

# Functional Analysis of Two Maize cDNAs Encoding T7-like RNA Polymerases

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We have characterized two maize cDNAs, *rpoTm* and *rpoTp*, that encode putative T7-like RNA polymerases. In vivo cellular localization experiments using transient expression of the green fluorescent protein suggest that their encoded proteins are targeted exclusively to mitochondria and plastids, respectively. An antibody raised against the C terminus of the *rpoTp* gene product identified mitochondrial polypeptides of ~100 kD. Their presence was correlated with RNA polymerase activity, and the antibody inhibited mitochondrial in vitro transcription activity. Together, these results strongly suggest that the product of *rpoTm* is involved in maize mitochondrial transcription. By contrast, immunoblot analysis and an antibody-linked polymerase assay indicated that *rpoTp* specifies a plastid RNA polymerase component. A quantitative reverse transcription-polymerase chain reaction assay was used to study the transcription of *rpoTp* and *rpoTm* in different tissues and under different environmental conditions. Although both genes were constitutively expressed, *rpoTm* transcripts were generally more prevalent in nonphotosynthetic tissues, whereas an increase in *rpoTp* transcripts paralleled chloroplast development. We suggest that these two genes encode constitutive components of the organelle transcription machinery but that their expression is nonetheless subject to modulation during plant development.

## INTRODUCTION

Plastid-containing eukaryotes are unique in possessing two extranuclear transcriptionally active compartments. Although the chloroplast genomes of photosynthetic eukaryotes encode subunits of an *Escherichia coli*-like RNA polymerase (PEP, for plastid-encoded polymerase) and some organisms, such as the protist *Reclinomonas americana* and the brown alga *Pylaiella littoralis*, encode RNA polymerase subunits in their mitochondrial genomes (Lang et al., 1997; Rousvoal et al., 1998), there is a clear and pervasive reliance on nucleus-encoded proteins for organelle transcription.

In chloroplasts, there are two types of nucleus-encoded proteins required for transcription. First, the nucleus encodes subunits of PEP or accessory transcription factors, such as sigma-like factors (Tanaka et al., 1996, 1997; Isono et al., 1997; Kestermann et al., 1998; Oikawa et al., 1998) or DNA binding proteins (Kim and Mullet, 1995; Trifa et al., 1998). Second, the nucleus encodes the RNA polymerase it-

self (NEP, for nucleus-encoded polymerase). Evidence for the existence of NEP has come from studies in which chloroplast translation (and therefore synthesis of PEP) has been inactivated, leading to the interpretation that residual transcription results from NEP (Hess et al., 1993; Silhavy and Maliga, 1998), or in which PEP has been directly inhibited (Kapoor et al., 1997). In addition, PEP has been inactivated by the deletion of chloroplast genes, revealing sites of NEP-mediated initiation (Allison et al., 1996; Serino and Maliga, 1998). It has been suggested that NEP activity results from a phagelike polymerase, because it has been observed that T7-like RNA polymerase activity is present in spinach chloroplasts (Lerbs-Mache, 1993). Indeed, the transit peptide of a T7-type RNA polymerase from *Arabidopsis*, when fused to the green fluorescent protein (GFP), can direct import into spinach chloroplasts in vitro (Hedtke et al., 1997) and to *Arabidopsis* chloroplasts in vivo (Hedtke et al., 1999). Taken together, these data suggest that a nuclear-encoded T7-like RNA polymerase contributes to chloroplast transcription in higher plants.

In plant mitochondria, evidence for a nucleus-encoded RNA polymerase is more indirect. One finding is that in the

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two completely sequenced mitochondrial genomes, those of liverwort and *Arabidopsis*, there are no open reading frames that encode homologs of known RNA polymerase subunits (Oda et al., 1992; Unseld et al., 1997). Second, in yeast and other fungi as well as in mammals, mitochondrial transcription is known to involve RNA polymerase core enzymes related to T7 and T3 RNA polymerases (reviewed in Tracy and Stern, 1995), suggesting that if mitochondrial RNA polymerase were organelle encoded in plants, the genes would be easily identified. Third, in vitro characterization of plant mitochondrial promoters shows that they resemble those of yeast mitochondria (Rapp et al., 1993; Binder et al., 1995; Caoile and Stern, 1997). Finally, recent data indicate that genes potentially encoding T7-like RNA polymerases are widely found in eukaryotes (Cermakian et al., 1996), including plants such as *Arabidopsis* (Hedtke et al., 1997), *Chenopodium* (Weihe et al., 1997), wheat (Ikeda and Gray, 1999), and maize (Young et al., 1998). A product of one of the *Arabidopsis* nuclear genes, when fused to the GFP, can be imported into pea mitochondria in vitro (Hedtke et al., 1997) and into *Arabidopsis* mitochondria in vivo (Hedtke et al., 1999), and a protein isolated from wheat mitochondria contains sequences corresponding to one of the wheat cDNAs (Ikeda and Gray, 1999).

In this report, we have characterized two maize cDNAs that encode putative T7-like RNA polymerases. In vivo cellular localization studies suggest that as in *Arabidopsis*, one gene product plays a role in chloroplast transcription, whereas the other functions in mitochondria. We also provide biochemical data to support transcriptional roles for these protein. Quantitative analysis of mRNAs accumulating from these two genes suggests that they may be regulated at the transcriptional level during plant development and by environmental signals.

## RESULTS

### Molecular Cloning of Two Genes Encoding Putative T7-like RNA Polymerases

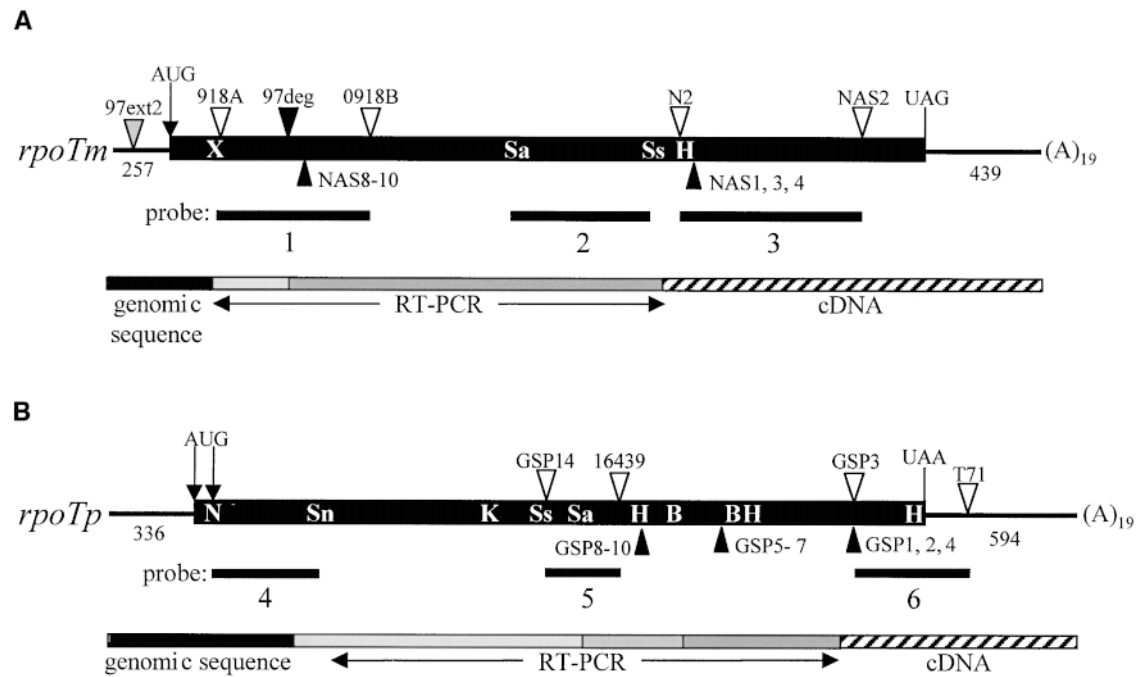
We initially screened a maize ear shoot cDNA library with the insert of a rice expressed sequence tag clone showing similarity to T7-like RNA polymerases, including that of yeast mitochondria. Two different classes of cDNA clones were obtained. The longest contained 0.95- and 1.4-kb inserts, including an open reading frame, a putative 3' untranslated region, and a poly(A) tail, suggesting that they were derived from nuclear genes and contained the complete 3' ends of their respective coding regions. Based on an informal consensus for nomenclature between groups working in this area, these genes were named *rpoTm* and *rpoTp* (for T7-like RNA polymerases). The "m" and "p" indicate the mitochondrial and plastid localization of the gene products, as shown below. To obtain upstream cDNA sequences, we used 5'

rapid amplification of cDNA ends (RACE), as described in Methods and shown schematically in Figures 1A and 1B, and obtained 3.0 and 3.1 kb of the sequence. Having at this point encountered sequences refractory to reverse transcription, we obtained additional sequence information by screening a maize genomic library with probes from the 5' end of each cDNA sequence. Where possible, the existence of the sequences in RNA was verified using reverse transcription-polymerase chain reaction (RT-PCR; data not shown). Expression data shown below strongly indicate that our sequences contain the full coding regions for each T7-like RNA polymerase gene, and the cDNA structures are summarized in Figures 1A and 1B. Single-copy probes from each cDNA were sent to the University of Missouri-Columbia maize restriction fragment length polymorphism laboratory for mapping; the gene referred to below as *rpoTm* mapped to chromosome 9, and *rpoTp* mapped to chromosome 7 (see Methods).

To estimate the copy number of *rpoT* genes in maize, we conducted a series of DNA gel blot analyses by using the cDNA probes shown below each gene map in Figure 1; the results are shown in Figure 2. Figure 2A shows that by using probes derived from the N-terminal regions of the two genes, which are least conserved at the amino acid level, single hybridizing bands were detected for *rpoTm* by using four different restriction endonucleases, and one or two bands were detected for *rpoTp*. By using probes from the centers of the coding regions, however, different results were obtained, as shown in Figure 2B. For *rpoTm*, two hybridizing fragments were observed with all restriction endonucleases used, whereas many fragments were detected with the short *rpoTp* probe 5. By using probes from the highly conserved C-terminal portions of the cDNAs, however, the genes appeared to be single copy, as shown in Figure 2C.

These results can be interpreted in several ways. For *rpoTm*, two of the three probes support the idea that this gene is single copy. Because probe 2 identifies two fragments, we must assume that sequences within this probe are repeated elsewhere in the genome. One possibility we initially considered is that the probe was cross-hybridizing with *rpoTp*. However, the region of *rpoTp* corresponding to the amino acid sequence within probe 2 is contained within 6.6- and 2.5-kb *SacI* fragments, which are clearly absent in lane Sa at left in Figure 2B. A second possibility is that a domain within *rpoTm* is shared by another protein with different N and C termini or that sequences within probe 2 are duplicated elsewhere in a noncoding region. Finally, we cannot rule out that probe 2 spans one or more introns that fortuitously contain sites for each restriction endonuclease that we have tested, because we have not obtained the complete genomic sequence for *rpoTm*.

In some ways, the results for *rpoTp* are similar to those obtained for *rpoTm*, although interpretation is easier because the genomic sequence for *rpoTp* has been published (Young et al., 1998). Results with an N-terminal probe (probe



**Figure 1.** Molecular Maps of *rpoTm* and *rpoTp*.

**(A)** Cloning and analysis of *rpoTm* cDNA. The filled rectangle indicates the coding region, with the unique translation initiation site (AUG) indicated with an arrow. UAG is the stop codon, and the lengths of the 5' and 3' untranslated regions are given in nucleotides. Selected restriction sites indicated in boldface white letters are HindIII (H), SacI (Sa), SspI (Ss), and XhoI (X). Primers used for RT-PCR cloning are indicated by black triangles; open triangles represent the primers used for amplification of probes 1 and 3, and the gray arrow represents the primer used for 5' end mapping. The portions of the composite cDNA sequence obtained from genomic clones, RT-PCR (5' RACE; two clones), and cDNA library screening are indicated.

**(B)** Cloning and analysis of *rpoTp* cDNA. Symbols are as in **(A)**, except that two potential translation initiation sites separated by 81 bp are shown (see Figure 3), and three 5' RACE clones were used for sequencing the gene. Additional restriction sites shown are BamHI (B), KpnI (K), NcoI (N), and SnaBI (Sn).

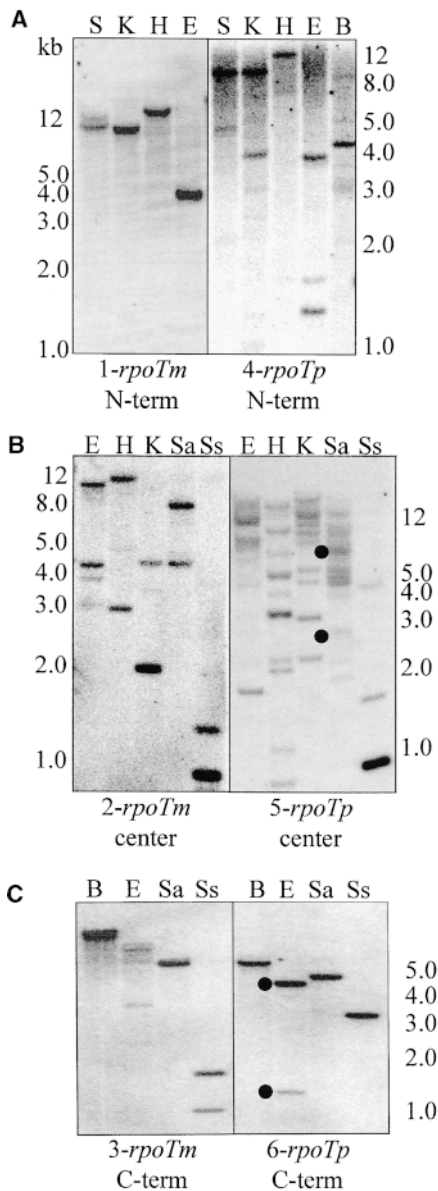
4) resembled those obtained with probe 2 for *rpoTm*, and the possible interpretations are similar, because we cannot explain the second hybridizing fragment in each lane (except for HindIII) as an adjacent fragment of the *rpoTp* gene. For instance, there are no EcoRI sites in the genomic sequence corresponding to probe 4. On the other hand, the C-terminal probe is strongly indicative of a single-copy sequence, because two hybridizing fragments are seen only with EcoRI. In this case, the result is explained by the genomic sequence, which predicts that a 1.2-kb fragment internal to the gene and a second downstream fragment of unknown size would hybridize with probe 6.

Probe 5 gave surprising results, identifying multiple bands with all endonucleases, except for SspI. This probe corresponds to parts of exons 8 and 9 and lies within a probe used by Young et al. (1998). This probe, which spans exons 7 to 16, yielded a similar result. Although the fragments predicted by the genomic sequence are clearly present (e.g., 0.8 kb for SspI and 2.5 and 6.6 kb for SacI), the other hybridizing bands must correspond to a repeated element. The

low-copy-number hybridizations obtained with the flanking probes 4 and 6 suggest that most of these do not represent additional closely related *rpoT* genes. Taken together, our results suggest that *rpoTm* and *rpoTp* are unique in the maize genome, although they contain some sequences repeated elsewhere that may be present in noncoding regions or in coding regions of partially related genes.

### Conservation of Critical Amino Acid Residues

Several alignments of eukaryotic T7-like RNA polymerases have been published recently (Cermakian et al., 1996, 1997; Hedtke et al., 1997; Young et al., 1998); therefore, we selected representative members of this gene family for comparison with the proteins predicted by the cDNA sequences of *rpoTm* and *rpoTp*. Rather than an evolutionary analysis, our objective was to identify conservation of functionally important residues. Figure 3 presents such an alignment, using the products from two maize genes and three Arabidopsis



**Figure 2.** DNA Gel Blot Analysis.

**(A)** Twenty-five micrograms of total maize DNA was digested with the indicated restriction endonucleases: Sall (S), KpnI (K), HindIII (H), EcoRI (E), BamHI (B), SacI (Sa), and SspI (Ss). Probes (Figure 1) were from the N-terminal (N-term) portions of each gene, as indicated.

**(B)** Analysis as given in **(A)**, except that probes were from the centers of the genes. Probe 5 for *rpoTp* (235 bp) is internal to that (1.2 kb) used by Young et al. (1998).

**(C)** Analysis as given in **(A)**, except that probes were from the conserved C-terminal (C-term) portions of the genes.

In **(A)** to **(C)**, numbers at left and right indicate sizes of DNA fragments in kilobases. In **(B)** and **(C)**, hybridizing DNA fragments referred to in the text are marked by filled circles.

genes as well as products from the dicotyledonous plant *Chenopodium*, the yeast mitochondrial RNA polymerase, and T7 RNA polymerase itself. Within this gene family, there are 11 well-conserved domains (I to XI; McAllister and Raskin, 1993), and the alignment shows that within each of these regions, there are from several to many residues conserved across all the analyzed sequences. This is especially notable for domains I to III, in which cross-kingdom sequence identity is poor in the flanking regions. T7 RNA polymerase also contains "thumb," "palm," and "finger" motifs that are structurally similar to domains of the Klenow fragment of DNA polymerase I and reverse transcriptase (Sousa et al., 1993), and motifs A, B, and C that are well conserved among RNA and DNA polymerases and form part of the catalytic pocket (McAllister and Raskin, 1993). Reasonable conservation is observed in the thumb motif, as shown in Figure 3. Asp-537, Lys-631, Tyr-639, and Asp-812 are located in the catalytic pocket of T7 RNA polymerase (McAllister and Raskin, 1993), and all are conserved universally among the sequences aligned in Figure 3. However, residues that confer promoter specificity for T7 RNA polymerase (the specificity loop; Rong et al., 1998) were poorly conserved when compared with surrounding sequences, as we had expected given the divergence of promoter sequences among phage, yeast mitochondria, and plant organelles.

Important residues in yeast mitochondrial RNA polymerase also were examined. Mutations in Ser-938 and Pro-679 cause temperature-sensitive instability of the mitochondrial genome (Lisowsky and Michaelis, 1989; Lisowsky et al., 1996), and these two residues are conserved in the plant sequences but not in T7 RNA polymerase. In conclusion, the amino acid sequence alignments are consistent with the maize genes *rpoTm* and *rpoTp* performing RNA polymerase functions related to those of the T7 family.

### Expression of *rpoTm* and *rpoTp* mRNAs

To examine the transcription of *rpoTm* and *rpoTp*, we isolated total or poly(A)<sup>+</sup> RNA from etiolated maize seedlings and analyzed them by using gel blots. Figure 4A shows that we could detect 3.6- and 3.8-kb transcripts by using probes from the C-terminal part of *rpoTm* or *rpoTp* cDNAs, respectively, using poly(A)<sup>+</sup> RNA, but these transcripts could be only weakly detected using 15  $\mu$ g of total RNA. Their presence as poly(A)<sup>+</sup> transcripts is consistent with the nuclear location of the genes, and the weak hybridization with total RNA suggests that both transcripts are of relatively low abundance. We saw no evidence for transcript size heterogeneity, which is consistent with them being single-copy genes; however, comigration of transcripts from other related genes certainly cannot be excluded. To map the 5' ends of the transcripts, primer extension was performed. Figure 4B shows that a single putative transcription start site could be detected for *rpoTm*, giving a 5' untranslated region length of 257 nucleotides and a total transcript length of

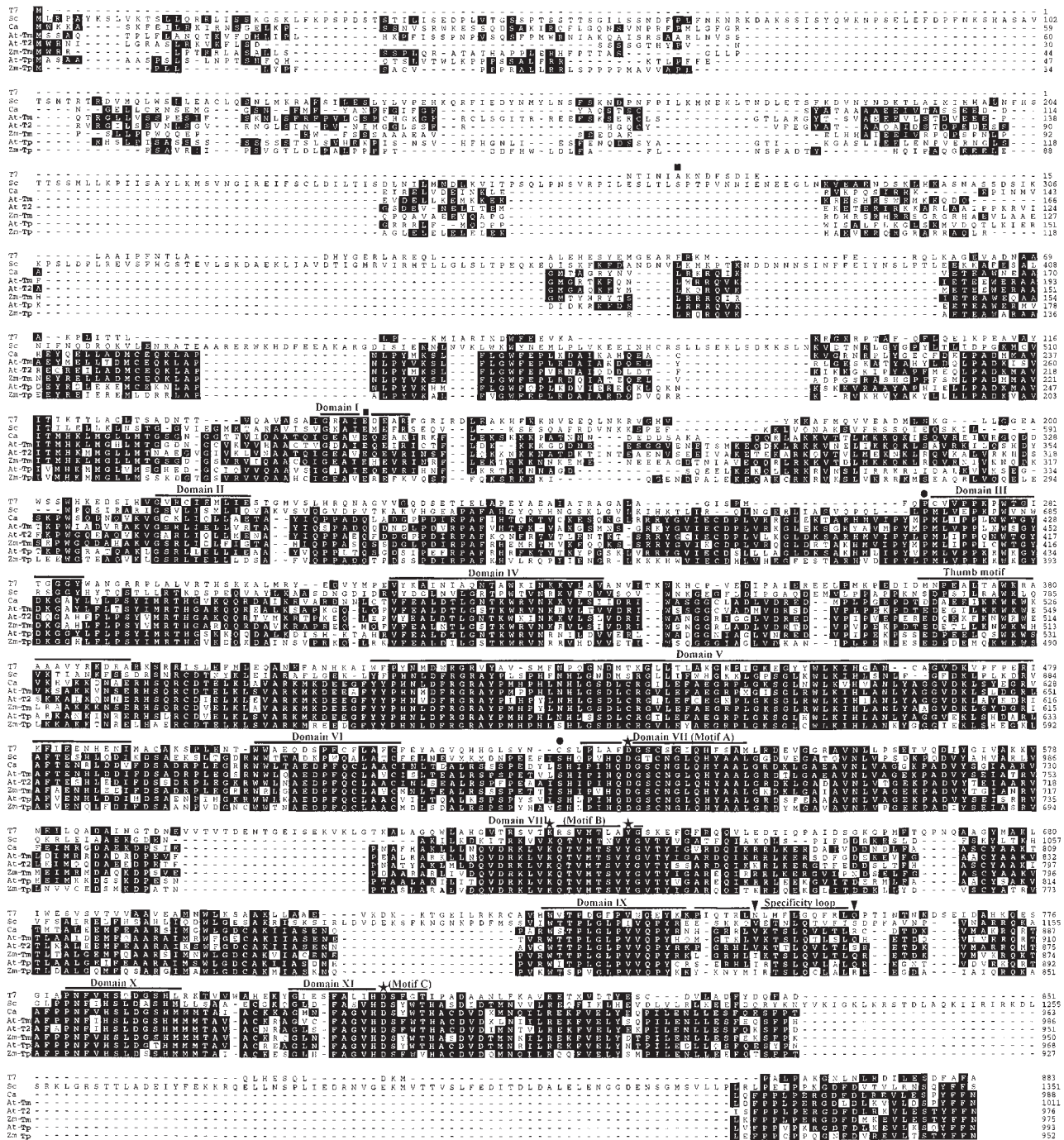
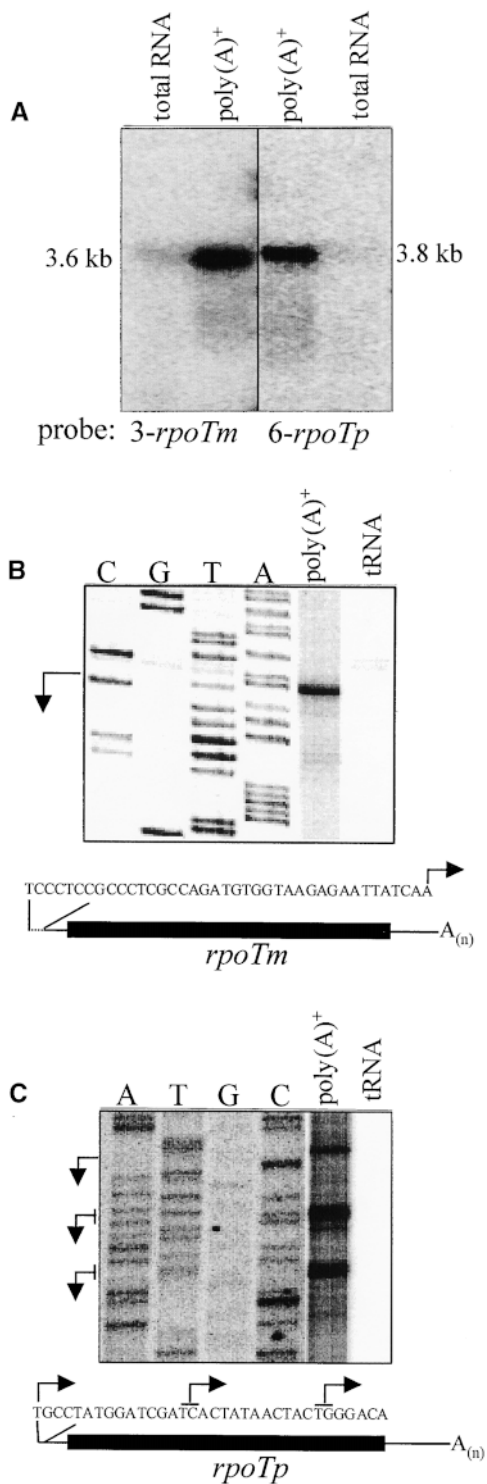


Figure 3. Amino Acid Sequence Alignment.

Amino acid sequences were compared among seven T7-like RNA polymerases by using the Clustal method with the Megalign program of DNASTar. Sequences of T7-like RNA polymerases were obtained from the databases and abbreviated as follows, with the GenBank accession number indicated: T7 (T7 RNAP; M38308); Sc (*Saccharomyces cerevisiae* mitochondrial RNAP, product of the *RPO41* gene; M17539); Ca (*Chenopodium album*; Y08137); At-T2 (Arabidopsis; AJ001037); At-Tm (Arabidopsis; Y08137); At-Tp (Arabidopsis; Y08463); and Zm-Tp (*rhoTp*) from this study. Sc, Ca, At-Tm, At-T2, and Zm-Tm are known or presumed to be localized in the mitochondrion; At-Tp and Zm-Tp are presumed to be plastid localized. Black lines indicate conserved domains (I to XI) between T7 RNA polymerase and other T7-like RNA polymerases; motifs A, B, and C (within parentheses) are conserved among nucleotide polymerases; and residues indicated by stars in motifs A to C are important catalytic residues (reviewed in McAllister and Raskin, 1993). Filled circles indicate functionally important residues in yeast mitochondrial RNA polymerase (Lisowsky and Michaelis, 1989; Lisowsky et al., 1996). Filled squares indicate residues involved in RNA binding by T7 RNA polymerase (He et al., 1997). The two arrowheads in the specificity loop indicate residues involved in promoter recognition by T7 RNA polymerase (Raskin et al., 1993; Rong et al., 1998). Dashes were used to optimize alignment.



**Figure 4.** Transcript Analysis for *rpoTm* and *rpoTp*.

**(A)** RNA gel blot analysis. Fifteen micrograms of maize total RNA or 1  $\mu$ g of poly(A)<sup>+</sup> RNA was separated by gel electrophoresis and analyzed as described in Methods. Probes are indicated below each gel and in Figure 1. Transcript lengths are indicated at left and right in kilobases.

3621 nucleotides (see Figure 1), which is fully consistent with the hybridization results shown in Figure 4A. Figure 4C shows that multiple 5' ends were detected for *rpoTp*; however, we could not distinguish between multiple start sites and primer extension artifacts. Taking the most upstream band as the putative start site, our sequence data yielded a transcript length of 3786 nucleotides, which is also consistent with the results shown in Figure 4A.

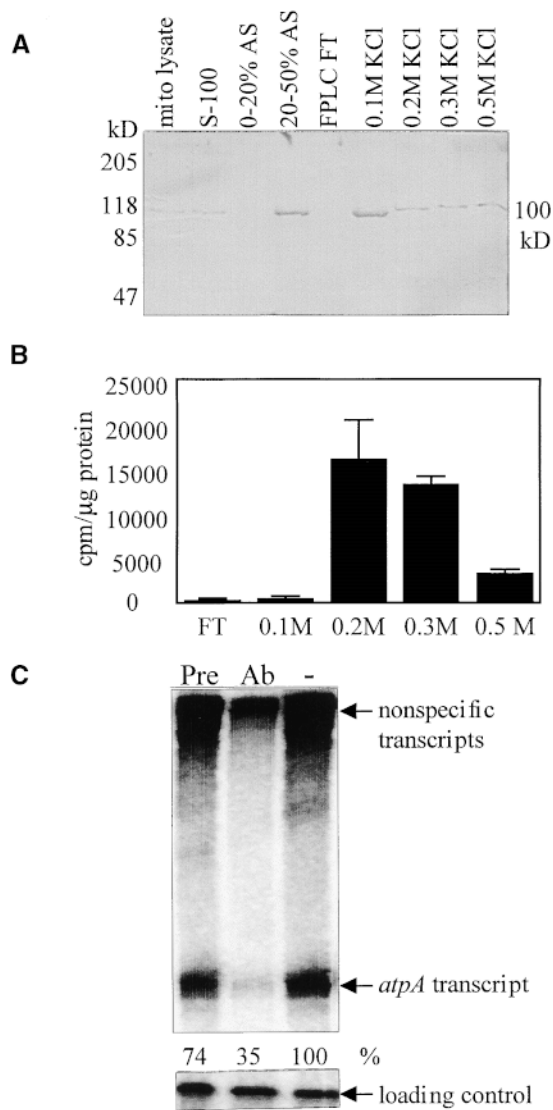
**Protein Detection and Immunoinhibition Assay for *rpoTm***

A histidine-tagged protein corresponding to a highly conserved part of the C terminus of *rpoTp* was overexpressed in *E. coli*, and antibodies were raised against it in rabbits. We expected that these polyclonal antibodies would recognize the products of both *rpoTm* and *rpoTp* if they were present in vivo. To detect any cross-reacting proteins in maize mitochondria, we used a crude lysate as well as more highly purified fractions, according to the protocol we developed to conduct in vitro transcription of maize mitochondrial promoters (Rapp and Stern, 1992). Figure 5A shows that whereas a protein migrating at ~95 kD was detected in some of the cruder fractions, the most highly purified fast protein liquid chromatography (FPLC) fractions contained immunoreactive species of 98 to 100 kD. Because preimmune serum also identified a 95-kD protein in the same fractions, we concluded that this reaction was nonspecific (data not shown). However, the preimmune serum did not react with 98- to 100-kD proteins in the 0.2, 0.3, and 0.5 M KCl fractions obtained by anion exchange FPLC. We concluded that these species might represent the product of the *rpoTm* gene.

To correlate the presence of the 98- to 100-kD proteins seen in Figure 5A with RNA polymerase activity, we performed activity assays for each FPLC fraction by using poly(dAT) as a template. This assay was used previously during purification of wheat mitochondrial transcription activity (Hanic-Joyce and Gray, 1991). The results shown in Figure 5B indicate that RNA polymerase activity is present in the 0.2, 0.3, and 0.5 M KCl FPLC fractions but is essentially undetectable in the flow-through fraction and the 0.1 M KCl

**(B)** 5' End mapping of *rpoTm* mRNA. The 5' end-labeled primer 97Ext2 (Figure 1) was extended using poly(A)<sup>+</sup> RNA or yeast tRNA as a control. The extension products were separated in a 5% denaturing polyacrylamide gel along with a sequencing ladder generated by using the same primer. The deduced start site is indicated by a bent arrow at the right, and the corresponding DNA sequence is presented below the gel.

**(C)** 5' End mapping of *rpoTp* mRNA, as described in **(B)**, using primer GB5S4. The multiple start sites were regularly observed. Arrows are as given in **(B)**.



**Figure 5.** Biochemical Analysis of *rpoTm*.

**(A)** Immunoblot analysis. Eight micrograms of mitochondrial proteins from different steps of fractionation (see Rapp and Stern, 1992) was separated in a 10% SDS-polyacrylamide gel. Antiserum raised against the RpoTp fusion protein, described in Methods, detected 100- and 98-kD proteins present in the 0.2, 0.3, and 0.5 M KCl fractions. A 95-kD protein detected in several other fractions is probably nonspecific, because it was also detected with preimmune serum (data not shown). Mito lysate, crude mitochondrial lysate; S-100, supernatant from centrifugation of lysate; AS, ammonium sulfate cuts; FPLC FT, flow-through fraction from Resource Q chromatography of proteins from 20 to 50% AS fraction; remaining fractions are proteins eluted from the Resource Q column at the indicated KCl concentrations. Numbers at left and right indicate protein molecular masses in kilodaltons.

**(B)** RNA polymerase activity assay. Protein (3.5 μg) from different steps of fractionation was assayed for incorporation of radioactively labeled  $^{32}\text{P}$ -UTP with poly(dAT) as a template. The bar graph shows the mean of two experiments with the standard deviation indicated.

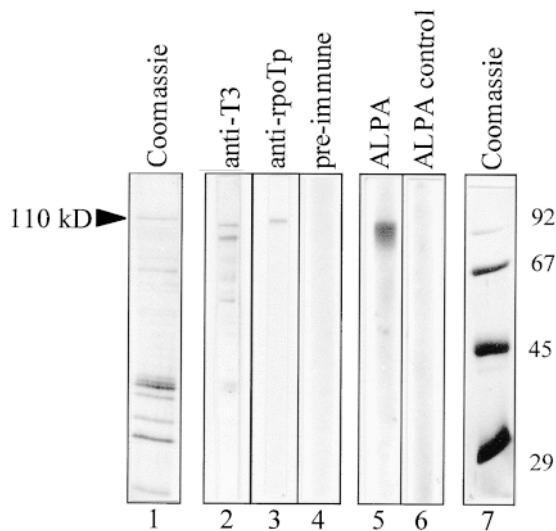
eluate, which is consistent with our earlier results and with a potential role of the 98- to 100-kD proteins as a mitochondrial RNA polymerase.

To demonstrate further that a T7-like RNA polymerase activity is present in transcriptionally active mitochondrial protein fractions, we performed an immunointerference assay. IgG was purified either from immune serum or preimmune serum and incubated with the 0.3 M KCl fraction, which has specific initiation activity, before the addition of a DNA template containing the *atpA* promoter. After in vitro transcription, the reaction products were isolated using as an internal standard a pre-labeled synthetic transcript (see Methods). The results shown in Figure 5C indicate that relative to the preimmune serum control, there was an ~50% reduction in both *atpA* promoter activity and nonspecific initiation activity (presumably from initiation at the ends of the linearized plasmid) when IgG from immune serum was used. Nearly identical results were obtained using immunodepletion analysis (data not shown). Incomplete inhibition could have a number of different causes, but the results still suggest that there is a T7-like transcription activity in the 0.3 M KCl fraction. Furthermore, the results imply that the same type of activity is responsible for both specific and nonspecific initiation. We suggest that this activity may be the product of *rpoTm*, which, as shown below, most likely encodes a mitochondrial protein.

#### Protein Detection and an Antibody-Linked RNA Polymerase Assay for *rpoTm*

To obtain evidence for functionality of an *rpoTm*-like product in chloroplast transcription, we again used the antibody raised against its C terminus. Although experiments using crude protein preparations from maize chloroplasts were unsuccessful (data not shown), immunoblot analysis shown in Figure 6, lanes 3 and 4, using a spinach chloroplast protein fraction enriched (Trifa et al., 1998) for a previously characterized T7-like polymerase activity (Lerbs-Mache, 1993), revealed a protein of the expected size (110 kD). When the same protein preparation was tested for RNA polymerase activity by using an antibody-linked polymerase

**(C)** Immunoinhibition of in vitro transcription activity. Six micrograms of IgG from the antiserum raised against the RpoTp fusion protein or from preimmune serum used as a control was incubated with the mitochondrial extract (0.3 M KCl fraction) for 1 hr on ice before being added to the transcription reaction mixture. Pre, preimmune serum; Ab, antiserum; (-), control transcription reaction. The loading control was a  $^{32}\text{P}$ -labeled synthetic RNA added in equal amounts to each reaction at its termination. It was used to control for loss of reaction products during phenol extraction and precipitation, and the indicated percentage of control transcription has been normalized to the loading control.



**Figure 6.** Immunological Characterization of the Spinach Chloroplast NEP Enzyme.

Transcriptionally active fractions were purified by heparin-Sepharose and phosphocellulose chromatography as described in Methods. Aliquots of phosphocellulose-purified RNA polymerase corresponding to 5  $\mu$ g of protein were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R 250 (lanes 1 and 7), or the proteins were transferred to nitrocellulose membranes (lanes 2 to 6). After transfer, filters were incubated with antibodies raised against T3 RNA polymerase (lane 2), maize plastid phagelike RNA polymerase (RpoTp; lanes 3, 5, and 6), or preimmune serum (lane 4). Antibodies were revealed either by horseradish peroxidase (lanes 2 to 4) or by antibody-linked polymerase assays (ALPAs; lanes 5 and 6). For ALPAs, the phosphocellulose-purified RNA polymerase preparation was used in the second binding reaction. Lane 6 corresponds to control reactions in which either DNA or nucleotides were omitted from the transcription reaction. In lane 7, molecular mass markers are given in kilodaltons.

assay (Lerbs-Mache, 1988, 1993), we found that the anti-RpoTp antibody identified a transcriptionally linked protein of the same size (Figure 6, lanes 5 and 6). Although these data do not prove unequivocally that the *rpoTp* gene specifies an NEP activity in maize chloroplasts, they do demonstrate that an antibody against its gene product recognizes chloroplast RNA polymerase activity.

#### Cellular Localization of *rpoTp* and *rpoTm*

To determine the probable subcellular localization of the *rpoTm*- and *rpoTp*-encoded polypeptides, we fused their putative N-terminal transit peptides to the *GFP* coding region, transfected the plasmids into maize mesophyll protoplasts by electroporation, and examined the transformed

protoplasts by using confocal laser scanning microscopy (see Methods). Along with the RpoTm-GFP (255 amino acids) and RpoTp-GFP (89 amino acids) fusions, we used a series of controls in which GFP targeting could be predicted.

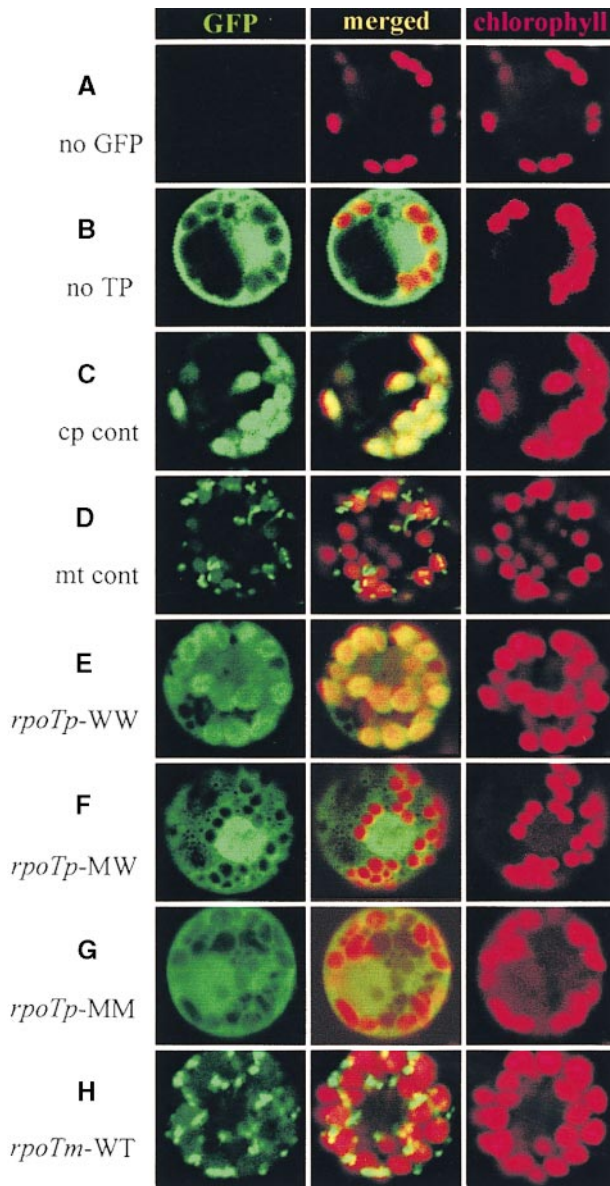
Figure 7 shows the results of these experiments, with panels at left showing the GFP (green) channel, those at right showing chlorophyll autofluorescence (red) channel, and those in the center representing a combination of the two images, with yellow indicating colocalization of the GFP and chlorophyll. In Figure 7A, results are shown for a construct lacking the GFP, and as expected, the green channel was negative. Figure 7B shows results for the GFP lacking a specific targeting sequence; in this case, nonspecific localization to the nucleus and cytosol was expected and seen. Figure 7C shows a chloroplast control in which the GFP was fused to the transit peptide of the Arabidopsis small subunit of ribulose biphosphate carboxylase (Chiu et al., 1996); as expected, the green and red channel images were coincident. As a mitochondrial control, the GFP was fused to the transit peptide of an Arabidopsis cDNA that encodes the  $\gamma$ -subunit of the mitochondrial  $F_1F_0$  ATP synthase, and Figure 7D shows a punctate GFP localization that is distinct from that of chloroplasts and typical of mitochondrial localization in plant cells (Kohler et al., 1997).

Figure 7E shows the results from the RpoTp-GFP fusion, and the results clearly indicate that the GFP is localized to chloroplasts. We conclude that *rpoTp* probably specifies a plastid T7-like RNA polymerase. Because there are two in-frame AUG codons separated by 81 bp in *rpoTp*, we mutated either the first (Figure 7F) or both (Figure 7G) to test whether the first 27 amino acids were required for chloroplast targeting. The results show that these amino acids are indeed required for correct targeting and suggest that the first AUG is used *in vivo*, because otherwise we would have expected to see dual chloroplast-cytosol/nucleus targeting for the construct WW (Figure 7E). Figure 7H shows results for the RpoTm-GFP fusion, and this chimeric protein is clearly targeted to mitochondria, suggesting that *rpoTm* encodes a mitochondrial RNA polymerase. No evidence for dual targeting was seen for either RpoTm-GFP or RpoTp-GFP fusions.

#### Modulation of *rpoTm* and *rpoTp* Transcript Accumulation in Plants

Because the energetic roles of the mitochondria and chloroplasts vary according to the plant developmental program and environmental conditions, and because synthesis of the respiratory and photosynthetic electron transport chains requires organelle transcription, we decided to investigate how *rpoTm* and *rpoTp* gene expression might vary among plant tissues and in response to illumination. To study *rpoT* expression, we measured steady state RNA accumulation, because sensitivity was too low for detection of the polypeptides by immunoblotting of total proteins (Figure 5





**Figure 7.** Subcellular Localization of the *rpoTp* and *rpoTm* Gene Products Revealed by Using GFP Fusions.

Transient expression of GFP was observed using a Bio-Rad MRC600 confocal microscope 16 to 24 hr after electroporation. At left, green channel for the GFP; at right, red channel for chlorophyll autofluorescence; at center, the merged images of those at left and right, with the yellow color indicating colocalization of the GFP and chloroplasts. The constructs are indicated at left.

(A) Negative control lacking the GFP (*adh*- $\beta$ -glucuronidase).

(B) Nucleus/cytosol control in which the GFP lacks a transit peptide (TP; HBTP5sGFPTYG).

(C) Chloroplast control (cp cont) with the GFP fused to the transit peptide of the Arabidopsis ribulose biphosphate carboxylase small subunit (35S- $\Omega$ -TP-sGFPTYG).

(D) Mitochondrial control (mt cont) with the GFP fused to the transit

and data not shown). Transcript accumulation from the *rpoT* genes was also low (Figure 4A), and we initially isolated poly(A)<sup>+</sup> RNA from different samples and analyzed RNA accumulation by using filter blots. However, because of variable yields, this method failed to yield quantitative results.

Therefore, we exploited quantitative RT-PCR to measure steady state mRNA levels under the conditions described in Methods. Briefly, this entailed showing that the amount of PCR product was directly proportional to the amount of input cDNA, using a relatively low number (25) of cycles, as shown in Figure 8A. In addition, multiple experiments were conducted for each type of sample, and every experiment was run in duplicate. In our experiments, the levels of *rpoTm* and *rpoTp* transcripts were measured relative to that of actin. Although we cannot assume that actin RNA levels themselves did not change during our experiments, this approach provides a basis for measuring the relative (rather than absolute) expression of each *rpoT* gene. However, actin is a ubiquitous protein in plant cells, and we used primers from conserved regions of maize actin genes to avoid amplifying mRNA from specific members of this gene family (Shah et al., 1983; Moniz de Sa and Drouin, 1996).

Figure 8B shows the levels of *rpoT* transcripts in different tissues normalized to those of actin. We found that *rpoTm* transcripts were more abundant than those of *rpoTp* in the nonphotosynthetic silks and roots, whereas in contrast, *rpoTp* transcripts were more abundant than those of *rpoTm* in leaves. Relative to actin, *rpoTp* transcripts were several-fold higher in leaves than in the other tissues. The results for *rpoTp* are consistent with NEP activity being required even in older photosynthetically active tissues.

During maize leaf development, cell division occurs primarily in the basal meristem, and older cells are displaced by younger cells below them. This developmental process results in a positional gradient of cell age, from less differentiated, younger cells at the base to more differentiated, older cells at the tip. Chloroplast differentiation also follows this gradient (Leech et al., 1973). Figure 8C shows the steady state levels of *rpoT* transcripts in this positional gradient. For *rpoTm*, mRNA is most abundant in the region closest to the basal meristem and reduced  $\sim 10$ -fold near the tip, which is the same pattern obtained for several mitochondrially encoded transcripts in a wheat leaf gradient (Topping and Leaver, 1990). In contrast, *rpoTp* transcripts increase gradually, with

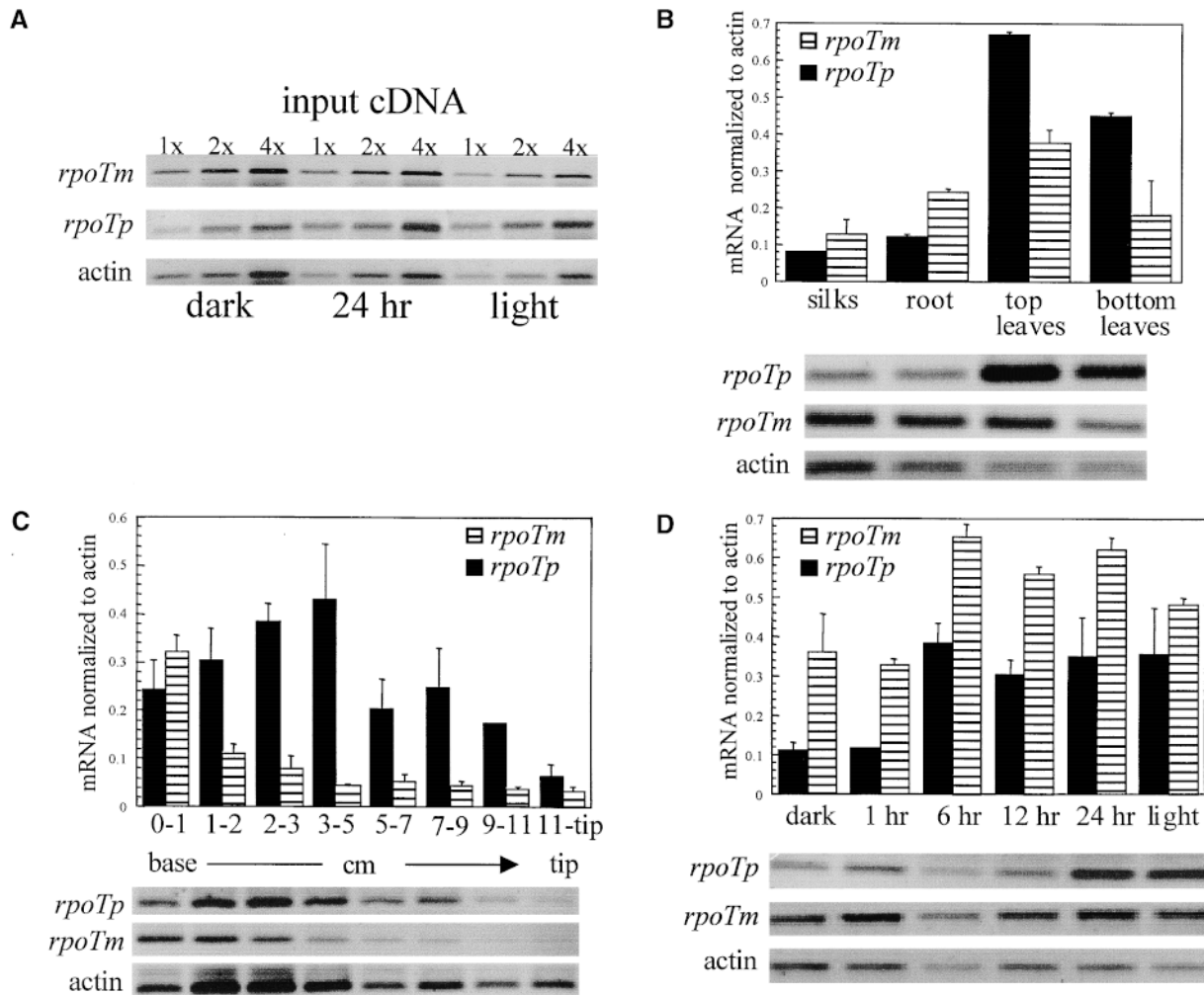
peptide from the Arabidopsis mitochondrial F<sub>0</sub>F<sub>1</sub> ATPase  $\gamma$ -subunit (35S- $\Omega$ -pre-sGFP).

(E) Wild-type *rpoTp* transit peptide fused to the GFP (*rpoTp*-WW).

(F) Mutation of the first AUG of *rpoTp* (*rpoTp*-MW).

(G) Mutation of both AUGs of *rpoTp* (*rpoTp*-MM).

(H) Wild-type *rpoTm* transit peptide fused to GFP (*rpoTm*-WT).



**Figure 8.** Measurement of *rpoTm* and *rpoTp* Transcript Accumulation by Using Quantitative RT-PCR.

(A) Control reactions for RT-PCR, which was performed as described in Methods. In this example, first-strand cDNA synthesis was conducted using total maize RNA from plants grown in continuous darkness (dark), plants exposed to 24 hr of light after growth in darkness (24 hr), or plants grown in continuous light (light). PCR was performed with the primers described in Methods, using increasing amounts of input cDNA (1×, 2×, or 4×) for each set of reactions. Although the ratios of cDNA input to each set of reactions was 1:2:4 as shown, the absolute amount of input cDNA was twofold (2×) lower for actin, which is a much more abundant mRNA. In doing this, the actin signal remained within linear range. (B) Duplicate PCR reactions were conducted for each trial, and the products were stained with ethidium bromide after gel electrophoresis and quantified by densitometry. The bar graphs represent the amounts of *rpoT* transcripts from the mean of two trials relative to actin, with the standard deviation indicated. The gels shown below the graph are ethidium bromide–stained examples of a representative experiment. (C) Measurement of *rpoT* transcripts in leaf sections, as described in Methods and in (B). (D) Measurement of *rpoT* transcripts upon illumination of dark-grown plants, as described in Methods and in (B). Light indicates plants that were grown in continuous light; dark indicates etiolated seedlings.

a peak 3 to 5 cm from the base, and then decrease near the leaf tip. Overall, the expression pattern of *rpoTm* is consistent with mitochondrial activity, whereas *rpoTp* transcription is consistent with a role of NEP early in chloroplast development. Both transcripts decrease relative to actin in the oldest cells near the leaf tip.

Plastid transcription activity increases early in chloroplast

development in monocots, such as barley (Baumgartner et al., 1989), and illumination of etiolated plants stimulates chloroplast development. To determine whether illumination has any effect on the accumulation of *rpoT* transcripts, we germinated maize seedlings in the dark for 3 days and then illuminated them for different amounts of time. Continuously dark- or light-grown plants were used as controls. The re-

sults in Figure 8D show that *rpoTp* transcripts increased approximately threefold after 6 hr of illumination and then remained stable. Transcripts of *rpoTm* increased approximately twofold during the same period. These results may reflect a general increase in cell metabolism, including division, shortly after illumination.

## DISCUSSION

### T7-like RNA Polymerase Genes in Plants

We have reported the cloning and sequencing of two maize cDNAs that could encode T7-like RNA polymerases. The protein sequence predicted from the *rpoTp* gene was identical to one reported independently (Young et al., 1998), with one amino acid difference (Val-717→Ala). This mutation may have come from infidelity in PCR-based cloning methods or may have been due to a polymorphism between the maize lines used. Our RNA gel blot analysis (Figure 4A) revealed 3.8- and 3.6-kb transcripts. They are similar in length to those of homologous genes from other plant species (Hedtke et al., 1997; Weihe et al., 1997). Interestingly, T7-like RNA polymerase genes are encoded by the mitochondrial genome in the brown alga *P. littoralis* (Rousvoal et al., 1998), whereas the primitive protist *R. americana* has retained the ancestral bacterial RNA polymerase ( $\alpha_2\beta'\beta''\sigma$ ) genes in its mitochondrial genome (Lang et al., 1997). At this point, there is no evidence for *E. coli*-like RNA polymerases in vascular plant mitochondria. However, a more thorough examination of plant mitochondrial transcription, particularly of those promoters not conforming to established consensus sequences or those that are inactive in vitro, may be informative. Instances of both nonconforming promoters (Mulligan et al., 1991) and nonfunctional promoters (e.g., *rrn18*; W.D. Rapp and D.B. Stern, unpublished data) have been found in maize. In contrast, T7-like RNA polymerases are expected for plastids, because both *E. coli*-like PEP and nucleus-encoded NEP activities are well characterized (reviewed in Gray and Lang, 1998).

The number of T7-like RNA polymerase genes in vascular plants is still an open question, albeit one that should be resolved in the next few years for at least one species, when the Arabidopsis genome sequence is completed. To date, the most information is available for Arabidopsis for which three genes have been reported, with two apparently specifying mitochondrial proteins (Hedtke et al., 1997, 1999; Weihe et al., 1997). In the case of maize, our DNA gel blot results were somewhat ambiguous (Figure 2), because some probes detected apparently single-copy genes, whereas others detected two or more fragments. An independent analysis of *rpoTp* (Young et al., 1998) suggested that a gene family was present, but this probe turned out not to be representative of our findings. Screening for more cDNAs with these multiprobe probes should address the issue, as will reverse ge-

netic analysis. If multiple gene family members exist and are functional, this could represent redundancy or specialization for promoter recognition. In the case of the canonical T7 RNA polymerase, promoter recognition is reliant on specific amino acid sequences (Rong et al., 1998).

### Sequence Comparison and Origin

Our sequence alignment revealed that amino acid identity increases toward the C terminus (Figure 3). Apart from the unique N-terminal region, there is a remarkable conservation of functional domains and important catalytic residues. Relative to the plant proteins, those of yeast (150 kD) and *Neurospora crassa* (156 kD) mitochondria are large (Masters et al., 1987; Chen et al., 1996); however, the roles of their N- and C-terminal extensions have not been fully elucidated. Deletion studies suggest that the N-terminal part of yeast Rpo41p is required for interaction with the specificity factor mtTFB (Cliften et al., 1997), whereas a role for this region, or at least Rpo41 as a whole, in DNA replication has been proposed by others (Clayton, 1991; Van Dyck and Clayton, 1998).

Structural features of T7-like RNA polymerases include domains that are also present in DNA polymerase and reverse transcriptase (Sousa et al., 1993). From an evolutionary point of view, one might imagine a mitochondrial ancestor or early endosymbiont with a bacterial type of RNA polymerase, which was later replaced by a T7-like enzyme (Rousvoal et al., 1998) for which the gene was eventually transferred to the nucleus. How the T7-like polymerase sequence was acquired will take time to resolve; however, it has been suggested that it may have originated from duplication and divergence of DNA polymerase or reverse transcriptase-encoding genes (Cermakian et al., 1997).

### Cellular Localization and Functional Analysis

Our results for intracellular targeting are based on in vivo expression of GFP fusions (Figure 7). Whereas there is little primary sequence conservation among the transit peptides of chloroplast or mitochondrially targeted proteins, they usually possess certain characteristics, such as richness in hydroxylated amino acids. Mitochondrial transit peptides usually form an  $\alpha$  helix, whereas chloroplast transit peptides usually contain a consensus cleavage site and form an amphiphilic  $\beta$  sheet in their C-terminal domain (Glaser et al., 1998; Soll and Tien, 1998). The putative transit peptide of *rpoTp* does not have clear chloroplast targeting signal (e.g., the immediate N terminus is rich in proline); neither is it predicted to sort to chloroplasts according to the commonly used computer program PSORT (<http://psort.nibb.ac.jp:8800/>). In contrast, the N terminus of *rpoTm* does possess the expected characteristics of a mitochondrial transit signal and is predicted to do so by PSORT. The undependability of PSORT emphasizes

the importance of using *in vivo* targeting experiments to verify or supplement computer predictions.

The results shown in Figure 7 do not reveal evidence for dual targeting of either chimeric protein, although this interpretation is limited by the resolution of the method. Although chloroplast NEP and mitochondrial promoter sequences appear to be sufficiently divergent to rule out a single protein functioning in both compartments, promoter specificity also might be conferred by  $\sigma$ -like factors rather than by the polymerases themselves. In addition, dual targeting of a single precursor to mitochondria and chloroplasts has been found for several proteins (reviewed in Small et al., 1998).

Immunoblots using antibodies raised against the C-terminal portion of the *rpoTp* gene product revealed 98- to 100-kD proteins in transcriptionally active protein fractions from maize mitochondria (Figure 5). This size is consistent with that predicted by the cDNA sequence, although we were limited by not knowing the mature N terminus of the protein. The two slightly different molecular masses seen could be due to a second T7-like RNA polymerase present in the mitochondria, as appears to be the case for Arabidopsis, to a post-translational modification, or to protein processing. Although there is not a complete correlation between RNA polymerase activity and the amount of immunoreactive material, we emphasize that these impure fractions may have other proteins that influence transcription either as inhibitors or activators. Alternatively, there may be additional polymerases in the more active 0.2 M and/or 0.3 M KCl fractions. In our immunoinhibition experiment, mitochondrial *in vitro* transcription activity was not completely abolished, which again could be due to a second polymerase or because the antibody, raised against the 116 amino acids from the C-terminal end of *rpoTp*, may not completely neutralize the catalytic domain. Overall, however, the results strongly support a role for a T7-like polymerase in our *in vitro* transcription system.

Our evidence for the role of *rpoTp* in chloroplast transcription was based on reactivity of a 110-kD protein in both immunoblot and antibody-linked polymerase assays, using spinach chloroplast proteins (Figure 6). The 110-kD size corresponds to that of a protein previously shown to have T7-like RNA polymerase activity (Lerbs-Mache, 1993). Whereas there is no direct biochemical evidence for NEP activity in maize chloroplasts, maize *iojap* mutants, which lack chloroplast ribosomes and therefore the potential to translate PEP, have significant residual chloroplast transcription activity (Silhavy and Maliga, 1998). The evidence for transcription of *rpoTp*, along with its *in vivo* targeting and expression pattern, makes it a strong candidate for a component of this activity.

### Transcription of *rpoTm* and *rpoTp*

Using quantitative RT-PCR, we observed that *rpoTm* and *rpoTp* were expressed in all maize tissues examined (Figure 8). In nonphotosynthetic tissues, *rpoTm* had a higher ex-

pression level, whereas in photosynthetically active tissues, *rpoTp* mRNA levels were generally higher. Although mRNAs encoding many chloroplast proteins increase in response to illumination, this was not necessarily expected for NEP, which is the presumed product of *rpoTp*. Most models invoke NEP as an enzyme that has its highest activity early in plastid development, yielding later to PEP as the principal transcription activity (reviewed in Stern et al., 1997). Thus, we had expected *rpoTp* transcription to parallel that of genes for phytochrome A and protochlorophyllide oxidoreductase, for example, whose mRNAs decrease in response to illumination (Sharrock and Quail, 1989; Reinbothe et al., 1996). The constitutive or even increased expression of *rpoTp* in photosynthetic tissues implies an important role in mature chloroplasts, over and above maintaining the supply of rRNAs and ribosomal proteins. Candidate genes dependent on *rpoTp* include *accD* and *clpP*, whose transcription appears to rely exclusively on NEP (Hajdukiewicz et al., 1997; Silhavy and Maliga, 1998) and whose activities (fatty acid metabolism and protease, respectively) would be expected to be required irrespective of developmental status.

Because the major role of mitochondria is the production of ATP by oxidative phosphorylation, plant mitochondrial function is likely to be modulated as the chloroplast develops, while the ability of plant cells to produce ATP by photophosphorylation increases. We thus expected a decrease in *rpoTm* expression in green or illuminated tissues, somewhat akin to glucose repression of *RPO41* transcription in yeast (Wilcoxon et al., 1988). Although we did find a substantial decrease in *rpoTm* transcripts along the leaf gradient (Figure 8C), in other experiments *rpoTm* transcripts did not vary greatly. This suggests that the product of *rpoTm* is required for constitutive mitochondrial functions. The 10-fold decrease in *rpoTm* transcript accumulation along the leaf gradient might be expected to result in a decrease in the accumulation of mitochondrially encoded mRNAs, much as yeast mitochondrial transcripts decline as *RPO41* expression decreases (Ulery et al., 1994).

Indeed, mitochondrial gene expression has been found to decrease in successive sections from the basal meristem to the distal tip in wheat (Topping and Leaver, 1990). Although to our knowledge this phenomenon has not been examined in maize, mitochondrial gene expression does vary at the RNA level in different maize cell types (Li et al., 1996). It will be interesting to determine whether *rpoTm* can be implicated in this transcriptional or post-transcriptional regulation.

## METHODS

### Screening of cDNA and Genomic DNA Libraries and 5' Rapid Amplification of cDNA Ends Extension of cDNA Sequences

A maize ear shoot cDNA library in the vector  $\lambda$  ZapII was a gift of B. Veit (Plant Gene Expression Center, Albany, CA) and was screened

with the rice expressed sequence tag clone R2175 by using standard procedures. A maize B73 inbred line genomic library in the vector  $\lambda$  Gem12 was obtained from D. Braun (University of Missouri, Columbia) and screened with the 5' end of each cDNA obtained using 5' rapid amplification of cDNA ends (RACE), with the probes indicated in Figure 1. Positive clones were identified by plaque hybridization, and the inserts were recovered by *in vivo* excision.

To conduct 5' RACE, we isolated total RNA from 4-day-old etiolated seedlings of Pioneer Hi-Bred (Johnston, IA) line B3733, and we obtained poly(A)<sup>+</sup> RNA using the Poly-A-Tract mRNA isolation system (Promega). Reverse transcription-polymerase chain reaction (RT-PCR)-based cloning was performed using a 5' RACE kit according to the instructions provided by the manufacturer (Life Technologies Inc., Rockville, MD). Briefly, first-strand synthesis was primed using the oligonucleotides shown in Figure 1 (GSP series for *rpoTp* and NAS series for *rpoTm*). PCR was performed first using the upstream anchor primer AAP and subsequently the amplification primer AUAP. The products were cloned and sequenced, and each part of the cDNA was sequenced multiple times from multiple clones. The sequences have GenBank accession numbers AF127021 (*rpoTm*) and AF127022 (*rpoTp*). Chromosomal mapping data can be found in the maize database (<http://www.agron.missouri.edu/query.html>) under the following locus and bin numbers: *rpoTp*, *rpo2*, and bin 9.03; and *rpoTm*, *rpo3*, and bin 7.04. The DNA sequence alignments shown in Figure 3 were conducted using the Megalign program of the DNASTar package (DNASTar, Inc., Madison, WI).

#### DNA and RNA Gel Blots

Total DNA or RNA was extracted from 4-day-old etiolated seedlings of Pioneer Hi-Bred line B3733. Twenty-five micrograms of DNA was digested with restriction endonucleases and electrophoresed in 0.75% agarose gels. Gels were transferred to nylon membranes and hybridized at 65°C (Church and Gilbert, 1984) with fragments labeled with phosphorus-32 by random priming. The blots were washed twice for 15 min at 65°C with 40 mM NaPO<sub>4</sub>, and hybridizing fragments were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Poly(A)<sup>+</sup> RNA was obtained using the Poly-A-Tract system. Fifteen micrograms of total RNA or 1  $\mu$ g of poly(A)<sup>+</sup> RNA was subjected to electrophoresis in 6% formaldehyde-0.75% agarose gels. After electrophoresis, the gels were transferred and hybridized, as was done for the DNA blots.

#### Primer Extension

Primer GB5S4 (5'-GGATTGGATTGAGGTGGAAG-3') was used to map the 5' end of *rpoTp* mRNA, and primer 97Ext2 (5'-GTAGATTGTCGCTTCGTCG-3') was used to map the 5' end of *rpoTm* mRNA. The primers were end labeled with  $\gamma$ -<sup>32</sup>P-ATP and used for reverse transcription in a 15- $\mu$ L reaction containing 2 ng of end-labeled primer, 1  $\mu$ g of poly(A)<sup>+</sup> RNA, or 10  $\mu$ g of tRNA, 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM deoxyribonucleotide triphosphates, and 100 units of Superscript II (Life Technologies, Inc.). The reactions were incubated at 42°C for 30 min, and the extended products as well as a sequence ladder generated with the same primer were analyzed in 5% denaturing polyacrylamide gels.

#### Antibody Preparation, Immunoblotting, and Immunoinhibition of *In Vitro* Transcription

The DNA encoding the C-terminal 116 amino acids from *rpoTp* (see Figure 3) was amplified by PCR and inserted into the expression vector pQE30 (Qiagen Inc., Valencia, CA), which generates a histidine-tagged fusion protein in *Escherichia coli*. This protein was overexpressed, purified using a Ni-nitrilotriacetic acid-agarose resin (Qiagen), and used to immunize rabbits. Polyclonal antisera were obtained and used directly in immunoblotting and immunoinhibition experiments.

For immunoblotting, mitochondrial proteins were fractionated as previously described (Rapp and Stern, 1992). Equal mass amounts of proteins (8  $\mu$ g) from different fractions were separated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blocking was performed in 1  $\times$  TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk. Blots were incubated with primary antibody (1 to 4000 dilution) in 1  $\times$  TBST at 4°C for 10 hr and washed three times with 1  $\times$  TBST, and reactive bands were detected using an alkaline phosphatase-conjugated secondary antibody.

The isolation of transcriptionally active mitochondrial proteins and *in vitro* transcription assays were performed as previously described (Rapp and Stern, 1992), except that a Pharmacia 1-mL Resource-Q column was used in the final chromatography step. Protein concentrations were determined by using the Bradford method (Bio-Rad). For immunoinhibition, IgG was purified from the antiserum by using protein A-agarose beads, according to standard protocols. Six micrograms of IgG from preimmune or immune serum was incubated with the transcriptionally active mitochondrial extract (0.3 M KCl fraction) for 1 hr on ice, before being added to the standard *in vitro* transcription reaction mixture.

#### RNA Polymerase Activity Assays

Mitochondrial RNA polymerase activity was measured as the incorporation of <sup>32</sup>P-UTP by using poly(dAT) as a template (Hanic-Joyce and Gray, 1991). Each reaction contained 3.5  $\mu$ g of mitochondrial protein from fast protein liquid chromatography (FPLC) fractions, 1  $\times$  MTB (10 mM Tris-HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 20 mM KCl), 0.5 mM each ATP, GTP, and CTP, 25  $\mu$ M UTP, 500  $\mu$ Ci/mL UTP, 3200 units per mL of RNasin (Promega), and 4 mg/mL poly(dAT). The reactions were incubated for 30 min at 30°C. An aliquot of each reaction was then spotted onto a GF-C filter (Whatman, Inc., Clifton, NJ), which was washed with 10% trichloroacetic acid and 0.1% sodium pyrophosphate three times under suction and twice with 70% ethanol. Incorporation of phosphorus-32 was determined by scintillation counting of the air-dried filters.

Transcriptionally active fractions of spinach chloroplast proteins were purified by heparin-Sepharose and phosphocellulose chromatography as previously described (Trifa et al., 1998). Aliquots of phosphocellulose-purified RNA polymerase corresponding to 5  $\mu$ g of protein were separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and used for immunoblotting, with antibody binding revealed by horseradish peroxidase or by using the antibody-linked polymerase assay, as previously described (Lerbs-Mache, 1988, 1993), with the phosphocellulose-purified RNA polymerase preparation used in the second binding reaction.

### Subcellular Localization

The putative N-terminal transit peptides of RpoTp (89 amino acids) or RpoTm (255 amino acids) were inserted into the BamHI site of the vector 35S-C4PPDK-sGFP(S65T), which has been previously described (Chiu et al., 1996). Briefly, this cassette is designed for high expression in maize mesophyll protoplasts and contains the cauliflower mosaic virus 35S promoter, a translational enhancing region from the maize pyruvate orthophosphate dikinase gene, and an enhanced fluorescence version of the green fluorescent protein (*GFP*) gene. Controls for intracellular targeting included a mitochondrial construct carrying the N-terminal 78 amino acids of the  $\gamma$ -subunit of the Arabidopsis  $F_1F_0$  ATPase inserted into the Sall and NcoI sites of 35S- $\Omega$ -sGFP(s65T) (Chiu et al., 1996), a nucleus/cytosol construct lacking a specific targeting sequence (HBTP5sGFPTYG), a chloroplast control carrying the ribulose biphosphate carboxylase small subunit transit peptide (35S- $\Omega$ -TP-sGFPTYG), and a negative control lacking the GFP (*adh*- $\beta$ -glucuronidase) (Sheen, 1990; Sheen et al., 1995; Chiu et al., 1996). Protoplast isolation and electroporation were performed as described previously (Sheen, 1990). Fifty micrograms of each construct was electroporated into freshly isolated maize mesophyll protoplasts, and expression of the GFP was monitored using a Bio-Rad MRC600 confocal microscope after 16 to 24 hr.

### Measurement of Steady State Transcript Level by Using Quantitative RT-PCR

For determination of *rpoT* transcript levels in different maize tissues, Pioneer Hi-Bred line B3733 plants were grown in a greenhouse for 60 days, and silks, roots, top leaves (the thirteenth leaf), and bottom leaves (the fourth leaf) were harvested. For measurement of transcripts along the leaf gradient, plants were grown in a greenhouse for 10 days. At this time, the third leaf to emerge from the coleoptile was ~15 cm long (Martineau and Taylor, 1985). The third leaf was cut into segments, as indicated in Figure 8C. For measurement of transcript levels upon illumination, seedlings were grown for 3 days in the dark and exposed to 1, 6, 12, or 24 hr of light or grown for 4 days in the light.

Total RNA was treated with DNase I (PCR controls showed that DNA had indeed been removed), and first-strand cDNA synthesis was performed as follows. Total RNA (2.5  $\mu$ g) was incubated with 500 ng of oligo(dT)<sub>12-18</sub> at 70°C for 10 min and chilled on ice, the reaction buffer (final concentration of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, and 10 mM DTT) was added, and incubation was continued at 42°C for 2 min, with 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) then being added. After incubation at 42°C for 50 min, the reaction was heated to 70°C for 15 min, and the total volume was brought to 200  $\mu$ L. Aliquots (5  $\mu$ L) of cDNA were PCR amplified in a range determined to be linear by using dilutions of cDNA and quantifying the PCR products by using densitometry; densitometry was performed by obtaining images of ethidium bromide-stained gels with EagleSight software (Stratagene Inc., La Jolla, CA) and analyzing them with ImageQuant software (Molecular Dynamics).

The primers used as markers for total poly(A)<sup>+</sup> RNA quantity were from conserved regions of maize actin genes. Primers ZmACa (5'-GGCAGCTCGTAGCTCTTCTC-3') and ZmACb (5'-AACAGGGAG-AAGATGACCCA-3') have no mismatches with genes *maz56*, *maz63*, *maz81*, *maz83*, and *maz89* and have a single or two internal mis-

matches in one of the two primers for genes *maz65* and *maz95*, respectively. There are three mismatches in ZmACb for *maz87* and several insertions or deletions for both primers relative to *MAC1*. PCR conditions for a 50- $\mu$ L reaction were 2.5  $\mu$ M of primers, 1.5 mM MgCl<sub>2</sub>, 1  $\times$  PCR buffer (Promega), and 1 unit of Taq DNA polymerase (Promega). PCR cycles were at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min for 25 cycles and then 72°C for 5 min. For *rpoTp* and *rpoTm*, 10- $\mu$ L aliquots of cDNA were used, with the upstream primer (rpZmB, 5'-CTGGCTTGGTACTGTGC-3') being in a sequence identical to the two genes, and the other primers (GSPC1, 5'-CTCACCATGTTTCATGTGCAG-3'; and 97cDNA, 5'-CAGCAGCGTGTATAATGCAACTG-3' for *rpoTp* and *rpoTm*, respectively), being gene specific. PCR conditions were identical to those used for actin; however, to remain in linear range, the amount of input cDNA was always twofold less for actin.

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