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Maize chloroplast RNA polymerase: The 180-, 120-, and 38-kilodalton polypeptides are encoded in chloroplast genes

(plastid genes/*rpoA*/*rpoB*/*rpoC2*)

JIM HU AND LAWRENCE BOGORAD*

Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

Contributed by Lawrence Bogorad, November 27, 1989

ABSTRACT **Prominent polypeptides with apparent molecular masses of 180, 120, 85, and 38 kDa are found in an extensively purified preparation of maize chloroplast DNA-dependent RNA polymerase that retains the capacity to initiate transcription of the cloned chloroplast gene *rbcL* correctly and the requirement for a supercoiled DNA template for specific and active transcription. Amino-terminal amino acid sequences of the 180-, 120-, and 38-kDa polypeptides have been determined and found to correspond precisely to the sequences deduced from the 5′ ends of the maize chloroplast *rpoC2*, *rpoB*, and *rpoA* genes, respectively. These experiments show that these chloroplast *rpo* genes encode the prominent polypeptides in the highly enriched maize chloroplast RNA polymerase preparation and support the conclusion that these polypeptides are functional components of the enzyme. The *rpoB*, *rpoC1*, and *rpoC2* genes have been mapped on the maize chloroplast chromosome.**

Chloroplasts of algae and higher plants contain DNA coding for their own tRNAs, rRNAs, and mRNAs whose translation products constitute perhaps one-third to one-fifth of plastid proteins (1). Although the transcriptional and translational systems of chloroplasts are, in some respects, similar to those of bacteria (2), not much is known about the mechanism of regulation of chloroplast gene expression. It appears to be controlled at many levels, such as DNA copy number, DNA topology, transcription, posttranscription, translation, and posttranslation (3), and seems to vary from gene to gene and from species to species. Understanding the transcriptional apparatus of chloroplasts is crucial to understanding how chloroplast genes are expressed.

Chloroplast RNA polymerases have been partially purified from a few species (4–8). The structure of the chloroplast RNA polymerase(s) is still unknown, but preparations from maize chloroplasts enriched in polypeptides of apparent molecular masses of 180, 140, 100, 95, 85, and 40 kDa are active in RNA synthesis (4), and polypeptides of similar sizes (taking into account the use of different gel systems and the markers) have been found in RNA polymerase preparations from pea (6) and spinach chloroplasts (9).

The chloroplast chromosomes of several species (10–15) have been shown to contain genes encoding proteins with segments about 26–50% homologous to the *Escherichia coli* RNA polymerase subunits. One objective of this research was to determine whether chloroplast *rpo* genes encode polypeptides in the polymerase preparation as a means of establishing whether they resemble bona fide polymerase subunits.

Recently, we separated and identified two types of RNA polymerase activities (16) in maize chloroplast extracts by chromatography on a DEAE anion-exchange column. One activity, designated PF (the DEAE peak activity fraction), is the RNA polymerase activity that had been partially purified earlier and used for *in vitro* studies (5, 17–19). It actively transcribes supercoiled but not relaxed cloned chloroplast DNA templates (17, 19) and does not bind to linear maize chloroplast promoter sequences (16). The other type of activity is found in the DEAE fraction designated BF (the DNA-binding fraction); proteins in this fraction have been shown to bind to the maize chloroplast *psbA* promoter specifically (16) and to transcribe relaxed templates as well as supercoiled ones. The present work concerns the PF enzyme.

In this paper, we show that the products of the *rpoC2*, *rpoB*, and *rpoA* (15) genes† on the maize chloroplast chromosome are the 180-, 120-, and 38-kDa polypeptides found in highly enriched maize chloroplast PF RNA polymerase. The presence of these polypeptides has been shown to correlate well with the RNA polymerase activity at different stages of purification. This shows that the chloroplast *rpo* genes are functional and thus provides strong evidence that these polypeptides are functional components of the RNA polymerase.

MATERIALS AND METHODS

Preparation of Maize Chloroplast RNA Polymerase PF. The procedure for purifying maize chloroplast PF (i.e., DEAE peak fraction) RNA polymerase was modified from that described earlier (17). Maize chloroplasts were purified by sucrose density gradient centrifugation, and RNA polymerase was extracted with 0.5 M KCl as described by Stirdivant *et al.* (17). Frozen purified chloroplasts from 4 kg of *Zea mays* (FR9cms × FR37; Illinois Foundation Seeds, Champaign, IL) leaves were used for each RNA polymerase preparation. The 0.5 M KCl extract was diluted 4-fold with DEAE column buffer [40 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol, phenylmethylsulfonyl fluoride at 25 μg/ml, and 10% (vol/vol) glycerol] and treated with micrococcal nuclease (10 units/ml) at room temperature for 10 min. Then one-fourth volume of 50% (wt/vol) PEG 8000 [in 40 mM Tris·HCl (pH 8.0), 200 mM KCl, and phenylmethylsulfonyl fluoride at 25 μg/ml] was added to precipitate the enzyme, and the mixture was shaken gently at 4°C for 1 hr.

The PEG step offers several advantages: it eliminates at least 60% of the unwanted proteins as well as leftover membrane debris; moreover, it serves as a convenient break point for the long operation because the enzyme can be stored in 10% PEG at 0°C for a few days without losing activity.

After centrifugation at 12,000 × *g* for 15 min at 4°C, the supernatant was discarded, and the pellet was dissolved in 5 ml of DEAE column buffer containing 100 mM KCl. The enzyme solution was centrifuged again at 12,000 × *g* for 10

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Abbreviation: PF, peak activity fraction from the DEAE column.

*To whom reprint requests should be addressed.

†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M31206–M31208).

min at 4°C. The supernatant was applied directly, at 0.5 ml/min, to a Protein-Pak glass DEAE-5PW anion-exchange column (8.0 mm × 7.5 cm; Nihon Waters, Tokyo) previously equilibrated with the DEAE column buffer containing 100 mM KCl on a Waters 650 advanced protein purification system (16). The pellet from the previous centrifugation was washed twice more, and the washes were loaded onto the same column in succession. The column was washed first with the DEAE column buffer containing 100 mM KCl and 0.1% Triton X-100 (Sigma) for 10 min at 0.5 ml/min, then with the same buffer without Triton X-100 for 20 min at the same pumping rate, and finally with the DEAE column buffer containing 180 mM KCl for 30 min. The enzyme was eluted with a linear 0.1–0.5 M KCl gradient as described (16). The fractions with highest RNA polymerase activity were pooled, concentrated by centrifugation in a Centricon-10 (Amicon), and applied to a 10–30% glycerol gradient (5, 20) containing 0.5 M KCl, 40 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol, and phenylmethylsulfonyl fluoride at 25 μg/ml. The gradient was centrifuged at 34,000 rpm in a Beckman SW40Ti rotor for 18 hr at 4°C. The fractions with the bulk of the RNA polymerase activity were pooled and applied slowly to a 1-ml PEI-cellulose column (21) equilibrated with the DEAE column buffer containing 0.5 M KCl. The column was washed with 0.88 M KCl in the same buffer, and the enzyme was eluted with 1.8 M KCl in the above buffer.

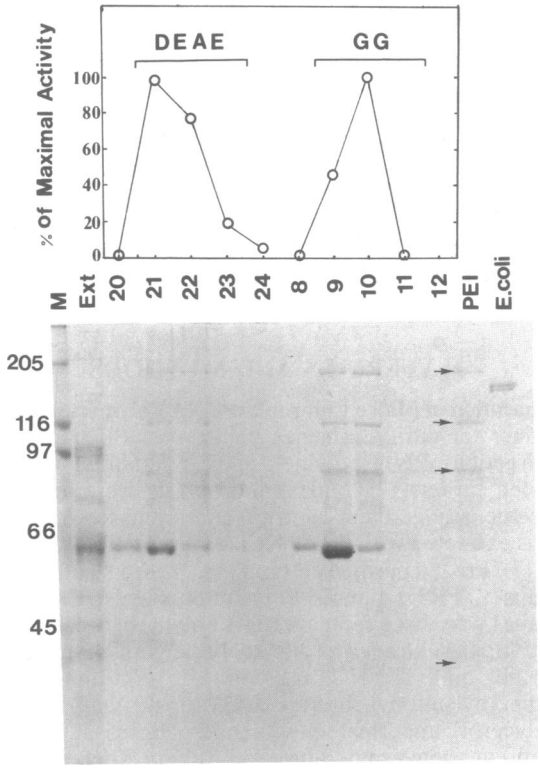

FIG. 1. Polypeptide composition and the transcription activity of fractions of maize chloroplast extracts after DEAE chromatography and subsequent glycerol density gradient centrifugation. A PEG fractionation step preceded DEAE chromatography. (*Upper*) RNA polymerase activity, assayed with denatured calf thymus DNA template, of peak fractions eluted from the DEAE column with 0.1–0.5 M KCl (left peak) and of fractions recovered after glycerol gradient centrifugation of pooled DEAE fractions (right peak). (*Lower*) Maize chloroplast RNA polymerase PF was partially purified, and the proteins from different purification steps were resolved by electrophoresis on a SDS/polyacrylamide gel. M, protein molecular mass markers (205, 116, 97, 66, and 45 kDa); Ext, chloroplast extract; DEAE, peak fractions 20, 21, 22, 23, and 24 of maize chloroplast RNA polymerase activity eluted from the DEAE column; GG, peak fractions 8, 9, 10, 11, and 12 of the RNA polymerase activity recovered from the glycerol gradient; PEI, RNA polymerase activity eluted from the PEI column; E. coli, purified *E. coli* RNA polymerase. The bands containing the 180-, 120-, 85-, and 38-kDa polypeptides are indicated by arrows in the column designated PEI.

There was no detectable loss of enzyme activity after extraction of the enzyme from chloroplasts through the DEAE chromatography step. However, more than half of the activity was lost during glycerol gradient centrifugation, and <20% of the total activity was recovered after the PEI-cellulose chromatography. The enzyme has no topoisomerase or nuclease contaminants after the glycerol gradient centrifugation (data not shown) and therefore is pure enough for many kinds of studies.

Proteins in fractions at different purification stages were precipitated with 8% (vol/vol) trichloroacetic acid, dissolved in a protein gel loading buffer, and separated on a SDS/polyacrylamide gel (22).

RNA Polymerase Assays. To monitor purification, RNA polymerase activity was assayed as described (4, 5), except that the reactions were carried out with 10 μCi (1 Ci = 37 GBq) of [^{3}H]UTP, instead of [^{14}C]ATP, in a 100-μl volume for 10 min.

The S1 nuclease assay of transcription products labeled with ^{32}P *in vitro* (16, 17, 23) was used to assess the correct initiation of transcription from the maize *rbcL* promoter by the RNA polymerase. The template was pZmc460 (17), which consists of the maize chloroplast *Bam*HI fragment 9 cloned in pBR322. The DNA probe was a recombinant M13 phage containing a *Sma* I–*Ava* I fragment from pZmc460 (17). The supercoiled template was isolated from *E. coli* HB101, and

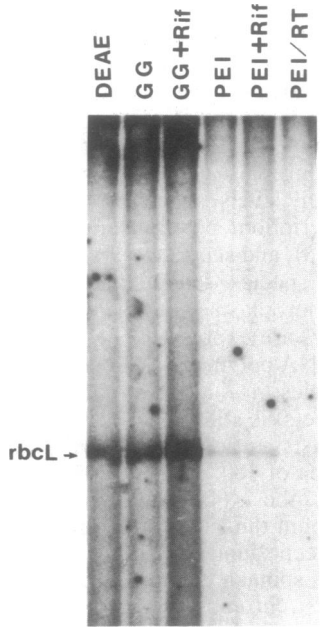

FIG. 2. Autoradiogram showing the transcription of the maize *rbcL* gene (cloned in pZmc460) by maize chloroplast PF RNA polymerase at different stages of purification. In the first five lanes, transcriptions were performed with supercoiled DNA templates, whereas in the last lane a relaxed DNA template was used. The position of the S1 nuclease-resistant hybrid of ^{32}P-labeled RNA synthesized *in vitro* and the single-stranded DNA probe is labeled *rbcL*. DEAE, transcription by RNA polymerase in the DEAE peak fraction; GG, transcription by RNA polymerase in the glycerol gradient peak fractions; PEI, transcription by RNA polymerase eluted from the PEI column; PEI + Rif, transcription by RNA polymerase eluted from the PEI column in the presence of rifampicin at 20 μg/ml; PEI/RT, transcription from the relaxed DNA template by RNA polymerase eluted from the PEI column.

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M:        ATG GCG GAA CGG GCC AAC CTG GTC TTC CAT AAT AAA GAG ATA GAT GGA
          Met Ala Glu Arg Ala Asn Leu Val Phe His Asn Lys Glu Ile Asp Gly
   ------------------------------------------------------------------------
R:          :   :   :   :   :   :   :   :   : Gln :   :   :   :   :   :
T:(M E V L) :   :   :   :   :   :   :   :   :   :   :   : Ala : Asn :
S:(M E V L) :   :   :   :   :   :   :   :   :   :   :   : Ala :   :   :
L:(M+16aa)  :   :   : Phe Val :   :   : Ile : Tyr :   :   : Val Met :   : Arg
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FIG. 3. DNA sequence of the 5′ end of the coding region of the maize chloroplast *rpoC2* gene and its deduced amino acid sequence. The amino-terminal sequence of the 180-kDa polypeptide (underlined) was determined by microprotein sequencing and is shown to match precisely the amino-terminal sequence deduced from the maize chloroplast *rpoC2* gene. The amino-terminal amino acid sequences deduced from the *rpoC2* genes of rice, tobacco, spinach, and liverwort are aligned under the maize sequence. M, maize; R, rice; T, tobacco; S, spinach; L, liverwort. The letters in parentheses represent single-letter designations of amino acids not present in maize.

the relaxed template was generated by treating the supercoiled pZmc460 with calf thymus topoisomerase I (17).

Mapping and Partial Sequencing of the Maize Chloroplast *rpoB*, *rpoC1*, and *rpoC2* Genes. *Bam*HI fragment 12 of the maize chloroplast DNA was isolated from Charon 1AZmcRI12 (24) and cloned in pUC18. A *Eco*RI–*Bam*HI fragment, which contains the 5′ end of the *rpoB* coding region, was isolated from the *Bam*HI fragment 12 sequence and subcloned into pUC18. *Bam*HI fragment 15 was cloned from pZmc539 into pUC18, and *Bam*HI fragment 25 (24) is harbored by pZmc573. DNA sequencing was done by the dideoxynucleotide chain termination method (25).

RESULTS

RNA Polymerase Activity Is Correlated with the Presence of Four Polypeptides. Fig. 1 *Lower* shows the proteins found in DEAE and glycerol gradient peak fractions, as resolved on a 10% polyacrylamide gel and stained with Coomassie blue. Transcriptional activity of the fractions (Fig. 1 *Upper*) is correlated with the presence of at least four polypeptides with apparent molecular masses of ≈180, 120, 85, and 38 kDa but not with the presence of the densely stained 55-kDa band (probably the ribulose biphosphate carboxylase large subunit). Therefore, at least these four polypeptides appeared likely to be subunits of the maize chloroplast RNA polymerase complex. Additional polypeptides were seen when the gel shown in Fig. 1 *Lower* was stained with silver (data not shown).

The PEI-Purified RNA Polymerase Retains the Ability to Initiate Correctly from a Negatively Supercoiled Template. Maize chloroplast RNA polymerase partially purified by DEAE and Sephacryl S-300 chromatography initiates transcription of the maize *rbcL* gene from a supercoiled template at the same position as occurs *in vivo* (23). Fig. 2 shows that both the capacity for specific initiation and the requirement for negatively supercoiled templates are retained through PEI purification. The enzyme is also insensitive to rifampicin (Sigma) up to at least 20 μg/ml.

Determination of Amino-Terminal Sequences of the 180-, 120-, and 38-kDa Polypeptides. After DEAE chromatography, proteins in the peak activity fractions were precipitated with 8% trichloroacetic acid and separated on a 10% polyacrylamide gel (22). After the proteins were blotted to a polyvinylidene difluoride membrane (26), bands containing the 180-, 120-, and 38-kDa proteins were excised for amino-terminal protein sequencing by the Harvard Biological Laboratories Microchemistry Facility. Excised bands to be sequenced were placed directly in the reaction cartridge of a model 470A gas-phase protein sequencer (Applied Biosystems) and subjected to automated Edman degradation by using the standard program 03RPTH. The resultant phenylthiohydantoin amino acids were analyzed on-line with an Applied Biosystems model 120A HPLC system. The sequences of 15 (Fig. 3), 11 (Fig. 4), and 16 (Fig. 5) amino acid residues were obtained for the 180-, 120-, and 38-kDa polypeptides, respectively.

The *rpoC2* Gene Encodes the 180-kDa Subunit of Maize Chloroplast RNA Polymerase. We have partially sequenced the maize chloroplast *rpoC2* gene. Fig. 3 shows that the sequence of amino acid residues 2–16 of the 180-kDa polypeptide of the chloroplast RNA polymerase, as revealed by protein sequencing, matches precisely the amino acid sequence deduced from the *rpoC2* gene. The first amino acid was not present in the mature protein. The closest relative of maize in which the *rpoC2* gene has been sequenced is rice (14). The rice *rpoC2* gene could encode a 173-kDa polypeptide. The first 16 amino acids encoded by chloroplast *rpoC2* genes are highly conserved among maize, rice (14), tobacco (10), and spinach (11), but the liverwort gene (12) encodes a somewhat divergent sequence.

Two ATG codons are found within the first five codons of the *rpoC2* genes of tobacco and spinach. However, sequences corresponding to the first four codons of the *rpoC2* genes of tobacco and spinach are absent from the genes of both maize and rice. This supports the proposition that the first ATG may not serve as the start codon for the spinach *rpoC2* gene (11).

The *rpoB* Gene Encodes the 120-kDa Subunit of Maize Chloroplast RNA Polymerase. Fig. 4 shows the DNA sequence of the 5′ end of the maize *rpoB* gene and the amino-terminal amino acid sequences deduced from DNA sequences of *rpoB* genes of maize, rice (14), tobacco (10), and spinach (11). The sequence of the first 11 amino acids deduced from the maize chloroplast *rpoB* gene sequence matches precisely the amino-terminal sequence of the 120-kDa chloroplast RNA polymerase subunit as determined by protein sequencing.

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M. ATG CTC CGG AAT GGA AAT GAG GGA ATG TCC ACA ATA CCC GGA TTT AGT CAG ATC
   Met Leu Arg Asn Gly Asn Glu Gly Met Ser Thr Ile Pro Gly Phe Ser Gln Ile
   ------------------------------------------------------------------------
R. :   :   :   :   :   :   :   :   :   :   :   :   :   :   :   :   :   :
T. :   : Gly Asp  :   :   :   : Ile :   :   :   :   :   : Asn :   :
S. :   :   : Asp  :   :   :   :   :   :   :   :   :   : Asn :   :
L. :  Glu Ile Phe Ile Leu  -   -   -   -   -   -   : Glu : Gly Lys :
```

FIG. 4. DNA sequence of the 5′ end of the maize chloroplast *rpoB* gene and its deduced amino acid sequence. The sequence of the first 11 amino acids of the 120-kDa polypeptide (underlined) was determined by microprotein sequencing and is shown to match precisely the amino-terminal sequence deduced from the *rpoB* gene. The amino-terminal amino acid sequences deduced from the *rpoB* genes of rice, tobacco, spinach, and liverwort are aligned under the maize sequence. The location of each of the six residues absent from the liverwort sequence is marked with a −. The abbreviations are as given in Fig. 3.

```
M: ATG GTT CGA GAA GAG ATA ACA GGA TCC ACT CAA ACA CTA GAG TGG AAG TGT GTT
   Met Val Arg Glu Glu Ile Thr Gly Ser Thr Gln Thr Leu Glu Trp Lys Cys Val
   ------------------------------------------------------------------------
R:  :   :   :   :   :  Val Ala  :   :   :   :   :   :  Gln  :   :   :   :
W:  :   :   :   :  Val Val Ala  :   :   :   :   :   :  Gln  :   :   :   :
T:  :   :   :   :  Lys Val  :  Val  :   :  Arg  :   :  Gln  :   :   :   :
S:  :   :   :   :  Lys  :  Arg Val  :   :   :   :   :  Gln  :   :   :   :
P:  :  Ile  :   :  Lys Leu Lys Val  :   :   :   :   :  Gln  :   :   :   :
L:  :  Ile Gln Asp  :   :  Lys Val  :   :   :   :   :  Gln  :   :   :  Ile
```

FIG. 5. DNA sequence of the 5′ end of the maize chloroplast *rpoA* gene (15) and its deduced amino acid sequence. The sequence of the first 16 amino acids of the 38-kDa polypeptide (underlined) was determined by microprotein sequencing and is shown to match precisely the amino-terminal sequence deduced from the *rpoA* gene. The amino-terminal amino acid sequences deduced from the *rpoA* genes of rice, wheat, tobacco, spinach, pea, and liverwort are aligned under the maize sequence. The abbreviations are as given in Fig. 3.

The maize *rpoB* gene is highly homologous to *rpoB* genes of rice (14), tobacco (10), spinach (11), and liverwort (12). On the basis of the size of the maize chloroplast *rpoB* gene (about 3.2 kilobase pairs) and the complete sequences of the *rpoB* genes of rice (14), tobacco (10), and spinach (11), the predicted molecular mass of the maize *rpoB* gene product should be 120 kDa. This corresponds well to the migration of the polypeptide in the SDS/polyacrylamide gel.

The *rpoA* Gene Encodes the 38-kDa Subunit of Maize Chloroplast RNA Polymerase. The maize *rpoA* gene has been sequenced by Ruf and Kössel (15). Fig. 5 shows that the amino-terminal sequence of the 38-kDa maize chloroplast polypeptide revealed by protein sequencing matches precisely the sequence deduced from the maize chloroplast *rpoA* gene. The *rpoA* genes of maize, rice (14), wheat (27), tobacco (10), spinach (11), pea (27), and liverwort (12) are conserved.

The *rpoB*, *rpoC1*, and *rpoC2* Genes Are Located on the Maize Chloroplast Genome Between the *trnC* and *rps2* Genes. A comparison of the organization of genes on the maize chloroplast chromosome with that of rice (14), spinach (11), tobacco (10), and liverwort (12) leads to the expectation that the maize chloroplast *rpoB/rpoC1/rpoC2* genes would map near coordinates 80–90 on the maize chloroplast DNA map (24). This has been confirmed by subcloning and partially sequencing this region. Fig. 6 shows a restriction map of the region and the location of segments that we have sequenced. The region between the *rpoB* and *rpoC1* is 39 base pairs.

DISCUSSION

Four genes found on the chloroplast chromosomes of liverwort (12), tobacco (10), rice (14), and spinach (11, 13) are homologous to the *E. coli* genes *rpoA*, *rpoB*, and *rpoC* and are predicted to encode proteins homologous to the *E. coli* RNA polymerase subunits α, β, and β'. As in liverwort (12), tobacco (10), rice (14), and spinach (11, 13), the maize chloroplast *rpoA* gene is located between the genes *rps11* and *petD* (24); the *rpoB/rpoC1/rpoC2* genes reside between the genes *trnC* (28) and *rps2* (Fig. 6; D. Stahl, S. R. Rodermel, A. Subramanian, and L.B., unpublished results). Although these chloroplast *rpo* genes have been sequenced from several species (10–13, 15, 29), it has been difficult to identify the gene products. Antibodies against fusion proteins of *E. coli* *trpE* and spinach *rpoA* as well as tobacco *rpoB* and *Euglena* *rpoC* have been reported to immobilize RNA polymerases from chloroplasts of spinach, pea, *Euglena*, and *E. coli* (30), but the antibodies could not be used for immunoblotting. Consequently, antibodies against a particular fusion protein could not be used to identify a specific polypeptide. However, it has been reported that an antibody against a pea chloroplast *rpoA–lacZ* fusion protein recognizes a 43-kDa polypeptide in transcriptionally active pea chloroplast preparations (27). Antisera have also been raised against the synthetic peptides corresponding to deduced amino- and carboxyl-terminal sequences from the maize *rpoA* gene (15). Although the antiserum against the amino-terminal sequence failed to recognize a polypeptide of the expected size despite the presence of amino-terminal sequence (Fig. 5), the antiserum against the carboxyl-terminal sequence did recognize a polypeptide of the predicted size in a maize chloroplast extract.

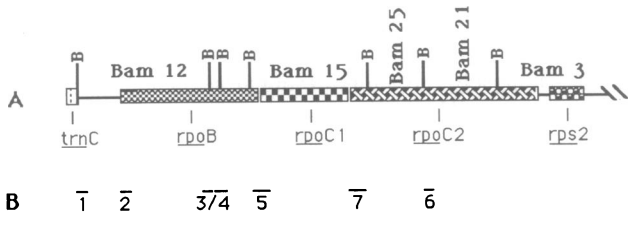

FIG. 6. Mapping of the maize chloroplast RNA polymerase genes. (*A*) A restriction map of the *rpoB/rpoC1/rpoC2* region. The *Bam*HI restriction sites are indicated by a B. (*B*) The segments that have been sequenced and used for constructing this map. Segment 1 is in the *trnC* and *rpoB* intergenic region; segment 2 contains the 5′ end of the *rpoB* coding region; segments 3 and 4 are within the *rpoB* coding region; segment 5 covers the 3′ end of the *rpoB* coding region through the 5′ end of the *rpoC1* gene; segment 6 is in the coding region of the *rpoC2* gene; and segment 7 contains the 5′ end of the *rpoC2* coding region.

We have approached this problem differently. First we enriched the maize plastid RNA polymerase activity and showed that the presence of four polypeptides, with sizes around 180, 120, 85, and 38 kDa in the preparation, is correlated quite well with the DNA-dependent RNA polymerase activity of maize chloroplasts (Fig. 1). Then we determined amino-terminal sequences of the 180-, 120-, and 38-kDa polypeptides recovered from a SDS/polyacrylamide gel and showed that they match the amino acid sequences deduced from the maize chloroplast *rpoC2*, *rpoB*, and *rpoA* gene sequences. On one hand, these data demonstrate that the maize *rpoC2* and *rpoB* genes are functional and encode plastid proteins and also support the previous reports (17, 27) that the *rpoA* gene is expressed. On the other hand, these observations bolster the conclusions drawn from our purification experiments that the 180-, 120-, and 38-kDa polypeptides are components of the maize chloroplast PF RNA polymerase and not contaminants that copurify with it.

The chloroplast genes *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* were so named (10, 12) because they are partially homologous to the genes for the α, β, and β' subunits of *E. coli* RNA polymerase. By extension, the 38- and 120-kDa polypeptides of maize chloroplast RNA polymerase PF are appropriately designated the α and β subunits of this enzyme. Ohyama *et al.* (12) noted that amino acid sequences in the amino-terminal and carboxyl-terminal portions of the *E. coli* RNA polymerase β' subunit resemble those deduced from liverwort chloroplast *rpoC1* and *rpoC2*, respectively. Hudson *et al.* (11) have suggested that the products of C1 and C2 be designated β' and β'' subunits, respectively. Therefore, the 180-kDa maize polypeptide is designated the β'' subunit.

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