, and the insert was sequenced. The sequence of the 5′-terminal part of the cDNA encoding human RtcB-like protein was further confirmed by inspection of two recent expressed sequence tag entries (GenBank AA090429 and AA 232068).

Computer Analysis—Unless indicated otherwise, sequence management and analysis were performed with the GCG Wisconsin package of programs (Genetics Computer Group, Madison, WI) on the UNIX platform. Protein sequence alignments were performed with the ClustalW 1.5 program (50), using default parameters; the alignment was improved manually. Shading of amino acids was performed with the BOXSHADE program at the BOXSHADE WWW server at the University of Lausanne (http://ulrec3.unil.ch/software/boxshade/boxshade.html). Phylogenetic analysis was performed with the PHYLIP package (51). The ClustalW 1.6 multiple-sequence alignment served as an input file for the PROTDIST program, which generated the distance matrix. The distance matrix was further analyzed by the NEIGHBOR program to obtain an evolutionary tree. The statistical significance of phylogenetic relationships was assessed by bootstrapping analysis with the BOOTSTRAP program; 500 data sets were analyzed by the PROTDIST program, generating distance matrices for all these data sets. Phylogenetic trees were generated from these matrices with the NEIGHBOR program. A consensus tree was generated with the CONSENSE program. Helix-turn-helix motifs were predicted with the helix-turn-helix motif program version 1.0.5 (52).

RESULTS

Requirements of the E. coli Cyclase—To compare the human and E. coli enzymes, we have studied requirements of the overexpressed and purified bacterial protein using oligoribonucleotides specifically labeled at the 3′-terminal phosphate, AAAAUAAGAp* or CCGCCACCCGp*, as substrates. The cyclization reaction with the E. coli enzyme showed a pH optimum of 8.0–8.5 (Fig. 1A). The reaction required the presence of divalent cations, either Mg2+ or Mn2+. In the presence of Mn2+, the enzyme activity was 50–70% higher than in the presence of Mg2+. With both cations, a broad optimum was found at 1–4 mM. No activity was seen when 2 mM Mg2+ or Mn2+ was replaced with 2 mM Ca2+, Zn2+, or Cu2+ (Table I and data not shown). The efficiency of Mn2+ as a cofactor distin-
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Guishes the E. coli and human enzymes. In the presence of Mn2+ ions, the human protein, either purified from HeLa cell extracts (39) or bacterially overexpressed (Table I), showed only 5 and 17%, respectively, of the activity seen with Mg2+. Activity of the E. coli enzyme was similar at 0 and 0.1 mM NaCl (in addition to 30 mM Hepes-KOH). Addition of NaCl to 0.2 or 0.4 mM inhibited cyclization by 30 and 70%, respectively. At 20 or 50 mM, sodium phosphate did not inhibit the reaction, but sodium pyrophosphate was strongly inhibitory (86 and 98% inhibition, respectively) (Table I and data not shown).

The activity of different nucleoside triphosphates as cofactors in the cyclization reaction was compared. ATP was found to be the most efficient cofactor, and considerable activity was also seen with GTP. UTP, CTP, and dATP were much less active (Fig. 1B). Kₐ values for ATP and GTP were 20 and 100 μM, respectively (see “Materials and Methods”). Precedently determined values for the human enzyme are 6 μM (ATP) and 200 μM (GTP) (38, 39). ADP and AMP were not active as cofactors (Table I). Likewise, ATP could not be replaced by either α,β-methylene (AMPPCP), β,γ-methylene (AMPPCP), or β,γ-imido (AMPPNP), all nonhydrolyzable analogs of ATP. ATPγS was about 20% more active than ATP (Table I). Similar observations were previously made for the human cyclase (37, 38, 40).

To test whether the cyclization reaction catalyzed by the bacterial cyclase proceeds via the formation of the covalent enzyme-NMP intermediate, the cyclase was incubated with different [α-32P]NTPs, and resulting complexes were analyzed by SDS-polyacrylamide gel electrophoresis. At a low cyclase concentration (12 ng/assay), radiolabeling of the enzyme could only be detected with [α-32P]ATP and [α-32P]CTP, but not with [α-32P]GTP, [α-32P]UTP, or [βγ-32P]DADTP (Fig. 2A and data not shown). The labeling with ATP was more efficient than with GTP (Fig. 2A, compare lanes 2 and 5). When 100-fold more protein (1.2 μg/assay) was used, covalent labeling of the cyclase was also detected with [α-32P]CTP, [α-32P]UTP, and [α-32P]DADTP (Fig. 2B).

**Substrate Specificity—**The foregoing experiments have shown that the E. coli enzyme can catalyze the cyclization of the 3′-terminal phosphate in synthetic oligoribonucleotides such as CCCCACCCCGp, AAAUUAAAGp, and AAAUAAAAAG Cp (41). We have tested the ability of two natural RNAs, the E. coli 5 S rRNA and human U14 snRNA, to act as substrates. The RNAs were modified by ligation of [5′-32P]ppGp to the 3′ terminus. Incubation with the E. coli cyclase resulted in the formation of 2′,3′-cyclic phosphate termini in both RNAs as determined by digestion with nuclease P1, followed by TLC on cellulose plates (data not shown; see “Materials and Methods”).

For the human cyclase, we have previously demonstrated that prolonged incubation of the 3′-phosphorylated oligodeoxyribonucleotides with an excess of the enzyme generates low amounts of products bearing dNp5ppp5A at the 3′ terminus. Competition experiments have shown that 3′-phosphorylated oligodeoxyribonucleotides are ~500-fold poorer substrates than oligoribonucleotides (41).

**TABLE I**

**Requirements of the E. coli cyclase**

| Experiment | Additions | Activity |
|------------|-----------|----------|
| 1          | Complete  | 100      |
| 2          | Omit MgCl₂ | 94       |
| 3          | Omit MgCl₂, add 2 mM MnCl₂ | 161      |
| 4          | Omit MgCl₂, add 2 mM CaCl₂ | 0        |
| 5          | Omit MgCl₂, add 2 mM ZnCl₂ | 0        |
| 6          | Omit MgCl₂, add 2 mM CuCl₂ | 0        |
| 7          | Complete, with human cyclase | 100      |
| 8          | Omit MgCl₂, add 2 mM MnCl₂ | 17       |
| 9          | Add 20 mM sodium phosphate | 99       |
| 10         | Add 50 mM sodium phosphate | 97       |
| 11         | Add 20 mM sodium pyrophosphate | 14       |
| 12         | Add 50 mM Na pyrophosphate | 2        |
| 13         | Omit ATP | 100      |
| 14         | Omit ATP, add ADP | 1.6      |
| 15         | Omit ATP, add AMP | 0        |
| 16         | Omit ATP, add AMPPCP | 0.7      |
| 17         | Omit ATP, add AMPPCP | 1.9      |
| 18         | Omit ATP, add AMPFNP | 5.3      |
| 19         | Omit ATP, add ATPγS | 115.2     |

**FIG. 2.** Labeling of the cyclase with α-32P-labeled ribonucleoside triphosphates and α-32PdATP. Assays contained 12 ng (A) or 1.2 μg (B) of the cyclase and were incubated in the presence of indicated labeled triphosphates for the time shown above the autoradiograms. Positions of protein size markers (in kDa) are indicated.

In the second assay, the oligoribonucleotides and oligodeoxyribonucleotides were compared for their ability to release AMP from the performed adenylated enzyme complex. The complex was formed by preincubation of the protein with [α-32P]ATP. Incubations were then continued in the presence of increasing quantities of different oligonucleotides. Addition of 33 fmol of RNAp3 decreased the amount of the complex by more than 50% (Fig. 3B, lane b), and no complex was detected when 330 or 3,300 fmol of RNAp3 was added in the second incubation (lanes c and d). In contrast, incubation in the presence of 330 or 3,300 fmol of RNAp5, DNAp5, or DNAp3OH (lanes e and f) did not result in the release of the label from the preformed complex. In the presence of even higher amounts (20 and 200 pmol), DNAp5 but not DNAp3OH resulted in AMP release from the complex (Fig. 3B, lanes e and f).

**Competition experiments** similar to those shown in Fig. 3A, were also carried out using nucleoside 3′-monophosphates (Np) and nucleoside 5′,3′-bisphosphates (pNp) or nucleoside 5′,2′-bisphosphates (pNp2) as competitors. No significant competition for the cyclization of AAAAUAAAAAGp3p was observed.
when 6,000-fold molar excess of each compound was used (Fig. 3A, inset). A small inhibitory effect of pC\(^3\)-p and pdCp was probably unspecific as incubation of radiolabeled [5\(^\prime\)-\(^32\)P]pC\(^3\)-p with an excess of the cyclase for 5 h at 25 °C did not result in detectable formation of pC\(^\text{p}\) as verified by TLC (data not shown). Identical inhibition with pC\(^3\)-p and pdCp also argues against these compounds acting as cyclase substrates because oligodeoxyribonucleotides or 2\(^\prime\)-deoxy-terminated oligoribonucleotides were found to be much less efficient substrates for the enzyme (Fig. 3 and data not shown).

Taken together, the results presented in this section indicate that E. coli cyclase can efficiently use as substrates 3\(^\prime\)-phosphate-terminated RNA molecules of different sequence and base composition and that ribonucleoside 3\(^\prime\)-phosphate-terminated DNA molecules are two to three orders of magnitude poorer substrates than RNAs.

**Structure of the Cyclase Operon—The gene encoding the cyclase, named \textit{rtcA} (for RNA terminal phosphate cyclase \textit{orfA}), is positioned at 76 min on the \textit{E. coli} K12 chromosome. \textit{rtcA} probably forms part of an uncharacterized operon, the structure of which is schematically shown in Fig. 4. Another ORF transcribed in the same direction, named \textit{rtcB}, is present upstream of \textit{rtcA}. As the termination codon of \textit{rtcB} is immediately followed by the AUG of \textit{rtcA}, it is very probable that the two genes are transcribed into a dicistronic mRNA. Inspection of the region positioned upstream of \textit{rtcB} suggested that transcription of the \textit{rtcB}/\textit{rtcA} unit may involve the alternative \(\sigma^{54}\) factor. The region likely to correspond to the \textit{rtcB}/\textit{rtcA} promoter contains TGGCA and TGGCA elements, the sequences and spacing of which are identical with the −12 and −24 elements constituting recognition signals for \(\sigma^{54}\) (53–55). Moreover, there are two putative binding sites for the integration host factor, known to be involved in transcription of many \(\sigma^{54}\)-specific promoters (56–59), further upstream. Finally, the ORF positioned upstream of \textit{rtcB}, named \textit{rtcR}, which is transcribed in the opposite direction, encodes a protein having all the features of \(\sigma^{54}\)-dependent regulators (Fig. 4; see below). Initiation of transcription by \(\sigma^{54}\)-RNA polymerase holoenzyme requires additional activator proteins that bind to enhancer-like sequences typically positioned 100–200 bp upstream from the transcription start site (reviewed in Refs. 58, 60–62).

A schematic structure of the \textit{rtcR}-encoded protein is shown in Fig. 5A. Generally, \(\sigma^{54}\)-specific regulators comprise three different domains: the N-terminal regulatory or sensory domain, the highly conserved central domain responsible for ATP hydrolysis and interaction with \(\sigma^{54}\), and the C-terminal DNA binding domain, which contains a helix-turn-helix motif (reviewed in Refs. 58, 60–62). The central domain of RtcR shows 28–39% identity and 56–61% similarity with counterparts in other regulators of this class and contains conserved regions C1–C7 found in other members of the family (61). The N-terminal domain does not have significant sequence similarity to any of the known \(\sigma^{54}\) class regulators or other proteins deposited in the data bases; it also does not contain conserved Asp residues characteristic of the members of two component systems (61, 63, 64). Alignment of C-terminal domains of RtcR and a representative selection of known \(\sigma^{54}\) regulators (Fig. 5B) suggests that RtcR has an atypical DNA binding domain with the helix-turn-helix motif containing a 20-amino acid-long turn. Helix-turn-helix motifs with turns as long as 20 amino acids have been identified in some structurally characterized DNA binding proteins (65). Phylogenetic analyses, performed with the PHYLIP program, indicated that within a family of \(\sigma^{54}\) class activators, RtcR does not resemble any particular known activator more than others (Fig. 5C). A similar conclu-
sion was reached when phylogenetic analyses were carried out separately for each of the three domains of the regulator (data not shown).

Involvement of RtcR and σ^{54} in Transcription of the Cyclase Operon—σ^{54}-specific regulators are usually constitutively expressed but not constitutively active (reviewed in Refs. 58, 61, 62, 64). For some regulators, it has been shown that deletion of the sensory domain derepresses ATPase activity of the regulator and makes it constitutively active (64). To investigate whether the RtcR regulator plays a role in expression of the cyclase operon, we have tested the effect of overexpression of wild-type and N-terminally truncated forms of RtcR on transcription of rtcA and rtcB in the strain YMC10 and its derivative YMC22, containing the inactivated σ^{54} gene. RNA isolated from either control bacteria or from bacteria transformed with plasmids expressing the full-length RtcR or its N-terminally truncated form, RtcR_{N}, was reverse transcribed using random hexamers as primers. Resulting cDNAs were utilized as templates for PCR, using pairs of oligodeoxynucleotide primers specific for the rtcA and rtcB genes. The primers encoding σ^{54} (rpsN) and a ribosomal protein S5 (rpsA). Expression of the rtcR gene was monitored with two pairs of primers, one specific for the region encoding the N-terminal part of RtcR and another specific for the C-terminal part.

Results of RT-PCR analysis are shown in Fig. 6. As expected, overexpression of the mRNA encoding the wild-type RtcR could be visualized with both the rtcA and rtcB transcription start sites, as established by primer extension analysis (Fig. 7), and is indicated by a bent arrow. Translation start sites for rtcB and rtcR are marked with arrows, and putative Shine-Dalgarno sequences are underlined. The −12 and −24 consensus sequences characteristic of σ^{54} promoters are boxed. Sequences resembling the most highly conserved regions of the consensus binding site, AATCAAN_4TTA, for the integration host factor (IHF) are indicated by boxes connected by the dotted lines. The boxed sequences are flanked by AT-rich regions, which contain at many positions nucleotides conforming with the extended integration host factor consensus (80, 81). Inspection of the upstream region of the rtcA/rtcB promoter did not identify obvious sequence repeats that could act as likely targets for RtcR.

Expression of rtcA and rtcB was observed in YMC10 cells expressing the N-terminally truncated (lanes 5) but not wild-type (lanes 4) RtcR. Neither rtcA nor rtcB was expressed in the YMC22 σ^{54} knockout strain transformed with pRtcR3N alone (lanes 6). However, transcription of both genes took place when YMC22 was additionally cotransformed with pTH7, which encodes σ^{54} (lanes 7). Activation of rtcA and rtcB transcription by the N-terminally truncated but not wild-type RtcR was also observed in E. coli BL21(DE)pLysS cells transformed with plasmids in which expression of the regulators is driven by the T7 promoter (data not shown).

To obtain additional evidence that expression of the cyclase operon involves σ^{54}, the transcription start site of the rtcB/rtcA mRNA was determined by primer extension. The analysis was performed with RNA isolated from both YMC10 and the YMC22 σ^{54} knockout strain, each overexpressing the N-terminally truncated form of RtcR. Consistent with the experiments presented in Fig. 6, the primer extension product was only identified when RNA from YMC10 was used as a template (Fig. 7). The 5'-end of the RNA mapped to the C residue positioned 11 nucleotides downstream of the putative −12 TT-GCA box present in the rtcB upstream region.

Taken together, the results presented above indicate that both σ^{54} and the σ^{54}-specific regulator RtcR are involved in expression of the cyclase operon.

rtcA Is a Nonessential Gene—To investigate whether the rtcA gene product is essential for E. coli growth, the central 90% of the rtcA coding region was replaced by homologous recombination with a gene coding for kanamycin resistance in the strain MC1061 (46). The replacement was confirmed by Southern and PCR analyses (data not shown; see “Materials and Methods”). No differences in growth of the parent and the rtcA null strain
FIG. 5. Structural properties and phylogenetic analysis of RtcR. A, schematic structure of RtcR. Positions of the N-terminal regulatory domain, central domain, and a putative DNA binding domain are indicated. B, alignment of the C-terminal portion of the putative DNA binding domain of RtcR with a representative selection of other known α58-specific regulators. Names of the regulators, corresponding to SwissProt annotations, and the bacteria from which they originate are indicated. Positions corresponding to the first amino acid shown are also specified. Identical amino acids and amino acids conserved in at least 50% of sequences are indicated by black and gray boxes, respectively. Regions corresponding to the helix-turn-helix motif and to the additional N-proximal helix (65) are marked with vertical lines. The LevR regulator, which most probably contains its DNA binding domain at the N terminus (67), is not included in the comparison. C, phylogenetic analysis of α58-specific transcriptional regulators, performed with the PHYLIP package of programs. Numbers at branch points indicate the percentage of trees in the data set matching the consensus tree. Branch lengths are proportional to phylogenetic distances. For additional information, see "Materials and Methods."
on either LB or a minimal M9 medium were observed (data not shown).

*Genes Similar to rtcB Are Present in Archaea and Eucarya—*
Genes encoding cyclase-like proteins are conserved among Eucarya, Bacteria, and Archaea (41). By searching sequence data bases, we have found that also genes similar to rtcB, the gene likely to be cotranscribed with the cyclase gene in *E. coli*, are present in other organisms such as *Methanococcus jannaschii* and *Caenorhabditis elegans*, representing two other kingdoms. Expressed sequence tags encoding RtcB-like proteins in humans have also been identified. The human cDNA was fully sequenced, and the protein encoded by it is included in the alignment shown in Fig. 8 (for sequences of RtcB-like proteins in other species, see legend). The *E. coli* RtcB and other related proteins listed in Fig. 8 show no significant sequence similarity or motifs in common with proteins of known function deposited in public data bases. The noteworthy feature of RtcB proteins is the presence of six conserved histidine residues, suggesting possible involvement of a metal ion in RtcB function.

The RtcB protein was overexpressed in *E. coli* as a C-terminal His<sub>6</sub>-tag fusion and purified on the Ni-nitrilotriacetic acid column. The recombinant protein contained no detectable RNase or RNA ligase activities (the latter assayed with 5'-hydroxyl and cyclic-phosphate-terminated RNA substrates). Likewise, it had no phosphodiesterase activity opening the cyclic phosphate in RNA or pG<sub>3</sub>-p to either a 3'- or 2'-monoester or a phosphatase activity removing the 3'-phosphate from RNA. Addition of RtcB had no effect on the cyclization reaction catalyzed by the *E. coli* RNA 3'-phosphate cyclase (data not shown).

**DISCUSSION**

Recent cloning of a cDNA encoding the RNA 3'-terminal phosphate cyclase from humans led to the identification of cDNAs and/or genes encoding cyclase-like proteins in diverse eukaryotes and also the bacterium *E. coli* and the archean *M. jannaschii*. The protein encoded by the *E. coli* gene was overexpressed and shown to have RNA 3'-phosphate cyclase activity (41). In this work, we have studied the requirements and substrate specificity of the overexpressed *E. coli* cyclase. We have also established that the cyclase gene forms part of a so far uncharacterized operon, expression of which is controlled by σ<sup>54</sup> and the transcriptional regulator RtcR.

The requirements and other enzymatic properties of the overexpressed *E. coli* cyclase are generally similar to the properties of the human protein, although some important differences are apparent. One of them is the ability of Mn<sup>2+</sup> ions to replace Mg<sup>2+</sup> ions in reactions catalyzed by the *E. coli* but not by the human cyclase. Both enzymes preferentially use ATP as a cofactor but are also able to utilize GTP and, much less efficiently, other ribonucleoside triphosphates. GTP is a relatively more efficient cofactor with the *E. coli* than with the human enzyme. Apparent *K<sub>m</sub>* values for ATP and GTP are, respectively, 20 and 100 μM for the *E. coli* protein and 6 and 200 μM for the human counterpart. Like the human protein (37–41), the *E. coli* enzyme undergoes adenylylation, and the adenylyl group can be released from the preformed cyclase-AMP complex protein upon incubation with the 3'-phosphorylated RNA but not the 3'-OH-terminated RNA. Covalent labeling of the bacterial cyclase with [α-<sup>32</sup>P]GTP and, much less efficiently, with [α-<sup>32</sup>P]CTP, [α-<sup>32</sup>P]UTP, and [α-<sup>32</sup>P]dATP was also observed. Although we have not directly validated the identity of nucleotidyl groups attached to the *E. coli* enzyme, previous demonstration that the human cyclase can undergo guanylylation, cytidylylation, and uridylylation (39) argues that the same is also true for the *E. coli* enzyme. Altogether, the results discussed above strongly suggest that the mechanism of 3'-phosphate cyclization by the *E. coli* enzyme is similar to that
established for the human protein (Refs. 37–39; reviewed in Ref. 40). This conclusion is further supported by the observation that E. coli cyclase, like its human counterpart (37, 41), can inefficiently convert the 3'-terminal phosphate in the oligodeoxyribonucleotide AAAATAAAAGp into the product terminated with dN3pppA.2

Substrate specificities of the bacterial and human enzymes seem to be similar. Both enzymes can use a variety of 3'-phosphorylated RNAs and oligoribonucleotides bearing either purine or pyrimidine 3'-terminal nucleotides (Refs. 11, 38, 40, and 41) and this work). It was demonstrated previously that the oligodeoxyribonucleotides were found to be at least 300-fold poorer substrates than the oligoribonucleotides.

The observation that overexpression of the N-terminally truncated form of RtcR induces transcription of the rtcA/rtcB unit and is transcribed in the opposite direction. It encodes a protein having features of α4-dependent regulators (Fig. 5; reviewed in Refs. 58, 61, 64). By overexpressing the N-terminally truncated form of RtcR, we have demonstrated that this regulator controls expression of rtcA and rtcB in a α4-dependent manner. Further evidence that α4 is involved in expression of rtcA and rtcB is provided by the presence of the TTGCA and TGGCA elements centered 13 and 24 bp upstream of the rtcA/rtcB transcription start site established by primer extension (Figs. 4 and 7). Both the sequence and position relative to the transcription start site of these two elements conform with the consensus –12 and –24 boxes characteristic of all studied α4-dependent promoters (53–55).

The observation that overexpression of the N-terminally truncated but not full-length RtcR induces transcription of rtcA and rtcB suggests that, as in the case of several previously characterized α4-specific regulators (e.g., XylIR, DmpR, DctD, and LevR (66–69)), the N-terminal domain represses activity of RtcR, and its deletion makes the regulator constitutively active. Physiologically, activation of most α4-specific regulators is brought about either by phosphorylation or by binding of specific effector molecules to the sensory domain that, in the induced state, represses the ATPase activity of the protein required for isomerization of the promoter complex (reviewed in Refs. 55, 58–61, 64). RtcR is not a member of the two-component system family of regulators (63, 64), so its activation is unlikely to involve phosphorylation. A potential effector interacting with RtcR remains to be identified. With the exception of Myxococcus xanthus in which rpoN null mutants are

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2 P. Genschik and W. Filipowicz, unpublished results.
be essential for growth. Products of genes controlled by
sequently also genes or operons dependent on it were found not to
results.
(72–75). Identification of physiological conditions leading
tors (72–75). Identification of physiological conditions leading
tus such as flagellum and pilus, or in the synthesis of viru-
nonviable (70), in all bacteria studied to date, Escherichia coli and Salmonella typhimurium. In certain
factors have very diverse physiological functions. They are
involved in specialized metabolic processes such as utilization of alternative carbon and energy sources or assimilation and
fixation of nitrogen, in the production of extracellular struc-
tures such as flagellum and pili, or in the synthesis of viru-
findings that two different eukaryotic RNA ligases (18–
and also the RNA ligase identified in Escherichia coli and Salmonella
to activation of the rtcA operon in Escherichia coli would greatly help in
establishing a biological function of the RNA 3′-phosphate
cy clase in bacteria.

The findings that two different eukaryotic RNA ligases (18–
also the RNA ligase identified in Escherichia coli and Salmonella
gene encoding the RNA ligase in Escherichia coli is not essential (33). In the archeon Desulfurococcus mobilis, the 23 S pre-rRNA contains an intron, and its cleavage by the endonuclease seems to generate splicing intermediates containing 5′-phosphomononucleotides (76). It is not known whether the terminal phosphate has to undergo cyclization prior to the ligation step. In eukaryotes, the spliceosomal U6 snRNA and other organisms.

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