Chloroplast rpoA, rpoB, and rpoC Genes Specify at Least Three Components of a Chloroplast DNA-dependent RNA Polymerase Active in tRNA and mRNA Transcription*

Michael C. Little and Richard B. Hallick

From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

The purpose of this study was to determine the relationship between putative chloroplast RNA polymerase subunit genes and known chloroplast transcriptional activities. We have prepared fusion polypeptide genes from fragments of chloroplast DNA homologous to bacterial RNA polymerase subunit genes and expression vectors carrying portions of the anthranilate synthetase gene (tpe). Fusion proteins for chloroplast homologs of the RNA polymerase α (rpoA), β (rpoB), and β′ (rpoC) subunits were obtained from these genes. The fusion polypeptides synthesized by Escherichia coli in vitro were purified and used as antigens for production of rabbit polyclonal anti-RNA polymerase subunit-specific antibodies. The purified antibodies were able to immobilize chloroplast DNA-dependent RNA polymerases from spinach, pea, and Euglena gracilis. In addition, the soluble chloroplast RNA polymerase activity in tRNA and mRNA synthesis was strongly inhibited by these antibodies under conditions which had little effect on transcription by the chloroplast transcriptionally active chromosome that preferentially transcribed rRNA genes (Greenberg, B. M., Narita, J. O., DeLuca-Flaherty, C., Gruissem, W., Rushlow, K. A., and Hallick, R. B. (1984) J. Biol. Chem. 259: 14880-14887). From these data we conclude that the chloroplast genes homologous to bacterial RNA polymerase subunit genes are expressed in vivo and that the protein products specify at least three of the components of the chloroplast RNA polymerase(s) involved in tRNA and mRNA transcription.

The DNA-dependent RNA polymerases of plants play a central role in the expression of plant genes. The expression of nuclear genes is governed by at least three classes of polymerase, formally designated as I, II, or III, which are believed to be responsible for the discrete synthesis of tRNA, hnRNA and some small nuclear RNA, and S and tRNA transcripts, respectively (12, 24, 32). The nuclear enzymes are distinguished by their sensitivity to amatoxins, with the relative K values being I > III > II (12), by the complex nature of their polypeptide composition (10-14 putative subunits), and by the unique promoter sequence each recognizes (26). These nuclear RNA polymerases share some antigenic deter-

minants with RNA polymerases from other eukaryotes, and also share relatedness to each other (12). Another characteristic of nuclear polymerases is that protein factors are required for accurate initiation of RNA synthesis with cloned templates in vitro (5).

DNA-dependent RNA synthesis from organellar genes is catalyzed by distinct DNA-dependent RNA polymerases that differ from the nuclear enzymes (1, 8-13, 31). In chloroplasts of Euglena gracilis and higher plants, there are at least two RNA polymerase activities that are each capable of selective transcription of different classes of genes (9). One of these enzymes, which is tightly bound to the chloroplast chromosome, is denoted the chloroplast TAC (for transcriptionally active chromosome). It is capable of faithfully transcribing the rRNA genes, in analogy to the nuclear class I enzyme (8, 9, 12). The other activity, denoted the “soluble” activity, may be readily dissociated from the chloroplast DNA with 0.5 M KCl. This soluble enzyme retains the ability to faithfully transcribe, in a template-dependent manner, mRNA (22) and tRNA genes (8, 10, 11). Aside from the biochemical differences between these two RNA polymerases, putative subunits for the TAC from Euglena (19), spinach (1), and mustard (2) have been reported that are different in some regard from subunits of the highly purified “soluble” enzyme (16). Collectively, these studies are consistent with the idea that at least two RNA polymerases are involved in the transcription of chloroplast genes.

Recently, several chloroplast DNA sequences with significant homology to bacterial RNA polymerase subunit genes have been reported (20, 27, 28). Sijben-Muller et al. (28) first reported the sequences of a gene with homology to the gene for the α subunit of Escherichia coli RNA polymerase (denoted rpoA) (Fig. 1). This was followed by the publication of the complete chloroplast DNA sequences of Marchantia (20) and tobacco (27), which also contained an α-like subunit gene. In addition, these plants contain two additional chloroplast genes, possibly contained within a single transcription unit, with homology to the β′ (rpoC gene product) and β (rpoB gene product) subunits of E. coli RNA polymerase. Euglena chloroplast DNA also contains both an rpoB and rpoC gene. The Marchantia (20), spinach, and Euglena chloroplast rpoC loci appear to encode two polypeptide genes that have been designated rpoC1 and rpoC2. These loci are homologous to the amino-terminal and carboxyl-terminal domains, respec-

* This work was supported by grants from the National Institutes of Health (to R. B. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: Bio-Rad Laboratories, 1414 Harbor Way South, Richmond, CA 94804.

§ To whom correspondence should be addressed.

1 The abbreviations used are: TAC, transcriptionally active chromosome; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SSC, 0.12 M NaCl, 0.12 M sodium citrate; TAE, 0.484% Tris base, 0.114% glacial acetic acid, 1 mm EDTA.

2 G. Yepiz and R. B. Hallick, unpublished observations.

3 C. Radebaugh and R. B. Hallick, unpublished observations.

4 G. Hudson and P. Whitfield, personal communication.
FIG. 1. Domains of homology of derived amino acid sequences from chloroplast rpoA and rpoB genes specifying RNA polymerase subunits. A, comparison of the RNA polymerase β subunit genes of spinach (upper, 335 codons) with E. coli (lower, 329 codons). The amino acid sequence alignments have been previously reported (28). B, comparison of the RNA polymerase β subunit of tobacco (middle, 1071 codons) with that of E. coli (lower, 1343 codons) and with RPβ2, the yeast RNA polymerase II second largest subunit (upper, 1225 codons). Regions of homology are indicated by the blackened areas. The length of the homology domains and the percent identical residues are indicated. Note the two deletions in the tobacco gene relative to the E. coli gene. Regions of the coding sequence used for the expression cloning are indicated by arrows.

The chloroplast rpoA, rpoB, rpoC1, and rpoC2 genes are all transcribed. The spinach chloroplast rpoA gene is expressed as determined by Northern hybridization and in vitro translation (28) of chloroplast RNA. Transcripts of the tobacco genes were also reported (27). The Euglena rpoB-rpoC(1-2) locus lies within the Bam-Sal fragment designated BS-4 of the chloroplast DNA. The transcripts from this locus were previously quantified in this laboratory as determined by Northern hybridization and in vitro transcription (15). The spinach rpoA fusion begins at an internal BamHI site and continues to the carboxyl terminus of the gene. This segment is approximately 28% homologous in amino acid sequence to the E. coli gene in the region (codons 152-320) used for the expression clone. Of the chloroplast rpoB subunit genes, this is the least conserved subunit.

In the present study, we have begun to analyze the relationship between the protein products of these putative chloroplast DNA-encoded RNA polymerase genes and the known transcriptional activities from chloroplasts (Fig. 2). In this paper we report that these genes do indeed specify at least three components of at least one of the chloroplast RNA polymerases.

MATERIALS AND METHODS AND RESULTS AND DISCUSSION

Chloroplast RNA Polymerase Gene Structure and Fusion Protein Expression—Defined segments of the spinach rpoA, tobacco rpoB, and Euglena rpoC loci were cloned as transla-

tional fusion genes joined to the regulatory region and amino terminus of the E. coli trpE gene (see "Materials and Methods"). The location of these segments relative to the homologous coding loci of the E. coli rpoA, rpoB, and rpoC are shown in Figs. 1 and 1s. The spinach rpoA fusion begins at an internal Xhol site and continues to the carboxyl terminus of the gene. This segment is approximately 28% homologous in amino acid sequence to the E. coli gene in the region (codons 152-320) used for the expression clone. Of the chloroplast rpoB subunit genes, this is the least conserved subunit.

The tobacco chloroplast rpoB gene (Fig. 1B) has four major domains of homology with the bacterial β subunit gene and five domains with that of the yeast RNA polymerase II second largest subunit (28). The region of the chloroplast rpoB that was cloned for expression is an internal BamHI fragment spanning codons 573-935. This region is 42% homologous to the bacterial rpoB gene. This chloroplast gene contains two large deletions relative to the bacterial gene of approximately 100 codons each. Interestingly, one of these deletions (codons 976-1080 in the E. coli gene) occurs in a region of tandem repeat of the E. coli rpoB amino acid sequence. It corresponds to codons 965-1083 in the chloroplast gene. An E. coli RNA polymerase mutant with deletions in this region of the rpoB gene has been isolated which has an altered promoter specificity relative to the wild type (7).

The third fusion gene construct involved the E. gracilis rpoC locus. This gene lies proximal to a trna gene cluster (21). The carboxyl-terminal DNA sequence has been previously reported, but the rpoC gene was not identified (21). The entire gene has subsequently been sequenced. The region of the gene that was used for the expression cloning begins at an internal HindIII site and continues to the carboxyl terminus. As shown in Fig. 1s, this region is 39% homologous in derived amino acid sequence to the bacterial gene from codons 1110 to 1363 and 38-42% homologous to the carboxyl ter-

minus of different plant rpoC2 loci.

Expression of Chloroplast RNA Polymerases Fusion Protein in E. coli—The segments of the chloroplast rpoA, rpoB, and rpoC genes that had been cloned as translation fusion polypeptide genes by joining with E. coli trpE gene were each...
expressed \textit{in vivo} upon induction of host cells with indoleacrylic acid. The proteins synthesized in response to induction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For each fusion gene construct, a major new polypeptide product was synthesized following induction. The fusion polypeptides were found to be the major protein components in the insoluble protein pellets from lysed cells (Fig. 3). Lanes 1, 2, and 3 are solubilized polypeptides derived from the insoluble pellets of cells expressing the \textit{rpoC}, \textit{rpoB}, and \textit{rpoA} fusion genes, respectively. The yield for \textit{rpoA} and \textit{rpoB} was 100 mg of fusion polypeptide/liter of culture, with that of \textit{rpoC} being approximately 4-fold lower. The reason for the lowered expression of the \textit{Euglena rpoC} fusion gene is not known. We have observed the yield to also depend on the host \textit{E. coli} strain, with HB101 giving much lower expression than RR1. The fusion polypeptides were purified by preparative electrophoresis and used as antigens in the preparation of anti-RNA polymerase subunit antisera (see “Materials and Methods” in the Miniprint Supplement for experimental details).

**Binding of Chloroplast RNA Polymerases with the Fusion Protein Antibodies**—The ability of RNA polymerases from different chloroplast sources and also from \textit{E. coli} to be immobilized by the fusion protein antibodies is shown in Fig. 4. In this experiment, the binding of filter-bound immobilized antibodies to solvent-accessible epitopes of the enzyme in solution was first accomplished. Then, the ability of antibody-bound enzyme to synthesize RNA \textit{in situ} was used as a “reporter” activity to assess the binding reaction. As a control, the \textit{in vitro} transcription products were all shown to be

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig3.png}
\caption{Expression of the chloroplast RNA polymerase subunit genes as fusion proteins. Lanes 1, 2, and 3 are from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the insoluble pellets of \textit{E. coli} cells expressing \textit{rpoC}, \textit{rpoB}, and \textit{rpoA} fusion polypeptides. Asterisks indicate the major protein expressed which is the fusion protein. Molecular masses in kDa are given to the right.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig4.png}
\caption{\textit{In situ} RNA synthesis by antibody-immobilized \textit{E. coli} and chloroplast RNA polymerases. A, filters A, B, C, and D were incubated with RNA polymerase from \textit{E. coli}, \textit{pea}, \textit{Euglena}, and spinach, respectively. Bound RNA polymerase was then used as “reporter” activity for enzyme-antibody complex formation. B, strips in panel B were prepared as for panel A but were incubated in RNase following transcription as indicated in the text. The following antibodies were used: lane 1, anti-spinach chloroplast \textit{rpoA} gene product; lane 2, anti-tobacco chloroplast \textit{rpoB} gene product; lane 3, anti-\textit{Euglena chloroplast rpoC} gene product; lane 4, preimmune serum; lane 5, anti-\textit{E. coli} RNA polymerase holoenzyme. Control blots lacking RNA polymerases showed no signals (not shown).

RNase-sensitive (Fig. 4B). RNA polymerases from \textit{E. coli}, \textit{pea}, \textit{Euglena}, and spinach were incubated with filter strips A, B, C, and D, respectively, containing immobilized antibodies. The enzymes were assayed for binding to anti-\textit{rpoA} (lane 1), anti-\textit{rpoB} (lane 2), anti-\textit{rpoC} (lane 3), or anti-\textit{E. coli} holoenzyme (lane 5) antibodies. Each of the chloroplast enzymes, as evidenced by \textit{in situ} RNA synthesis, was immobilized by the anti-chloroplast RNA polymerase subunit antibodies. This is not surprising since the higher plant (\textit{rpoA} and \textit{rpoB}) and algal (\textit{rpoC}) genes have a high degree of cross-homology. Adsorption of these antibodies against an \textit{E. coli} lysate removes the determinants against \textit{trpE}, but some cross-reactivity against the bacterial subunits is retained. The antibody to the bacterial enzyme also binds the chloroplast RNA polymerases from each source. This is expected since the chloroplast and bacterial genes are also homologous (Fig. 18 and Supplemental “Results and Discussion”). In contrast, the preimmune antibody (lane 4) shows little, if any, binding of the enzymes. Collectively, these data are a demonstration of the relationship between the potential protein products encoded by the chloroplast \textit{rpoA}, \textit{rpoB}, and \textit{rpoC} genes and the RNA polymerase enzymatic activity of the “soluble” enzymes from chloroplasts of \textit{Euglena}, pea, and spinach.

**Inhibition of Chloroplast Transcription by Fusion Protein Antibodies**—The transcription of the intron-containing chloroplast valine tRNA gene of spinach chloroplast DNA by the soluble chloroplast RNA polymerase activity has previously been described (11). As a second test of the relationship between the potential \textit{rpoA}-, \textit{rpoB}-, and \textit{rpoC}-gene products and the known chloroplast transcriptional activities, selective inhibition of chloroplast tRNA transcription by the highly purified fusion protein antibodies was assayed. Transcription of a spinach pre-trnV template by the spinach-“soluble” enzyme is sensitive to the addition of each of the fusion protein antibodies (Fig. 5A). No inhibition is observed when preimmune antibody is added. The inhibition of pre-tRNA\textsuperscript{val} transcription is not a result of ribonuclease contamination, since the incubation of antibody with radiolabeled transcripts following RNA synthesis did not result in a decrease in the intensity of the transcript band (Fig. 5B). The antibodies purified via DEAE-Affi-Gel Blue appear free of detectable RNase. The observed immunoinhibition of transcription by these antibodies is consistent with the results on \textit{in situ} RNA
spinach chloroplast TAC is shown in Fig. 6A. The RNA produced by the spinach chloroplast TAC in panel A shows hybridization to a cloned tobacco rRNA gene as well as to SalI-9 and BamHI-7 fragments of tobacco chloroplast DNA. The latter fragments of chloroplast DNA contain genes specifying tRNAs and psbE polypeptide, and a cluster of ribosomal proteins, respectively. Thus, the spinach chloroplast TAC is similar to that of mustard in that it produces in vitro transcripts from protein-coding loci and rDNA genes (25). In contrast, the Euglena TAC makes only rDNA, as seen in the Euglena TAC RNA product.

The effect of antibody concentration on both Euglena and spinach TAC RNA synthesis was determined (Fig. 7). Precipitation of antibody with the TAC has little if any effect on transcription from the rDNA genes. The same amount of any of the antibodies completely inhibited in vitro transcription by the soluble RNA polymerases that immuno-react with the TAC RNA polymerases. Whether this discrimination is due to a different subunit structure for the TAC versus the TAC RNA polymerases. Whether this discrimination is due to a different subunit structure for the TAC versus the TAC RNA polymerases.

Effect of Fusion Peptide Antibodies on rRNA Synthesis by the TAC —The effect of the antibodies on ribosomal RNA synthesis by the purified TAC of both Euglena and spinach chloroplasts was also determined. The selective transcription of rDNA by the Euglena chloroplast TAC has previously been reported (25). The characterization of the products by the

spinach chloroplast TAC as probes for Western blots of chloroplast RNA polymerase preparations from spinach.
pea, and *E. gracilis* as determined by an antibody-linked
in situ RNA polymerase assay. The tRNA-specific RNA poly-
merases from spinach, pea, and *Euglena* pea was also strongly
inhibited by these antibodies. By contrast, the same antibody
treatments had little effect on RNA synthesis by the tran-
scriptionally active chromosome RNA polymerases from both
*Euglena* and spinach chloroplasts. From these data we con-
tinue to treat the chloroplast genes homologous to the
transcriptionally active chromosome RNA polymerases from both
*rpoA*, *rpoB*, and *rpoC* as expressed in organello and that the
protein products specify at least three of the components of
the soluble RNA polymerase. The identification of genes for
subunits of the TAC RNA polymerase activity was not pos-
sible with the available antibodies.

REFERENCES

1. Briat, J. F., and Mache, R. (1980) *Eur. J. Biochem.* 111, 503-509
2. Bulow, S., Reiss, T., and Link, G. (1987) *Curr. Genet.* 12, 157-159
3. Chelm, B., Hallick, R. B., and Gray, P. W. (1978) *Biochemistry* 17, 4239-4244
4. Chelm, B., Hallick, R. B., and Gray, P. W. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 2258-2262
5. Cooke, R. M., Durand, R., Job, B., Penon, P., Tessiere, M., and Job, D. (1984) *Plant Mol. Biol.* 3, 217-225
6. Ellis, J. R., and Harty, M. R. (1971) *Nat. New Biol.* 233, 193-196
7. Glass, R. E., Jones, S. T., Nene, V., Nomura, T., Fujita, N., and Ishihama, A. (1986) *Mol. Gen. Genet.* 203, 487-491
8. Greenberg, B. M., Narita, J. O., DeLuca-Flaherty, C., Gruissem, W., Rushlow, K. E., and Hallick, R. B. (1984) *J. Biol. Chem.* 259, 14850-14887
9. Greenberg, B. M., Narita, J. O., DeLuca-Flaherty, C., and Hallick, R. B. (1985) in *Molecular Biology of the Photosynthetic Apparatus* (Steinbäck, K., Arntzen, C., Bogorad, L., and Bonitz, S., eds) pp. 393-399, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Gruissem, W., Greenberg, B. M., Zawarski, G., Prescott, D. M., and Hallick, R. B. (1983) *Cell* 35, 815-828
11. Gruissem, W., Greenberg, B. M., Zawarski, G., and Hallick, R. B. (1986) *Methods Enzymol.* 118, 253-270
12. Guilloye, T. J. (1981) in *The Biochemistry of Plants* (Stumpf, K., Conn, E. E., and Marcus, A., eds) Vol. 6, pp. 208-247, Academic Press, New York
13. Hallick, R. B., Rushlow, K. E., Orozco, E. M., Stiegler, G. L., and Gray, P. W. (1979) *JCN-UCLA Symp. Mol. Cell. Biol.* 15, 127-141
14. Hildebrand, M., Jurgenson, J. E., Ramage, R. T., and Bourque, D. P. (1985) *Plant Sci.* 14, 64-79
15. Leurs, S., Brautigam, E., and Parthier, B. (1985) *EMBO J.* 4, 1661-1666
16. Leurs, S., Briat, J-F., and Mache, R. (1983) *Plant Mol. Biol.* 2, 74
17. Lipman, D. J., and Pearson, W. J. (1985) *Science* 227, 1445-1451
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Narita, J. O., Rushlow, K. E., and Hallick, R. B. (1985) *J. Biol. Chem.* 260, 11194-11199
20. Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Usmeono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H., and Ozeki, H. (1986) *Nature* 322, 572-574
21. Orozco, E. M., and Hallick, R. B. (1982) *J. Biol. Chem.* 257, 3265-3275
22. Orozco, E. M., Mullet, J. E., and Chua, N-H. (1985) *Nucleic Acids Res.* 13, 1283-1302
23. Peichl, T., and Link, G. (1985) *Eur. J. Biochem.* 148, 207-212
24. Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., and Chamberlin, M., eds) pp. 285-329, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Rushlow, K. E., Orozco, E. M., Lipper, C., and Hallick, R. B. (1980) *J. Biol. Chem.* 255, 3786-3793
26. Shenk, T. (1983) *Curr. Top. Microbiol. Immunol.* 93, 25-40
27. Shinozaki, K., Ohme, M., Tunaka, M., Watanuki, T., Hayashi, N., Matsubayashi, T., Zaita, N., Chungwongse, J., Obokata, J., Moriyasu, K., Sugita, M., Deno, H., and Yamaguchi, K. (1984) *Nature* 312, 526-530
28. Sijben-Möller, G., Hallick, R. B., Alt, J., Westhoff, P., Herrmann, M. G. (1986) *Nucleic Acids Res.* 14, 1029-1044
29. Deleted in proof
30. Tanese, N., Roth, M., and Goff, S. G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 4944-4948
31. Tewari, K. K., and Goel, A. (1983) *Biochemistry* 22, 2142-2148
32. Zieve, G. W. (1981) *Cell* 25, 296-297
Preparation of Chloroplast Transcription Extracts. Intact chloroplasts were prepared from 4 week old hydropnic spinach (Spinacia). Arrow bean (Vicia faba) and pea (Pisum sativum) plants. The spinach chloroplasts were prepared using the method described by C. Sharp and E. M. Farrant, unpublished results. The arrow bean and pea chloroplasts were prepared using the method of R. J. Walls and D. R. Hauge, unpublished results. The chloroplasts were prepared from ground material using a Polytron (Brinkman) tissue homogenizer at settings of 4 without bursts. As described elsewhere, the material was repreated as above. This permitted maximal extraction with minimal breakdown of the photosynthetic apparatus and other chloroplast enzymes. The purified chloroplasts were suspended in 0.5 M sucrose, 10 mM HEPES, 10 mM MgCl2, pH 7.5. The purified chloroplasts were suspended in 5 ml buffer A/100 g leaves (11) and lyzed by 10 min sonication at full power in a Branson 2020 sonicator. The supernatant was collected by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor. The intact chloroplasts were recovered from the 45% sucrose interface.

Chloroplast transcription extracts were prepared by either of two methods. For each experiment the chloroplast extract was prepared as described by C. Sharp and E. M. Farrant, unpublished results. In brief, the purified chloroplasts were suspended in 5 ml buffer (0.25 M sucrose, 10 mM HEPES, 10 mM MgCl2, pH 7.5) and 15 ul of 1 M MgCl2 were added. Following a 10 min preincubation at 25°C, 30 ul of 4 M ammonium acetate and 20 ul of 100% ethanol were added and the mixture was incubated at -20°C for 2 h. The precipitate was collected by centrifugation at 15,000 rpm for 1 h at 4°C. The supernatant was discarded, the pellet was washed twice with 14 ml 100% ethanol, 100% cold acetone, and finally with 2 volumes of cold acetone. The precipitate was redissolved in 10 ul of water and used as template in the transcription reaction. This fraction also contains RNA polymerase, and the soluble chloroplast RNA polymerases may be further purified through DEAE-Sepharose and a Sephacryl S200 column. As described elsewhere, the purified RNA polymerase was used in the chloroplast RNA polymerase transcription assay.

Transmission Assays. The assay for RNA-specific transcription was as described, using the spinach RNA polymerase template. After incubation for 30 min, 1 ml of labeled RNA was added and incubated for 15 min at 25°C. The reactions were continued and samples were layered with aqueous phase (labeled) and measured for Cerenkov radiation. In vitro transcription of RNA polymerase was determined essentially as above. Twenty-five ul of pure RNA or Euglena high salt TAC, prepared as in (10), were covalently protected with 0.1 ul at 10°C. The covalent RNA by the chloroplasts was carried out by RNase H digestion. The RNase H reaction was carried out at 37°C for 5 min with 0.1 ul of 0.1 M Tris-HCl, pH 7.5, 10 mM MgCl2, and 0.25 M ammonium acetate and 10% sucrose. Following incubation the RNA was precipitated with 2 volumes of ethanol on powdered dry ice for 10 min. The mixture was then centrifuged at 15,000 rpm for 1 h at 4°C. The supernatant was discarded, the pellet was washed twice with 14 ml 100% ethanol, 100% cold acetone, and finally with 2 volumes of cold acetone. The precipitate was redissolved in 10 ul of water and used as template in the transcription reaction. This fraction also contains RNA polymerase, and the soluble chloroplast RNA polymerases may be further purified through DEAE-Sepharose and a Sephacryl S200 column.

Supplemental Results and Discussion

Nucleotide Antagonists. A polyribosome sedimentation pattern from Euglena TAC (10) was selected for the synthesis of full length RNA. The RNA was transcribed in a reaction mixture containing ATP, CTP, GTP, UTP, 3H-labeled UTP, and 0.5 M sodium acetate, 10 mM MgCl2, 10 mM HEPES (pH 7.5). The reaction mix was incubated at 37°C for 1 h. The RNA was isolated from the reaction mixture and purified by precipitation with 50% (v/v) ethanol and 0.1 M ammonium acetate.

Euglena chloroplast TAC vs. Marchantia (moss) Euglena carboxylic acid sequence. The TAC was incubated at 37°C for 1 h. The RNA was isolated from the reaction mixture and purified by precipitation with 50% (v/v) ethanol and 0.1 M ammonium acetate. The RNA was dissolved in water and used as a template for a reverse transcription reaction. The RNA was transcribed in a reaction mixture containing ATP, CTP, GTP, UTP, 3H-labeled UTP, and 0.5 M sodium acetate, 10 mM MgCl2, 10 mM HEPES (pH 7.5). The reaction mix was incubated at 37°C for 1 h. The RNA was isolated from the reaction mixture and purified by precipitation with 50% (v/v) ethanol and 0.1 M ammonium acetate. The RNA was dissolved in water and used as a template for a reverse transcription reaction.

Euglena chloroplast TAC vs. Marchantia (moss) Euglena carboxylic acid sequence. The TAC was incubated at 37°C for 1 h. The RNA was isolated from the reaction mixture and purified by precipitation with 50% (v/v) ethanol and 0.1 M ammonium acetate. The RNA was dissolved in water and used as a template for a reverse transcription reaction. The RNA was transcribed in a reaction mixture containing ATP, CTP, GTP, UTP, 3H-labeled UTP, and 0.5 M sodium acetate, 10 mM MgCl2, 10 mM HEPES (pH 7.5). The reaction mix was incubated at 37°C for 1 h. The RNA was isolated from the reaction mixture and purified by precipitation with 50% (v/v) ethanol and 0.1 M ammonium acetate. The RNA was dissolved in water and used as a template for a reverse transcription reaction.

Euglena chloroplast TAC vs. Marchantia (moss) Euglena carboxylic acid sequence. The TAC was incubated at 37°C for 1 h. The RNA was isolated from the reaction mixture and purified by precipitation with 50% (v/v) ethanol and 0.1 M ammonium acetate. The RNA was dissolved in water and used as a template for a reverse transcription reaction.