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Cyanobacterial RNA Polymerase Genes *rpoC1* and *rpoC2* Correspond to *rpoC* of *Escherichia coli*

WEN-QIN XIE, KARIN JÄGER,† AND MALCOLM POTTS*

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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The DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) of cyanobacteria contains a unique core component, γ, which is absent from the RNA polymerases of other eubacteria (G. J. Schneider, N. E. Tumer, C. Richaud, G. Borbely, and R. Haselkorn, J. Biol. Chem. 262:14633–14639, 1987). We present the complete nucleotide sequence of *rpoC1*, the gene encoding the γ subunit, from the heterocystous cyanobacterium *Nostoc commune* UTEX 584. The derived amino acid sequence of γ (621 residues) corresponds with the amino-terminal portion of the β′ polypeptide of *Escherichia coli* RNA polymerase. A second gene in *N. commune* UTEX 584, *rpoC2*, encodes a protein which shows correspondence with the carboxy-terminal portion of the *E. coli* β′ subunit. The *rpoBC1C2* genes of *N. commune* UTEX 584 are present in single copies and are arranged in the order *rpoBC1C2*, and the coding regions are separated by short AT-rich spacer regions which have the potential to form very stable secondary structures. Our data indicate the occurrence of divergent evolution of structure in the eubacterial DNA-dependent RNA polymerase.

The transcription of genes is directed through the activity of DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). In eubacteria, a single form of the core RNA polymerase, together with ancilliary sigma factors, is responsible for the synthesis of virtually all cellular RNAs (5). The RNA polymerase of *Escherichia coli* consists of at least four different subunits, β, β′, α, and σ, and is present in two main enzyme forms, core ($\beta\beta'\alpha_2$) and holoenzyme (core plus σ; 4). The two genes encoding the β (*rpoB*) and β′ (*rpoC*) subunits of this RNA polymerase are adjacent to one another and are cotranscribed from the major promoter PL10 (5). The basic ($\beta\beta'\alpha_2$) design has been found in the RNA polymerases purified from representatives of gram-positive and gram-negative eubacteria (17, 38). Recently, however, an additional core component, γ, has been described for the RNA polymerase ($\beta\gamma\beta'\alpha_2\sigma$) of the cyanobacterium *Anabaena* sp. strain PCC 7120 (32). The γ subunit is serologically unrelated to the other subunits of the cyanobacterial RNA polymerase, but anti-γ serum cross-reacts with both *E. coli* β′ subunit protein and subunit A of the RNA polymerase from *Sulfolobus acidocaldarius*, an archaebacterium (31). The γ subunit has since been detected in the RNA polymerases of 15 out of 15 taxonomically diverse cyanobacteria, including two *Nostoc* species (31).

Three different nuclear RNA polymerases are found in eucaryotes, each one responsible for the transcription of a different class of genes (17). Comparison of the amino acid sequences of the largest subunit, A, of RNA polymerases II and III from *Saccharomyces cerevisiae* and the β′ subunit of the *E. coli* RNA polymerase revealed six regions (I to VI) of marked conservation (1).

The RNA polymerases of archaebacteria appear to be more closely related to those of eucaryotes (6, 38). The subunit compositions of the enzymes from representatives of the halophilic or methanogenic and sulfur-dependent thermophilic branches of the archaebacteria differ. It is unclear whether archaebacteria, like eucaryotes, possess multiple species of RNA polymerase (6, 38).

The chloroplast DNA of higher and lower plants contains regions with sequence similarity to *rpoA*, *rpoB*, and *rpoC* of *E. coli* (11, 23, 24). In the present study, we show that in the cyanobacterium *Nostoc commune* UTEX 584 two separate and linked genes correspond to different portions of the single *rpoC* gene of *E. coli*. One of these genes, for which the complete sequence is presented, encodes the γ subunit.

MATERIALS AND METHODS

Microorganisms and growth conditions. *N. commune* UTEX 584 and *Anabaena variabilis* PCC 7118 were grown as described previously (25) in liquid BG-11_0 medium (28) and BG-11 medium, respectively.

Recombinant DNA analyses. Routine methods were used for the manipulation of DNA (8, 18). Restriction endonucleases were obtained from BRL/Life Technologies Inc. and were used according to the specifications of the manufacturer. Plasmid and bacteriophage DNAs were purified as described previously (18, 33).

Synthesis of biotinylated *rpo* DNA probes. The plasmids pPD489 and pPD490 were generous gifts of P. Dennis, University of British Columbia, Vancouver, British Columbia, Canada. These two plasmids are derivatives of pBR322. pPD489 carries an *Eco*RI fragment (2.6 kilobase pairs [kb]) from within *E. coli rpoC*, and pPD490 carries an *Eco*RI fragment (2.8 kb) from within *E. coli rpoB* (5). The *Eco*RI fragments were purified, and nick translation (27) was used to label the DNA fragments with biotin-11-dUTP. The biotin-11-dUTP, DNA polymerase I, and DNase I were obtained from BRL/Life Technologies Inc. Southern blotting (34) was used both to determine the sizes of those fragments of *N. commune* UTEX 584 genomic DNA which hybridized to these probes and to optimize the conditions for filter hybridization. Procedures for Southern transfer and hybridization were as described by Mason and Williams (19).

Screening of the λgt10 library. A recombinant library of genomic DNA from *N. commune* UTEX 584 was constructed with the phage insertion vector λgt10 (imm^{434} *b527*) and propagated in *E. coli* C600 (*hfl*) by standard methods (8).

* Corresponding author.
† Present address: Institute of Physiological Botany, University of Uppsala, S-751 21 Uppsala, Sweden.

The library was constructed with *Eco*RI restriction fragments of *N. commune* UTEX 584 genomic DNA in the size range of 3 to 7 kb. Fragments of DNA of the appropriate size were isolated by sucrose density gradient centrifugation (15). Plaques of recombinant phages were transferred from agar plates (with soft overlays) to nitrocellulose filters (diameter, 82 mm; 19), and the library was screened (13) with the biotinylated *rpoC* DNA probe. Biotinylated DNA-DNA hybrids (positive plaques) were visualized through the use of a colorimetric assay which used streptavidin-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, and Nitro Blue Tetrazolium (BRL/Life Technologies Inc.). Positive plaques were isolated and subjected to two rounds of plaque purification. The cloned *Eco*RI fragments of *N. commune* UTEX 584 DNA were isolated from the purified phage DNA and subcloned, in both orientations, in pGEM-4 (Promega Biotec).

Deletion cloning and DNA sequence analysis. Processive digestion of the cloned DNA with exonuclease III and exonuclease VII was used to generate overlapping deletion fragments (35, 36). The linear fragments were then treated with the Klenow fragment of DNA polymerase I and blunt end ligated (with T4 ligase) for 5 h at room temperature, and the deletion plasmids were used to transform (9) *E. coli* DH5-α (r_K^- m_K^+; BRL/Life Technologies Inc). Plasmid preparations from the different transformants were used in DNA sequencing reactions with [α-^{35}S-thio]dATP (>400 Ci mmol^{-1}; Dupont, NEN Research Products) and the Klenow fragment of DNA polymerase I, following the dideoxy-chain termination method of Sanger et al. (29).

DNA sequence analysis. DNA sequence data were manipulated with the IBI-Pustell software of International Biotechnologies and PCGENE (IntelliGenetics).

In vitro transcription-translation of *N. commune* UTEX 584 *rpo* genes. The expression of *N. commune* UTEX 584 *rpo* genes was studied in a procaryotic cell-free coupled transcription-translation system obtained from Dupont, NEN. DNA templates for use in the in vitro system were purified after two rounds of cesium chloride ultracentrifugation and dialysis (18). Proteins were synthesized in the presence of carrier-free L-[^{35}S]methionine (1,115 Ci mmol^{-1}). Conditions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the determination of incorporation of [^{35}S]methionine in protein were as described previously (14), with the exception that gels were prepared for autoradiography without the use of fluorographic enhancing agents.

In vivo transcription-translation of *rpo* genes. The 6.5-kb *Eco*RI DNA fragment of *N. commune* UTEX 584 DNA was subcloned in the pUC18 expression vector (BRL/Life Technologies Inc.) and a recombinant plasmid (pX1) was used to transform *E. coli* JM109. Expression from the *lac* promoter was induced with IPTG (isopropyl-β-D-thiogalactopyranoside). Gene products were analyzed through Western blotting (immunoblotting) and immunolabeling as described previously (26), with the exception that the buffer used in electroblotting was supplemented with 0.01% (wt/vol) sodium dodecyl sulfate, and the transfer time was 2 h (24 V/A).

Antibodies. Antiserum raised against the core ($\beta\gamma\beta'\alpha_2$) of the RNA polymerase of *Anabaena* strain PCC 7120 was a gift from G. J. Schneider and R. Haselkorn.

RESULTS

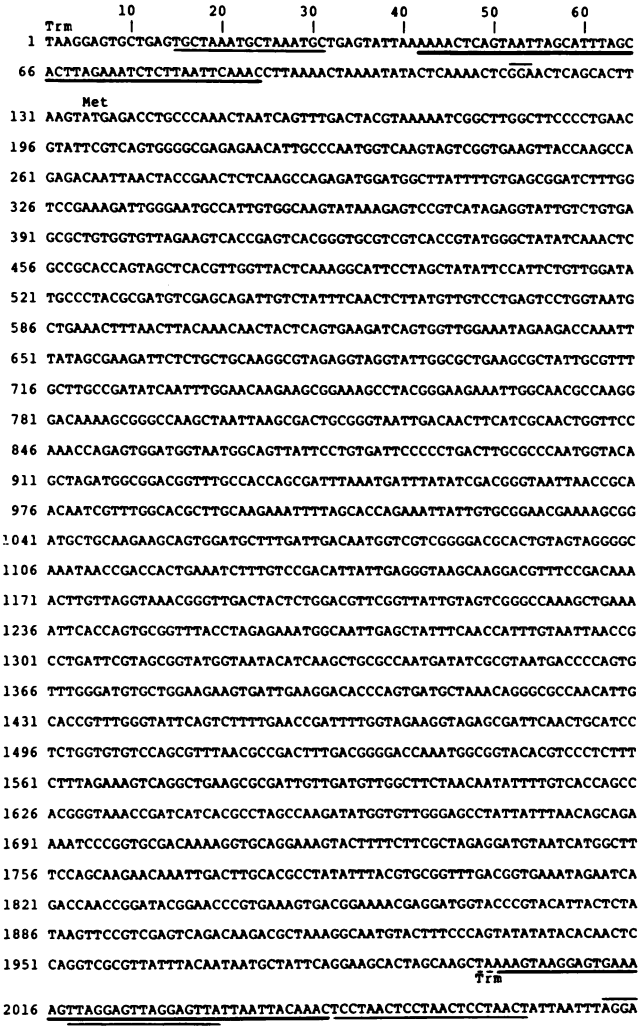
Isolation of *N. commune* UTEX 584 *rpo* genes. From Southern analyses, it was determined that the *E. coli rpo* probes hybridized with a single 6.5-kb *Eco*RI fragment of *N. commune* UTEX 584 genomic DNA. Subsequently, a recombinant phage, isolated from one positive-hybridizing plaque after screening of the gene library, was found to contain a DNA insert of 6.5 kb which gave strong hybridization signals under stringent conditions with both of the *E. coli rpo* probes used. Through the use of a set of deletion clones generated from one strand of the insert, it was determined that the *E. coli rpoB* probe hybridized only with those deletion fragments larger than 4.5 kb. The *rpoC* probe hybridized with all deletion fragments larger than 1.5 kb. It was assumed, therefore, that the 6.5-kb fragment contained sequences corresponding to both *E. coli rpo* genes. A nick-translated biotinylated probe generated from the purified 6.5-kb *N. commune* UTEX 584 DNA fragment hybridized, under stringent conditions, with a single 6.5-kb *Eco*RI fragment of *A. variabilis* PCC 7118 DNA (data not presented).

Organization of *N. commune* UTEX 584 *rpo* sequences. Sequence analysis was completed for both strands of the 6.5-kb DNA fragment by using 52 overlapping deletion clones. Three potential coding regions of 2,217, 1,866, and 2,151 base pairs (bp), separated by relatively short AT-rich sequences, were detected. The first, and incomplete, coding region showed extensive sequence similarity (data not presented) with portions of both the *rpoB* gene of *E. coli* and open reading frame 1070 isolated from tobacco chloroplast DNA (22). The second, and complete, region, of 1,866 bp (designated *rpoC1*; Fig. 1), represented an open reading frame of 622 codons which showed sequence similarity with the first 1,800 bp of *E. coli rpoC* (Fig. 2). The third region, of 2,151 bp (a portion of *rpoC2*; Fig. 1), showed sequence similarity with the remainder of *E. coli rpoC* (Fig. 2). For further discussion purposes, the fragment of DNA carrying the incomplete portions of *rpoB* and *rpoC2* and the complete sequence of *rpoC1* is referred to as *rpoBC1C2*. The amino acid sequences derived from *rpoC1* and *rpoC2* indicated that regions homologous to conserved domains within the β′ subunit of *E. coli* RNA polymerase were distributed between the two *N. commune* UTEX 584 gene products (Fig. 2, 3, and 4).

Intergenic sequences of the three *rpo* coding regions. The two nucleotide sequences separating the three *rpo* coding regions in *N. commune* UTEX 584 were AT rich and contained regions of extensive sequence similarity (Fig. 1). Several distinct components were recognized in the intergenic sequences of the *N. commune* UTEX 584 *rpo* genes through visual alignment. These components (TTAG repeat, TTAATT, and CAAAC sequences) were spaced at equivalent distances upstream from the presumed translational initiation codons of *rpoC1* and *rpoC2*. Potential ribosome-binding sequences (GGA and AGGA) were located 15 and 10 bases upstream from the translational initiation codons of *rpoC1* and *rpoC2*, respectively. The sequences from both intergenic regions have the potential to form very stable secondary structures (Fig. 5A and 5B).

Subunit composition of *N. commune* UTEX 584 core RNA polymerase. The β′, β, γ, and α subunits of *N. commune* UTEX 584 RNA polymerase, with M_rs of >180,000, approximately 159,000, 72,000, and 43,000, respectively, were identified through cross-reaction with *Anabaena* strain PCC 7120 core antiserum (data not presented).

Expression of *N. commune* UTEX 584 *rpoBC1C2* sequences in cell-free system and in *E. coli*. All of the ^{35}S-labeled products from the expression of *rpoBC1C2* in the cell-free system were precipitated with the *Anabaena* strain PCC 7120 core-specific antiserum, and immunoanalysis detected



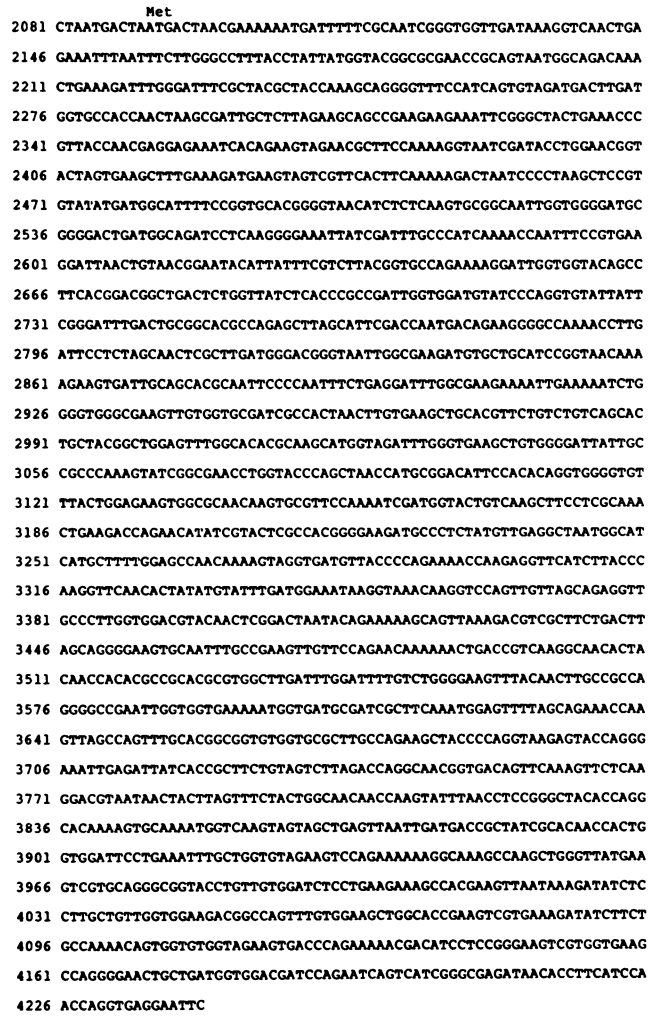


FIG. 1. Nucleic acid sequence of *rpoC1* and a portion of *rpoC2*. The sequence presented begins with the translational termination codon (Trm) of *rpoB*. Translational initiation codons for *rpoC1* and *rpoC2* are indicated (Met). In the sequences between the coding regions of *rpoB*, *rpoC1*, and *rpoC2*, light underlining indicates direct repeats and bold underlining indicates two regions of sequence similarity (see Fig. 5A and B). Putative Shine-Dalgarno sequences are indicated with overlining.

two strong bands, corresponding to polypeptides with M_rs of 71,000 and 94,000, when *rpoC1* and *rpoC2* were expressed from a *lac* promoter in *E. coli* JM109(pX1) (data not presented).

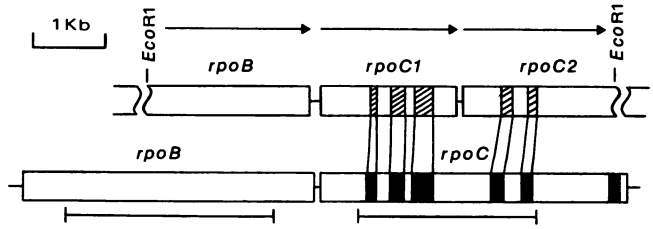


FIG. 2. Structural organization of *rpo* genes in *N. commune* UTEX 584 (upper) in comparison with *E. coli* (lower). The direction of transcription is indicated with arrows. The portions of the *E. coli* *rpo* genes used as probes are underlined. Regions of extensive sequence similarity (corresponding to segments I to VI [1]) are indicated by shading (*E. coli*) and hatching (*N. commune* UTEX 584).

DISCUSSION

Immunoanalysis has shown the cyanobacterial RNA polymerase to have a core structure of $\beta\gamma\beta'\alpha_2$ with what appears to be a single species of σ factor (31, 32). In the cyanobacterium *N. commune* UTEX 584, two genes, *rpoC1* and *rpoC2*, correspond to the single gene (*rpoC*) in *E. coli* which encodes the β' subunit of RNA polymerase. The amino acid sequences derived from *rpoC1* and the region of *rpoC2* which was sequenced in this study correspond, respectively, with the amino-terminal and carboxy-terminal portions of the *E. coli* β' subunit polypeptide. The γ subunit polypeptide contains 3 of the 6 highly conserved domains shared between the *E. coli* β' subunit and subunit A of eucaryotic RNA polymerases (1). Our data provide evidence of divergent evolution of structure in the genes encoding the large subunits of eubacterial RNA polymerase.

Expression of *N. commune* UTEX 584 *rpoC1* and *rpoC2* in a cell-free system and in *E. coli* generated translation products which cross-reacted with *Anabaena* strain PCC 7120 core antiserum, and the cross-reactive polypeptide with an M_r of 71,000 comigrated with the γ protein of *N.*

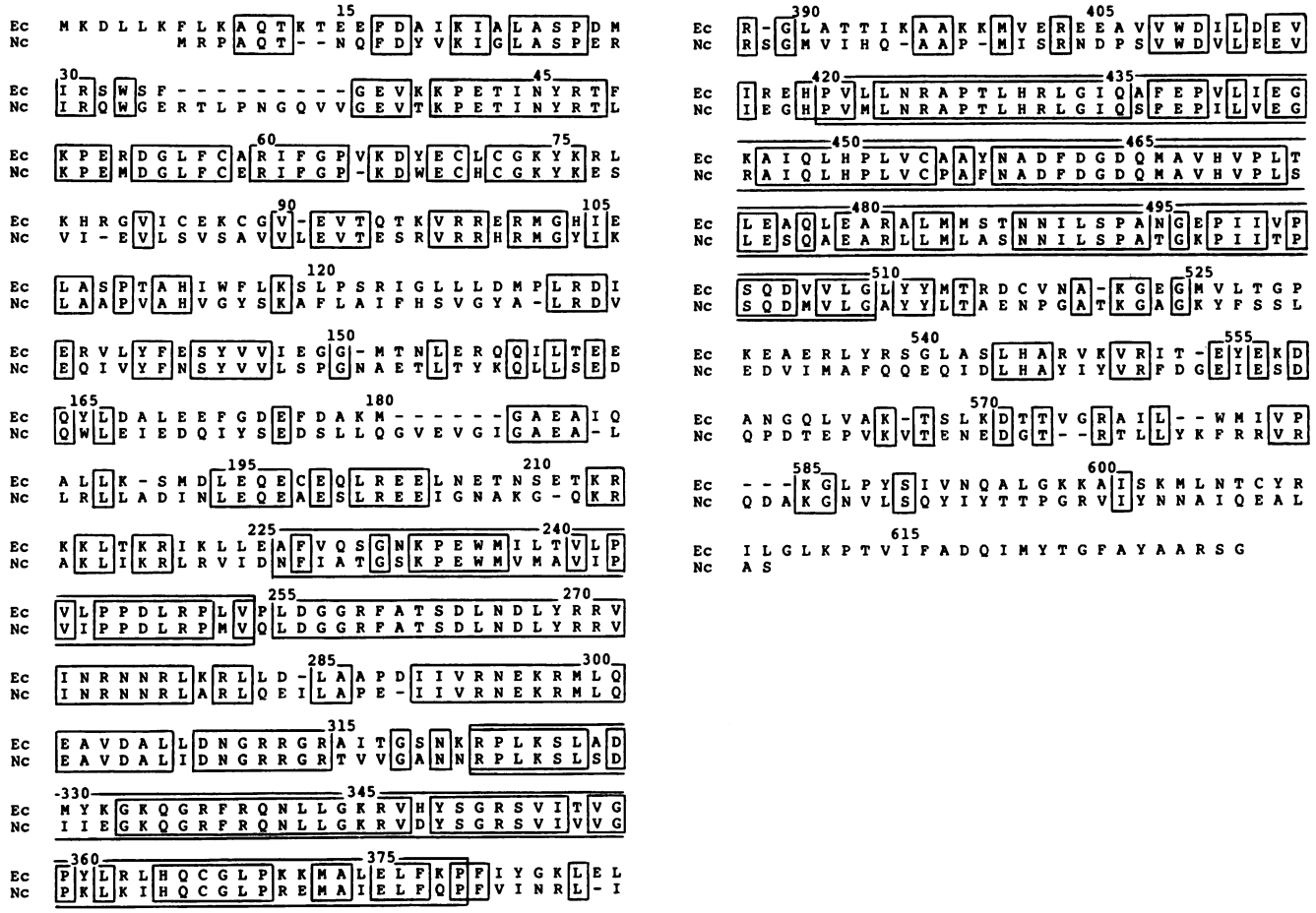


FIG. 3. Alignment of the derived amino acid sequence of γ from *N. commune* UTEX 584 (Nc) with the N-terminal portion of *E. coli* β′ (Ec). Dashed lines indicate a shifting of the sequences to permit the best fit. Numbering refers to the amino acid residues in the *E. coli* β′ subunit. Regions of sequence correspondence are enclosed in a single box. A double box around a region of sequence indicates the segments in the *E. coli* β′ subunit with extensive sequence similarity to component A of eucaryotes (1).

commune UTEX 584. In addition, the *rpoC1* gene from *Anabaena* strain PCC 7120 has been expressed from a T7 promoter of *E. coli*. It yields a protein with an M_r of 66,000 identified by γ-specific antibody in Western blot analysis; thus, it is clear that γ is the primary product of the *rpoC1* gene (K. Bergsland and R. Haselkorn, unpublished data). The *rpoC1* gene product of *N. commune* UTEX 584 has an M_r of 71,000, a value which agrees closely with the predicted M_r obtained from sequence analysis (M_r = 70,200). Both of these values are somewhat larger than the values for γ reported in a recent study of 15 taxonomically diverse strains of cyanobacteria (range in M_r, 65,000 to 68,500; 31) and are equivalent to the values reported (M_r = 72,000) for the γ subunits of two other cyanobacteria (10, 20).

The genes encoding the β, γ, and β′ subunits of *N. commune* UTEX 584 RNA polymerase are present in single copies, are transcribed in the same direction, and are arranged in the order *rpoB*, *rpoC1*, and *rpoC2*, respectively. As the γ subunit has been found in all those cyanobacteria tested to date (31), and as the 6.5-kb *Eco*RI DNA fragment harboring the *N. commune* UTEX 584 *rpoBC1C2* genes hybridized with a single 6.5-kb *Eco*RI fragment of *A. variabilis* PCC 7118 DNA, it seems likely that the arrangement of *N. commune* UTEX 584 *rpoBC1C2* genes described here will be found in other cyanobacteria. While the degree of sequence similarity between the intergenic regions of the *N. commune* UTEX 584 *rpo* genes is striking, the functional significance of these AT-rich sequences (with respect to transcription or translation), other than having the potential to encode very stable secondary structures, remains unclear. Short AT-rich spacer regions also separate *rpoC1* and *rpoC2* in the chloroplast genomes of *Marchantia polymorpha*, spinach, and tobacco (11, 23, 24).

Evidence for differences in the subunit compositions of archaebacterial RNA polymerases has been provided by immunochemical analyses (37–39). In halobacteria and the methanogenic bacteria, two subunits, B′ and B″, cross-react with a single subunit, B, present in the thermophile *Sulfolobus acidocaldarius* (37, 38). In addition, two subunits in archaebacterial RNA polymerases, A and C, are homologous with N-terminal and C-terminal portions of the β′ subunit of *E. coli*, respectively (37). Data from hybridization studies and DNA sequencing have shown that the order of the structural genes encoding A, B′, B″, and C in the methanogenic bacterium *Methanobacterium thermoautotrophicum* is B″, B′, A, and C (2, 30, 37). The division of β′ determinants between A and C in the archaebacterial enzymes is similar to the division of antigenic determinants between γ and β′ in *Anabaena* strain PCC 7120 (32), and the arrangement of the genes encoding archaebacterial A and C,

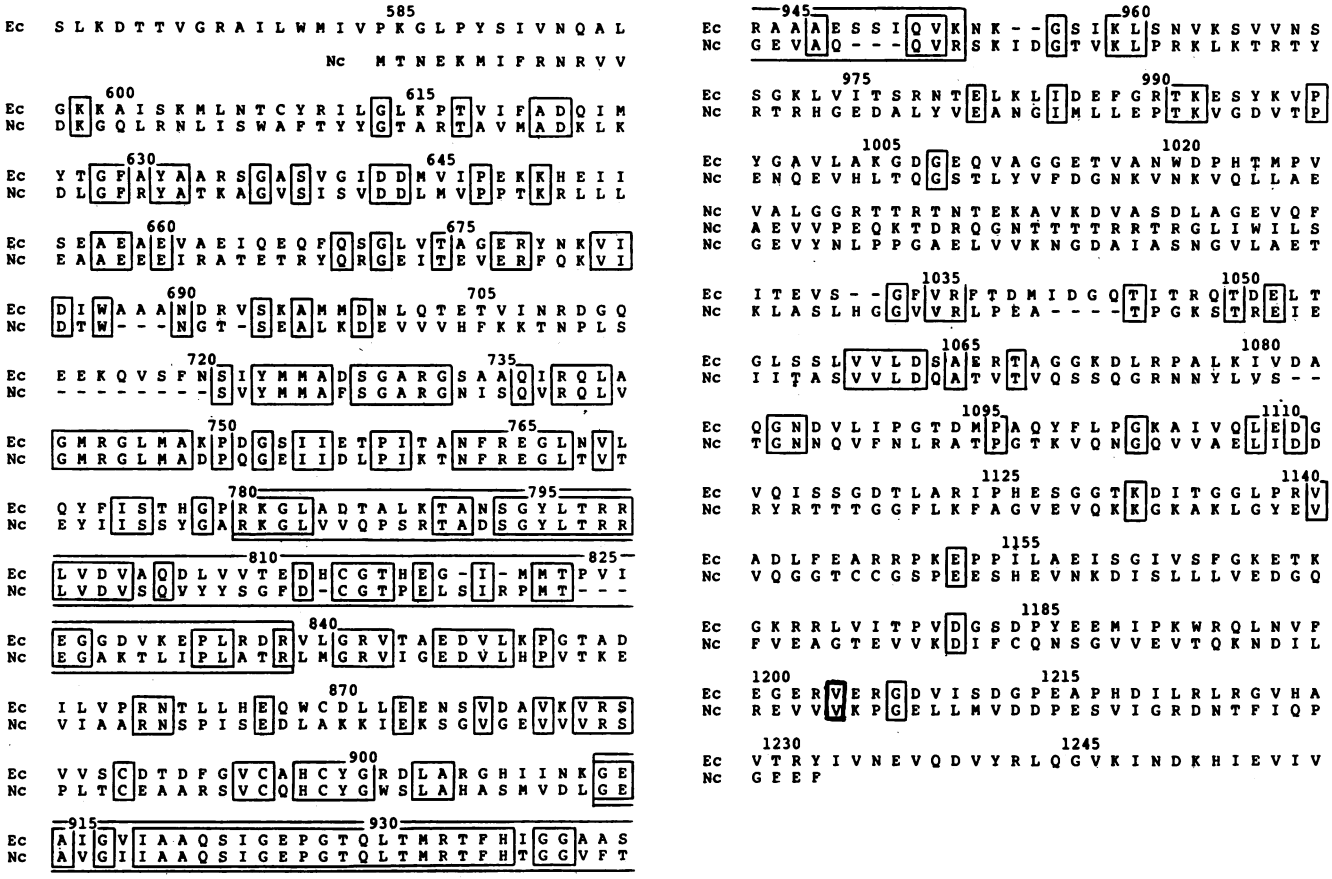


FIG. 4. Alignment of the derived amino acid sequence of RpoC2 (β′) with the carboxy-terminal portion of the *E. coli* β′ subunit. The conventions for numbering and labeling are as described in the legend to Fig. 3.

downstream of B (B″ + B′; 30), corresponds to the arrangement described here for *N. commune* UTEX 584. However, *Anabaena* strain PCC 7120 anti-γ subunit reacts with subunit A from *S. acidocaldarius* and *Anabaena* strain PCC 7120 anti-β + β′ reacts with both A and B of *S. acidocaldarius*, but neither of the *Anabaena* strain PCC 7120 sera react with the C subunit of *S. acidocaldarius* (31).

The form of structural organization of *rpo* genes described in this study has been conserved, with modifications, in the chloroplast genome. Sequences which correspond to *E. coli rpo* genes occur in the chloroplast genomes of higher and lower plants (11, 23, 24), and recent evidence suggests that these chloroplast *rpo* genes are expressed in vivo (16). Two separate chloroplast DNA sequences, *rpoC1* and *rpoC2*, correspond to *E. coli rpoC* but differ from it by the apparent insertion of an intron, an intergenic region (to create two genes), and a large section of nonhomologous coding sequence in *rpoC2* (11). RNA mapping data for spinach *rpo*

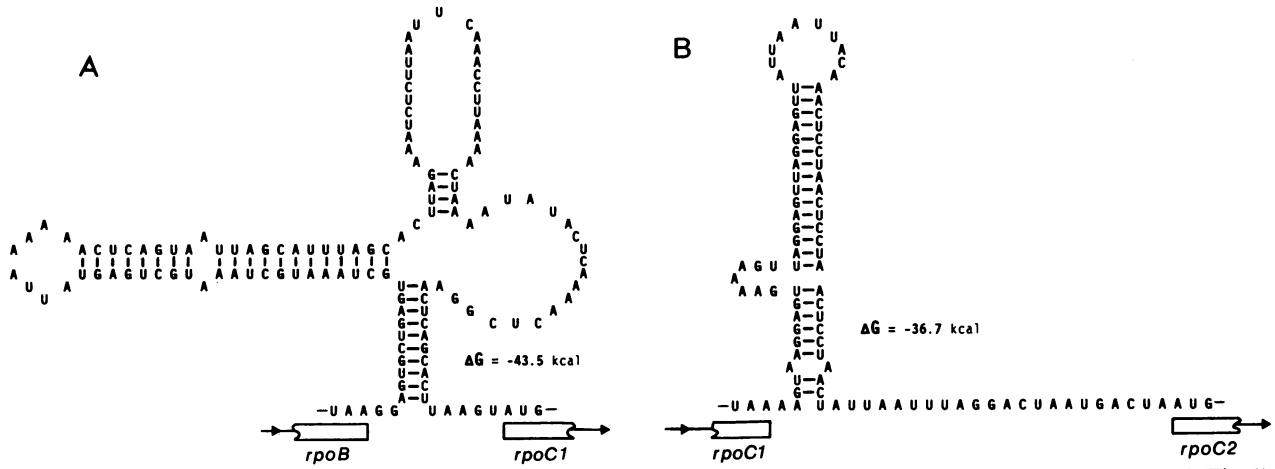


FIG. 5. Predicted secondary structures of the intergenic regions separating *rpoB* and *rpoC1* (Fig. 1) (A) and *rpoC1* and *rpoC2* (Fig. 1) (B).

sequences indicate that two isoforms of the B′ subunit may exist because of alternative splicing within the *rpoC1* sequence (11). The exact subunit composition of the spinach chloroplast RNA polymerase is uncertain.

The function(s) attributed to the large subunit of RNA polymerase has been ascertained mainly through detailed work with the RNA polymerase from *E. coli*. One conclusion from our study is that the function(s) which resides in the single β′ subunit of *E. coli* RNA polymerase (template binding; 21) may be divided between two different gene products in cyanobacteria. Such a division of function(s) could provide an additional level of control over the initiation of transcription in cyanobacteria. Although the cyanobacterial RNA polymerase shows promoter specificities similar to those of the *E. coli* enzyme, differences are apparent (32). Reconstitutions of subunits from both types of enzyme did not result in active hybrids, although these experiments were performed before the unique structure of the cyanobacterial enzyme became apparent (10). One question remains puzzling—what is the function(s) of the largest subunit of cyanobacterial RNA polymerase? Mainly because of its size, the largest subunit was considered to be the structural and functional equivalent of the *E. coli* β′ subunit (10, 20, 32). As shown here, the derived sequence of *N. commune* UTEX 584 *rpoC1* contained three segments (Fig. 2) which corresponded to three (I, II, and III) of the six highly conserved stretches of sequence observed in a comparison of the A subunit of eucaryotic and the β′ subunit of *E. coli* RNA polymerases (1). Furthermore, the derived sequence from *N. commune* UTEX 584 *rpoC2* contains two additional segments with extensive similarity to conserved regions IV and V of RNA polymerases (1; Fig. 4). The remainder of the derived sequence of RpoC2 and the carboxy-terminal region of the *E. coli* β′ polypeptide showed only minimal correspondence. A comparison of the sizes of the β′ subunits in RNA polymerases from diverse strains of cyanobacteria (10, 20, 31, 32) and that derived from our partial sequence analysis of *rpoC2* suggests that approximately 650 codons of *rpoC2*, with unknown function, remain to be analyzed.

The results from comprehensive immunochemical studies and data from DNA-sequencing studies provide convincing evidence for a common origin of the different RNA polymerases (1–3, 7, 12, 30, 37, 38). The structure of the original enzyme and the functions of its components can, however, only be speculated upon at present (37). Either the form of organization of *rpo* genes described here, which appears to have been conserved in the genome of chloroplasts, could have arisen through the splitting of a single ancestral gene in a fashion analogous to the splitting of component B into B′ and B″ in certain of the archaebacteria (37, 38) or, more likely, the genes may represent the vestiges of two separate primitive sequences, similar to those encoding A and C of the archaebacterial enzyme, respectively.

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