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In Vitro Synthesis and Processing of a Maize Chloroplast Transcript Encoded by the Ribulose 1,5-Bisphosphate Carboxylase Large Subunit Gene

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The large subunit gene (*rbcL*) of ribulose 1,5-bisphosphate carboxylase was transcribed in vitro by using maize and pea chloroplast extracts and a cloned plastid DNA template containing 172 base pairs (bp) of the maize *rbcL* protein-coding region and 791 bp of upstream sequences. Three major in vitro RNA species were synthesized which correspond to in vivo maize *rbcL* RNAs with 5′ termini positioned 300, 100 to 105, and 63 nucleotides upstream of the protein-coding region. A deletion of 109 bp, including the "−300" 5′ end (the 5′ end at position −300), depressed all *rbcL* transcription in vitro. A plasmid DNA containing this 109-bp fragment was sufficient to direct correct transcription initiation in vitro. A cloned template, containing 191 bp of plastid DNA which includes the −105 and −63 *rbcL* termini, did not support transcription in vitro. Exogenously added −300 RNA could be converted to the −63 transcript by maize chloroplast extract. These results established that the −300 RNA is the primary maize *rbcL* transcript, the −63 RNA is a processed form of the −300 transcript, and synthesis of the −105 RNA is dependent on the −300 region. The promoter for the maize *rbcL* gene is located within the 109 bp flanking the −300 site. Mutagenesis of the 109-bp chloroplast sequence 11 bp upstream of the −300 transcription initiation site reduced *rbcL* promoter activity in vitro.

Ribulose 1,5-bisphosphate carboxylase (RUBISCO) catalyzes CO_2 fixation, the first step of the Calvin cycle (40). In higher plants, the chloroplast enzyme is composed of eight identical, catalytic polypeptides of 50,000 to 60,000 kilodaltons and eight smaller polypeptides of 12,000 to 20,000 kilodaltons (2). The small subunit, whose function is unknown, is encoded in the nucleus by a small multigene family (7, 14, 19). The large subunit gene (*rbcL*) is present as a single copy on the multicopy chloroplast genome (5). Under most conditions, the synthesis of the two RUBISCO subunits is coordinated (55) and regulated by light (15, 50) and cytokinins (23). In maize and other C_4 plants, RUBISCO expression is leaf cell-type specific (30). The maize mRNAs encoding the two subunits are present in bundle sheath cells but not in mesophyll cells, which assimilate CO_2 via ribulose 1,5-bisphosphate and C_4 dicarboxylic acids, respectively (11, 35).

In chloroplast genomes of higher plants, the genes for the large subunit of RUBISCO and the beta subunit of ATP synthetase (*atpB*) are adjacent and transcribed divergently (31, 60). The 5′ ends of the two genes are separated by approximately 150 base pairs (bp) (52). Northern hybridization analyses of transcripts from a broad spectrum of angiosperms indicate that the *rbcL* gene is generally transcribed as a 1.6-kilobase mRNA, although larger transcripts are also observed (46). S1 nuclease protection experiments have revealed the presence of two 5′ termini for the *rbcL* RNAs of maize, barley, spinach, and peas (16, 21, 41, 49, 62.) Tobacco has a single *rbcL* RNA with its 5′ end at position −180 relative to the coding region (53). In maize and barley, the *rbcL* 5′ termini are located approximately 300 and 65 bp upstream of the coding region, whereas in spinach and peas, they are at positions −180 and −65. The discrepancy in size between the "−300" and "−180" transcripts (the transcripts with 5′ ends corresponding to positions −300 and −180) is due to a 130- to 140-bp insertion in the 5′ leader region of the *rbcL* genes of monocots relative to dicots (21, 49). In spinach, peas, and maize, the larger of the two *rbcL* transcripts can be radioactively labeled with [α-^{32}P]GTP and guanylyltransferase (16, 41). The chloroplast DNA sequences surrounding the −300 and −180 positions in monocots and dicots, respectively, are highly conserved and contain −10 and −35 regions which resemble procaryotic promoter consensus sequences (41). *Escherichia coli* RNA polymerase may recognize these conserved sequences in chloroplast DNA because it correctly initiates transcription of maize and spinach *rbcL* genes in vivo and in vitro (L. Hanley-Bowdoin, E. M. Orozco, and N.-H. Chua, *in* C. Arntzen, L. Bogorad, S. Bonitz, and K. Steinbeck, ed., *Molecular Biology of the Photosynthetic Apparatus*, in press). In contrast, there is less homology between the −65 regions of different species, and no procaryotelike promoter elements are evident (49). Based on in vitro capping experiments and DNA sequence homologies, it was concluded that the −300 and −180 regions define sites of transcription initiation. The −65 region was presumed to be an RNA cleavage site.

The development of a chloroplast transcription system capable of accurate initiation in vitro (45) has allowed us to analyze the biosynthesis of the −300 and −63 *rbcL* transcripts in maize. In this report, we establish that the −300 RNA is the primary maize *rbcL* transcript and that the −63 RNA results from processing of the larger RNA. We also show that a 109-bp sequence surrounding the −300 site contains all the sequences necessary for homologous and heterologous chloroplast RNA polymerases to initiate transcription in vitro.

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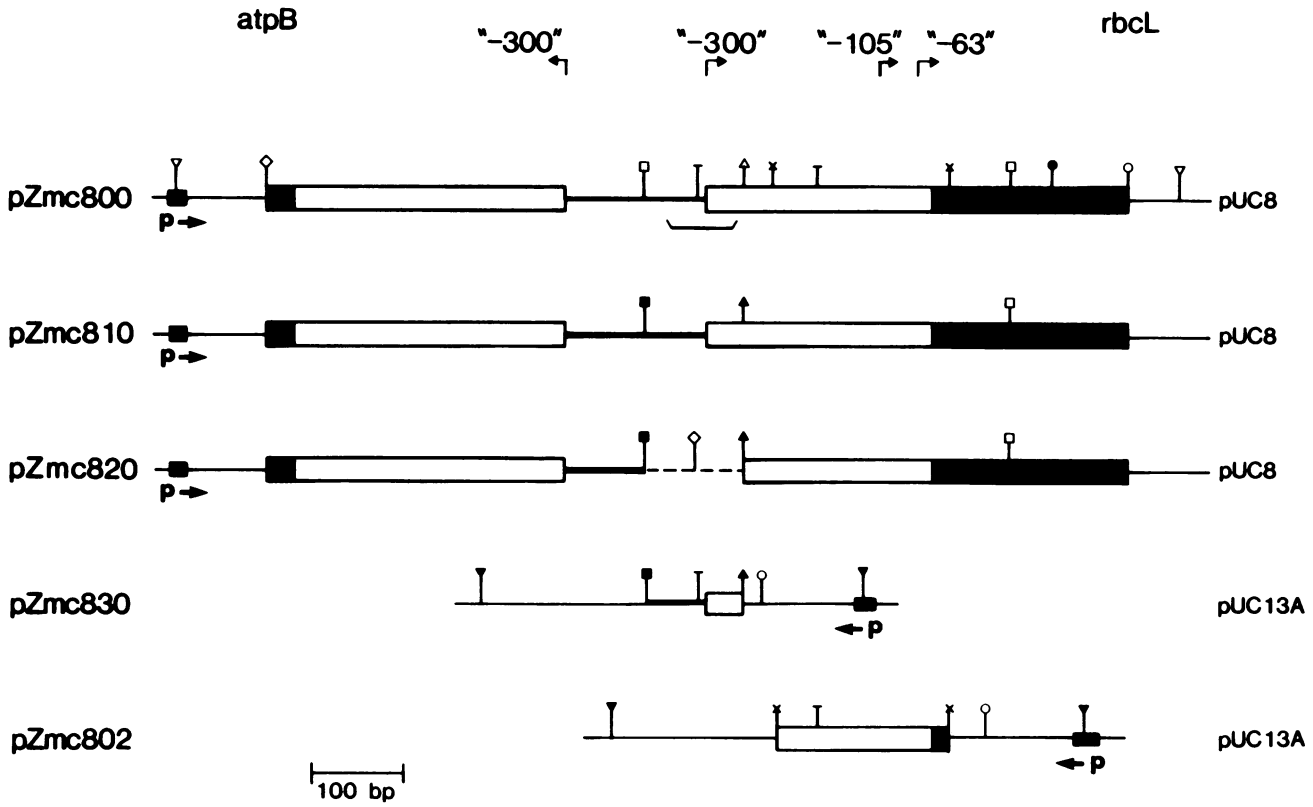


FIG. 1. Schematic diagram of cloned maize chloroplast DNAs used for in vitro transcription. Plasmid pZmc800 contains a 963-bp *Xma*I-*Pst*I fragment of maize plastid DNA, which includes the 5′ termini of the *rbcL* and *atpB* genes (41). The construction and cloning of plasmids pZmc810, pZmc820, pZmc830, and pZmc802 are described in detail in Materials and Methods. The vector DNA used in the cloning of each construct is given on the right; the *lacZ* promoter (p) and the direction of *lacZ* transcription are indicated. The solid boxes designate protein coding sequences, and the open boxes represent transcribed, untranslated regions. The heavy lines designate plastid DNA intergenic regions, and the dotted line (not to scale) corresponds to a 16-bp oligonucleotide derived from pUC13 (see Materials and Methods). The initiation sites (−300) relative to their respective open reading frames and the directions of transcription of the *rbcL* and *atpB* genes are indicated. The 80-bp homology sequence surrounding the *rbcL* transcription initiation site is bracketed for pZmc800. The positions of the 5′ ends (−105 and −63) of the other *rbcL* RNAs are also given. Only those restriction sites relevant to subsequent cloning or DNA probe preparation are indicated. Symbols: —, *Acc*I; □, *Aha*III; ▲, *Bam*HI; ▼, *Ban*I; ▽, *Bst*NI; X, *Eco*RI; △, *Hpa*I; ●, *Hpa*II; ○, *Pst*I; ◇*Xma*I; ■, *Sst*I.

MATERIALS AND METHODS

Enzymes and reagents. Avian myeloblastosis virus reverse transcriptase and DNA polymerase I (large fragment) were from Boehringer Mannheim Biochemicals. RNasin RNase inhibitor was from Promega Biotec. All other enzymes and reagents have been described previously (41, 45).

Plants. *Zea mays* (FR9 × FR37) and *Pisum sativum* (Progress no. 9) were purchased from The Illinois Seed Foundation and Burpee Seed Co., respectively. Plants were grown in vermiculite in a Conviron environmental growth chamber under the indicated conditions for maize (8 days dark: 16 h at 28°C, 8 h at 22°C; 1 day light-dark cycle: 16 h light at 28°C, 8 h dark at 22°C) and for peas (7 days: light 16 h at 22°C, dark 8 h at 18°C).

Plasmid DNAs. Plasmid pZmc800, a 963-bp *Xma*I-*Pst*I maize chloroplast DNA fragment cloned into pUC8 (41, 58), was modified by the insertion of an *Sst*I linker into one of five *Aha*III sites and a *Bam*HI linker into the unique *Hpa*I site (Fig. 1). The resulting plasmid, pZmc810, contains unique *Sst*I and *Bam*HI restriction sites separated by 117 bp (109 bp of chloroplast DNA and 8 bp of linker DNA). Plasmid pZmc820 was constructed by replacing the 117-bp *Sst*I-*Bam*HI sequence of pZmc810 with a 16-bp *Sst*I-*Bam*HI oligonucleotide derived from the polylinker region of pUC13. Plasmid pZmc802 contains a 191-bp *Eco*RI fragment from pZmc800. Plasmid pZmc830 contains a 117-bp *Sst*I-*Bam*HI fragment from pZmc810. Both fragments were cloned into a pUC13 derivative (pUC13A) from which the *Acc*I site was removed by digestion with *Sal*I, 3′-end repair with DNA polymerase I (large fragment), and religation. Plasmid pZmc830 was modified by digestion with *Acc*I, 3′-end repair, and religation to give pZmc831. The 119-bp *Sst*I-*Bam*HI chloroplast DNA insert of pZmc831 was cloned into the same restriction sites of pZmc820, resulting in plasmid pZmc821. *E. coli* JM83 was used in all cloning procedures.

Plasmid DNAs were isolated by the alkaline-sodium dodecyl sulfate method (8) and further purified by CsCl-ethidium bromide gradients (13) and Bio-Gel A-15m chromatography (51). Before use as templates for in vitro transcription, the plasmid DNAs were treated with proteinase K (45).

Radioactive single-stranded DNAs and sequencing. DNA restriction fragments for use as probes in S1 nuclease protection assays were purified by agarose gel electrophoresis and DEAE-cellulose paper elution (18). The purified fragments were treated with calf intestine alkaline phosphatase, 5′-end labeled by using [γ-^{32}P]ATP and T4 polynucle-

otide kinase, and strand separated as described previously (37, 45). Primers for reverse transcription were prepared from restricted plasmid DNAs and 5'-end labeled without prior isolation of the appropriate restriction fragment. Radioactive single-stranded primers were purified by electrophoresis through and elution from 8% polyacrylamide–8.3 M urea DNA sequencing gels (37). DNA sequencing reactions of single-stranded ^{32}P-labeled DNAs were performed by the method of Maxam and Gilbert (37).

Chloroplast in vitro transcription. Intact chloroplasts were purified on Percoll gradients (4), i.e., 40/94% and 40/85% step gradients for maize and pea plastids, respectively. The use of 24-h greened maize seedlings instead of plants grown on a normal light-dark schedule significantly increased the yields of intact chloroplasts. Preparation of transcriptionally active high-salt extracts and in vitro transcription procedures have been described elsewhere (45; E. M. Orozco, Jr., J. E. Mullet, L. Hanley-Bowdoin, and N.-H. Chua, Methods Enzymol., in press). Heterologous transcription reactions (40 μl) contained 5 μl of pea high-salt extract and 5 μg of cloned maize chloroplast template DNA per ml. In homologous maize transcription reactions (40 μl), 5 or 9 μl of high-salt extract and 10 μg of template per ml were used. The amount of high-salt extract added to a reaction was arbitrarily fixed at the concentration which produced optimal *atpB* gene transcription, and it varied between preparations (45).

In vitro RNAs were assayed by S1 nuclease protection and primer extension by using single-stranded DNA probes (41, 59). For S1 nuclease protection experiments, one sixth of the transcripts synthesized in a standard in vitro reaction were assayed with at least a 100-fold excess of probe. For primer extension experiments, one third of the in vitro RNAs transcribed in a standard reaction were analyzed with at least 500-fold primer excess. RNasin (500 U/ml) was included in primer extension reactions (17). The ^{32}P-labeled DNAs were analyzed on 6% polyacrylamide–8.3 M urea DNA sequencing gels (37).

For these studies, plastid high-salt extracts were used exclusively. Because of high levels of endogenous RNA present in these extracts, it was necessary to use in vitro-specific probes to analyze maize transcripts synthesized by the homologous system. A DEAE chromatographic technique has been described which removes contaminating nucleic acids from high-salt extracts (45), but the difficulty of isolating large quantities of intact maize plastids for extract preparation prohibited its application. Furthermore, the active components present in the chloroplast transcription extracts have not been characterized, and the less purified high-salt extract was deemed more likely to contain all the factors important for mRNA transcription and processing.

RESULTS

Chloroplast extracts accurately transcribe maize *rbcL* and *atpB* genes in vitro. Chloroplast *rbcL* transcripts have been examined in several higher plant species, and multiple 5' termini have been documented. Early studies of maize *rbcL* transcripts reported an RNA with its 5' end positioned 59 to 63 bp before the open reading frame (38). Recent experiments, with S1 nuclease protection probes which extend further upstream from the coding region, uncovered a second maize *rbcL* transcript with its 5' terminus at position −300 (21, 41). Both transcripts were detected (Fig. 2, lane 1) when total RNA prepared from 8-day-etiolated maize seedlings was analyzed by S1 nuclease protection, by using an *Hpa*II probe containing 789 nucleotides (nt) of upstream

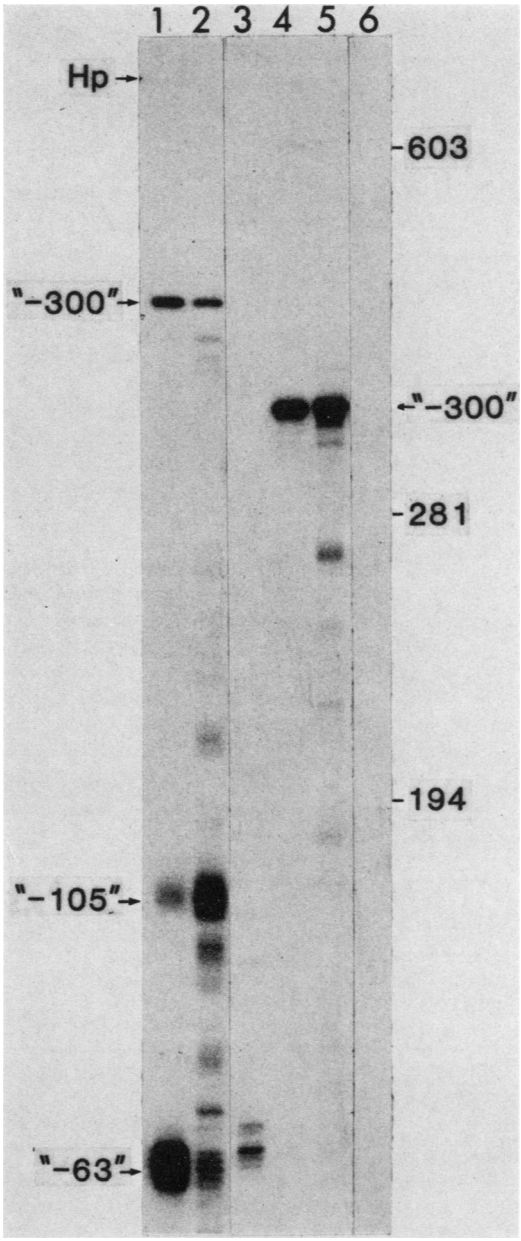


FIG. 2. In vivo and in vitro transcription of maize *rbcL* and *atpB* genes. In vivo maize RNA (2.3 μg of total RNA from 8-day-etiolated seedlings) and in vitro RNAs synthesized by pea high-salt extract (30°C, 60 min) were assayed by S1 nuclease protection by using individual strands of an 874-bp *Hpa*II restriction fragment (Hp; see Fig. 3C). Each strand was 5'-end labeled with ^{32}P within the coding region of either the *rbcL* or the *atpB* gene. Lanes 1 to 3: The slow-moving strand was protected from S1 nuclease digestion by in vivo *rbcL* transcripts (lane 1) and in vitro *rbcL* RNAs synthesized in reactions containing pZmc800 (lane 2) or no exogenous template (lane 3). Lanes 4 to 6: The fast-moving strand was protected from S1 nuclease digestion by in vivo *atpB* transcripts (lane 4) and in vitro *atpB* RNAs synthesized in reactions containing pZmc800 (lane 5) or no exogenous template (lane 6). The S1-protected products corresponding to −300, −105, and −63 *rbcL* transcripts are indicated on the left. The −300 *atpB* assay product is designated on the right. The sizes in nucleotides of three φX174-*Hae*III DNA fragments are also shown on the right.

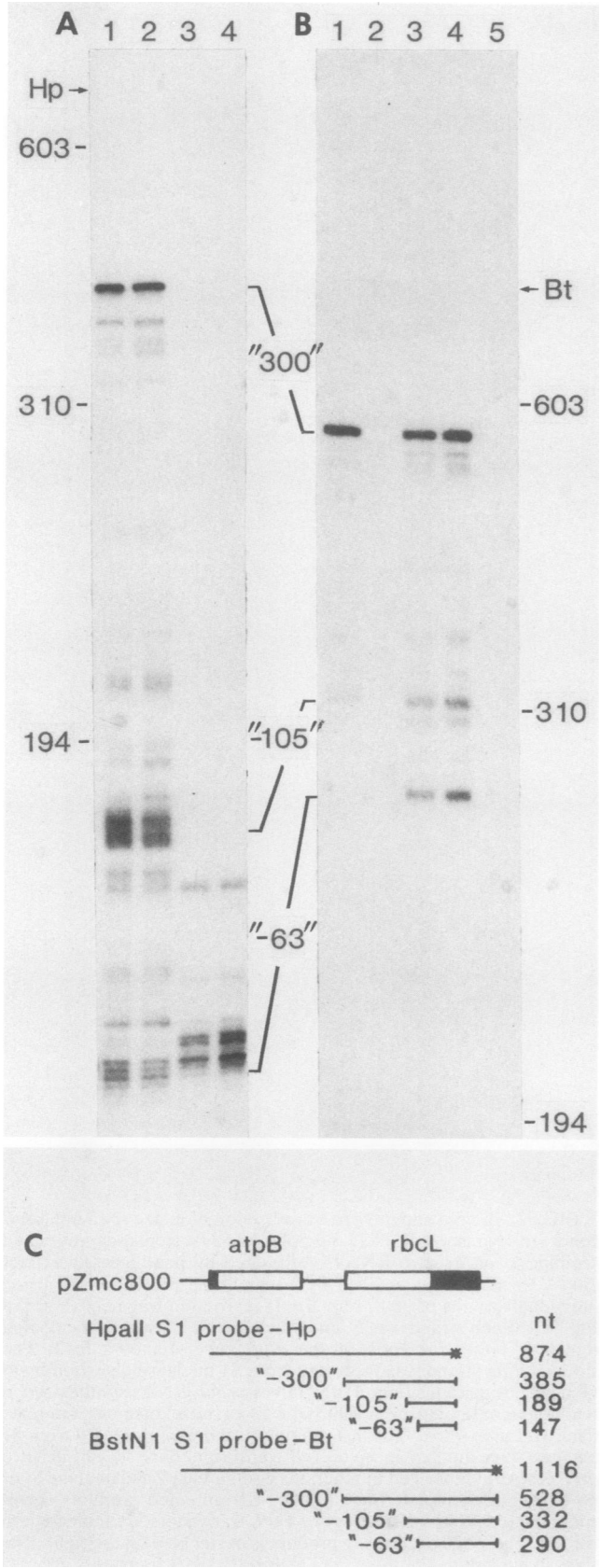


FIG. 3. In vitro transcription of maize *rbcL* gene constructions containing linker sequences and lacking the −300 region. (A) RNAs synthesized by pea high-salt extract in vitro (30°C, 60 min) were analyzed by S1 nuclease protection using the slow-moving strand of the *Hpa*II probe (Hp; panel C). DNAs protected by transcripts synthesized in reactions containing pZmc800 (lane 1), pZmc810 (lane 2), pZmc820 (lane 3), or no exogenous template (lane 4) are shown. (B) In vitro transcripts synthesized by pea or maize high-salt extracts in vitro (20°C, 60 min) were analyzed by S1 nuclease protection using the slow-moving strand of the *Bst*NI probe (Bt; panel C). Lane 1: S1 nuclease protection by the RNAs synthesized by using pea high-salt extract and pZmc800. Lanes 2 to 5: S1 protection by the RNAs synthesized by maize high-salt extract with no exogenous template (lane 2), pZmc800 (lane 3), pZmc810 (lane 4), or pZmc820 (lane 5). The different mobilities of the protected products in panels A and B, which are aligned between the two panels (−300, −105, and −63), are due to the protection of S1 probes 5′-end labeled at restriction sites located at different positions in pZmc800. The sizes in nucleotides of three ϕX174-*Hae*III DNA fragments are shown on the extreme left and right. (C) Plasmid pZmc800 (Fig. 1) and the two single-stranded S1 nuclease probes prepared therefrom are depicted. The position of the ^{32}P-labeled 5′ end of each probe is identified with an asterisk. Beneath each probe, the protected fragments which correspond to the *rbcL* transcripts indicated in panels A and B are shown. The sizes in nucleotides of the two probes and the protected products are listed on the right.

sequences and a ^{32}P-labeled 5′ end within the coding region (Fig. 3C). A third, previously undocumented *rbcL* RNA (−105) was also detected in vivo. The −63 RNA is the predominant maize *rbcL* transcript in vivo, and the −300 and −105 RNAs are much less abundant. In RNA isolated from purified, fully greened bundle sheath cells, the −105 *rbcL* transcript was barely detectable, whereas the ratio of −300 to −63 RNA remained approximately the same (data not shown). A single *atpB* transcript, with its 5′ end located 300 bp before its respective coding region, was detected in vivo by using the complementary strand of the *Hpa*II DNA as an S1 nuclease probe (Fig. 2, lane 4).

Earlier studies showed that extracts prepared from spinach chloroplasts can initiate transcription of the spinach *rbcL* and *atpB* genes correctly (45). By using extracts prepared from maize and pea chloroplasts, we confirmed that maize *rbcL* and *atpB* RNAs produced in vitro have the same 5′ termini as in vivo-synthesized transcripts. Plasmid pZmc800, which contains the 5′ ends of the maize *rbcL* and *atpB* genes (41; Fig. 1), was transcribed by pea chloroplast high-salt extract. Because the *rbcL* and *atpB* genes of peas and maize are only weakly homologous in their 5′ leader regions, it was possible to use the maize DNA probes (*Hpa*II) described above to assay the in vitro transcripts. Pea chloroplast RNA polymerase transcribed the maize *rbcL* gene accurately, resulting in the −300, −105, and −63 RNAs (Fig. 2, lane 2). The in vitro −63 signal was clearly distinct from the background owing to S1 protection of the probe by endogenous pea *rbcL* RNA (lane 3). The stoichiometry of the maize *rbcL* transcripts was altered in vitro relative to in vivo levels (cf. lanes 1 and 2). The −105 RNA was a major *rbcL* transcription product in vitro, and its relative abundance varied with transcription parameters, e.g., temperature and time, measured under constant S1 nuclease assay conditions. S1 protection analysis of the −300 *rbcL* RNA with increasing concentrations of the nuclease (125 to 2,000 U/ml) did not produce the −105 signal. These data indicate that the −105 product corresponds to an authentic *rbcL* transcript and is not an S1 artifact. We located the 5′ terminus of the RNA between positions −100 and −105 relative to the *rbcL* coding region by coelectrophoresis and size comparison with ^{32}P-labeled ϕX174-*Hae*III DNA frag-

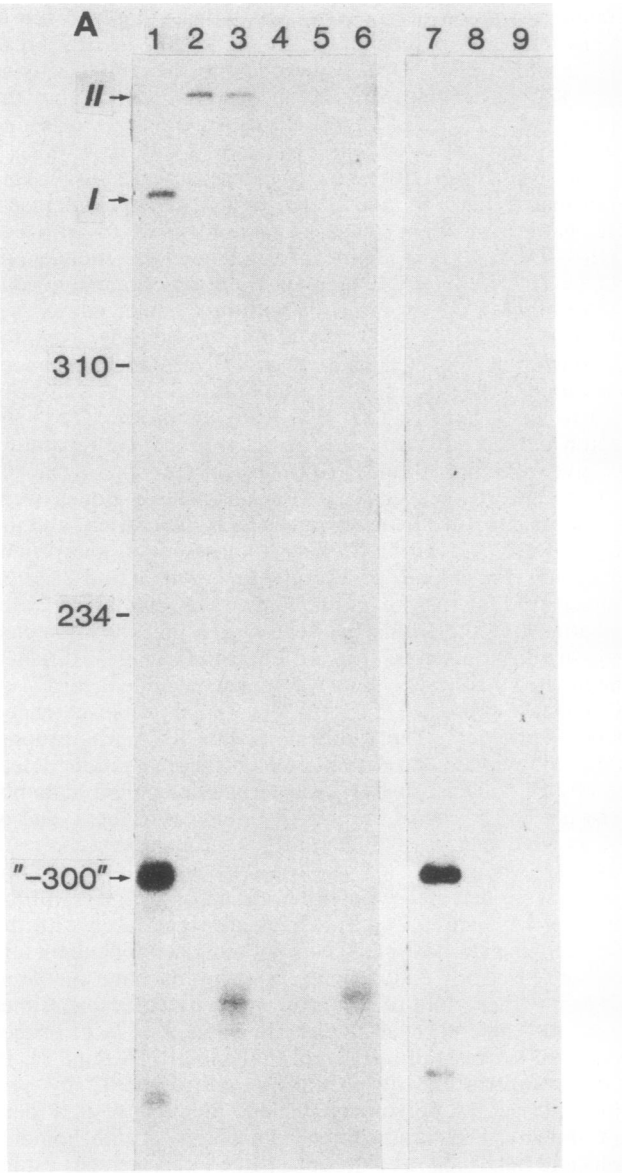
ments. The diffuse nature of the signal is most likely an S1 artifact owing to the high AT content (>90%) of the region. Pea chloroplast RNA polymerase also correctly transcribed the maize *atpB* gene in vitro, resulting in a single species with the same 5′ terminus as that observed in vivo (Fig. 2, cf. lanes 4 and 5).

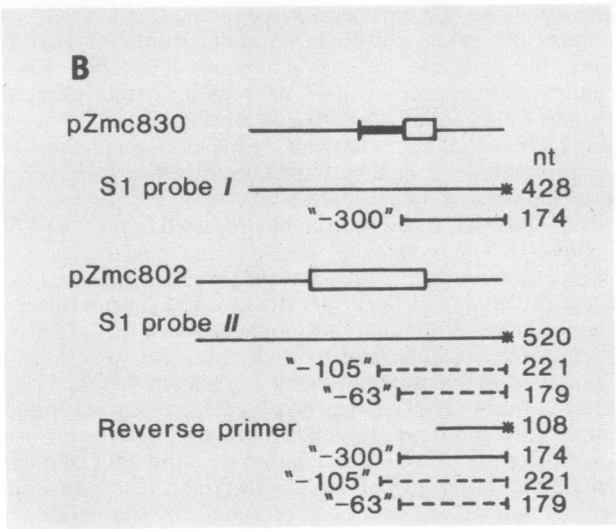
Homologous transcription of plasmid pZmc800 by maize chloroplast high-salt extract is shown in Fig. 3B. Maize (lane 3) and pea (lane 1) chloroplast RNA polymerases produced the same −300 *rbcL* transcript in vitro. The transcription assays in Fig. 3B were performed at 20°C to stabilize the −300 transcript. Plastid transcription extracts contained endogenous RNase activities which can catalyze both specific and nonspecific digestion of *rbcL* RNA, especially at higher temperatures. Consequently, the relative levels of in vitro −105 and −63 maize *rbcL* RNAs were substantially lower when the reactions were performed at 20 rather than 30°C (cf. lane 1, Fig. 3A, with lane 1, Fig. 3B). High levels of endogenous background resulting from the presence of in vivo *rbcL* RNAs in the maize high-salt extract precluded direct comparison of *rbcL* RNAs transcribed by maize RNA polymerase in vivo and in vitro. It was necessary to assay *rbcL* RNAs transcribed in the homologous in vitro system with an in vitro-specific *Bst*NI probe with a ^{32}P-labeled 5′ end within flanking vector DNA (Fig. 3C). This probe detected only RNAs containing vector sequences and, therefore, that had been transcribed from exogenous template. No signal owing to endogenous nucleic acid was observed (Fig. 3B, lane 2). The maize *atpB* gene was also transcribed accurately in vitro by homologous chloroplast RNA polymerase (results not shown), as determined by S1 nuclease analysis with the complementary strand of the in vitro-specific *Bst*NI probe.

Promoter for the maize *rbcL* gene is located in 109 bp surrounding the −300 5′ terminus. The three 5′ termini for maize *rbcL* mRNA may reflect three independent transcription initiation events or a combination of initiation and subsequent 5′-end processing of the primary transcript(s). In vitro capping experiments of spinach, pea, and maize *rbcL* mRNAs with [α-^{32}P]GTP and guanylyltransferase showed that, in each case, the longer transcript is the primary product of a transcription initiation event (16, 41). Failure to label the shorter RNAs in these experiments suggested that they are processed forms of the longer *rbcL* transcripts. However, these results could also reflect inaccessibility of the 5′ termini to guanylyltransferase because of RNA secondary structure. To resolve these questions, we examined transcription initiation and RNA processing separately in vitro.

Plasmid pZmc800 was modified by the insertion of *Sst*I and *Bam*HI linkers to give pZmc810 (Fig. 1). In pZmc810, the linkers are 109 bp apart and include the −300 5′ end of the maize *rbcL* gene. Plasmid pZmc810 was used as template in transcription reactions catalyzed by pea (Fig. 3A, lane 2) and maize (Fig. 3B, lane 4) chloroplast RNA polymerases. In both cases, the S1 nuclease-protected products were indistinguishable from those observed when pZmc800 was the transcription template (cf. lanes 1 and 2, Fig. 3A; cf. lanes 3 and 4, Fig. 3B). The discontinuity in the pZmc810 RNA:pZmc800 DNA hybrid owing to the transcribed 8-bp *Bam*HI linker was insensitive to S1 nuclease digestion under our assay conditions. However, when pZmc800 and pZmc810 *rbcL* transcripts were analyzed by primer extension, the pZmc810-derived −300 RNA was longer than its pZmc800 analog, as expected (data not shown). The similar *rbcL* transcription patterns observed with the two templates facilitated future analyses. The −300 site is flanked by unique restriction sites in pZmc810, which simplified subsequent template modifications. In pZmc820, the 109 bp of maize chloroplast DNA surrounding the −300 site of pZmc810 was replaced by 16 bp of pUC13 DNA, but the −105 and −63 sites were left intact (Fig. 1). Plasmid pZmc820 did not direct the synthesis of any *rbcL* mRNA species when transcribed in vitro by either pea (Fig. 3A, lane 3) or maize (Fig. 3B, lane 5) chloroplast RNA polymerases. The pattern of S1 nuclease protection observed with pea high-salt extract was identical to that seen for endogenous pea *rbcL* RNA (Fig. 3A, lane 4). There was no endogenous background in the experiments with maize high-salt extract because the in vitro-specific *Bst*NI probe was used for hybridization (Fig. 3C). These results suggest that sequences surrounding the −300 site are required for all *rbcL* transcription in vitro, not just for −300 RNA synthesis. The −300 region and the −105 and −63 regions were cloned separately to give plasmids pZmc830 (109-bp insert) and pZmc802 (191-bp insert), respectively (Fig. 1). These clones were transcribed in vitro by using maize high-salt extract, and the RNAs were assayed by S1 nuclease protection and primer extension (Fig. 4A). The S1 probes and reverse transcription primer used for these experiments were 5′-end labeled with ^{32}P at a *Ban*I site within the vector such that they detected only in vitro transcripts (Fig. 4B). Plasmid pZmc830 directed the in vitro synthesis of an RNA which gave identical 174-nt S1 nuclease-protected (Fig. 4A, lane 1) and primer-extended (lane 7) products. The synthesis of this RNA was proportional to template and extract concentrations, and its detection by S1 nuclease analysis was strand dependent (data not shown). The 5′ end of the RNA is identical to the 5′ end of the in vivo maize −300 *rbcL* RNA (41; see Fig. 7B). In contrast, when pZmc802 was transcribed in vitro, no transcripts were detected with either strand of a *Ban*I S1 probe (II, Fig. 4A, lanes 2 and 3). The weak signal seen with the slow-moving strand (lane 3) was not template dependent and was observed for endogenous transcription (lane 6). Similarly, no transcripts of pZmc802 were detected by primer extension (lane 8), even though the same *Pst*I-*Ban*I primer was used successfully to detect the −300 RNA (lane 7). In vitro transcription of pZmc830 and pZmc802 by pea chloroplast RNA polymerase yielded identical results (data not shown). The transcription data obtained with homologous and heterologous chloroplast RNA polymerases established that the 109-bp region surrounding the −300 5′ end contains the maize plastid DNA *rbcL* promoter. Furthermore, the synthesis of the −105 and −63 *rbcL* RNAs requires the presence of the −300 region; neither pZmc820 nor pZmc802 can support *rbcL* synthesis in vitro. The 191 bp, which include the −105 and −63 *rbcL* 5′ ends, does not contain promoter sequences, and these RNAs are unlikely to be primary transcripts.

The −300 *rbcL* transcript can be processed to the −63 RNA in vitro. In vitro synthesis of −105 and −63 maize *rbcL* RNAs is concomitant with −300 *rbcL* transcription and may reflect processing of the −300 RNA to the shorter species. The pea high-salt extract does not synthesize the −63 RNA as efficiently as does maize. Therefore, we were able to obtain preparative quantities of the −300 RNA (Fig. 5, lane 1), free of other *rbcL* transcripts, by adjusting transcription temperature and time. Plasmid pZmc800 (5 μg/ml) was used as template in a 300-μl transcription reaction (20°C, 30 min) containing 37.5 μl of pea high-salt extract. The transcripts were treated with DNase I and proteinase K and purified by normal procedures with 7.5-fold greater volumes (45). The in





vitro RNAs were redissolved in 88 μl of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA, and 8 μl of the RNA solution was added to each standard transcription reaction containing maize high-salt extract and no exogenous template. The reactions were incubated at 30°C for the indicated times, placed on ice for the remainder of the 60-min incubation period, and prepared for S1 nuclease analysis as usual (Fig. 5, lanes 2 to 8 and 17). Parallel in vitro transcription reactions containing maize high-salt extract and pZmc800 as template, but no exogenous in vitro RNA, were incubated at 30°C for the indicated times, placed on ice for the remainder of the 60-min incubation, and prepared for S1 nuclease analysis (Fig. 5, lanes 9 to 15). The in vitro-specific *Bst*NI probe (Fig. 3C) was used for S1 nuclease analysis.

In the homologous transcription assays (Fig. 5, lanes 9 to 15), very little *rbcL* gene transcription was detected at 5 min (lane 11), although it is significant that the earliest detected transcript was the −300 RNA. Within 15 min (lane 12), the level of the −300 transcript was maximal, and it remained constant during the 60-min incubation period (lanes 13 to 15). In contrast, the levels of the −105 and −63 *rbcL* RNAs continued to increase during the 60-min time course. The kinetics suggest a precursor-product relationship between the maize −300 *rbcL* transcript and the −105 and −63 RNAs. We confirmed this relationship for the −300 and −63 transcripts. In parallel assays, the −300 RNA precursor was converted to the −63 RNA with increasing incubation times at 30°C (Fig. 5, lanes 2 to 8). The in vitro processing of the −300 transcript was very rapid, with a significant amount of −63 RNA appearing as early as 5 min (lane 3), and was essentially complete by 45 min (lane 6). A small amount of −63 RNA was present at 0 min (lane 2) and most likely resulted from manipulation of samples before inactivation of the processing activity. The 5′-end processing of the −300 transcript is dependent on the presence of maize plastid extract during incubation. The −300 transcript was stable for 60 min at 30°C under standard transcription conditions in the absence of extract (lane 8). In lanes 1 to 8, the faint band which comigrates with the −105 signal is probably an S1

FIG. 4. In vitro transcription of maize *rbcL* −300 and −105 and −63 subclones. (A) Plasmids pZmc830 and pZmc802 were used as templates in transcription reactions (20°C, 60 min) containing maize high-salt extract. The in vitro RNAs were analyzed by S1 nuclease protection and primer extension. Lanes 1 to 6: S1 protection of the designated *Ban*I probe (panel B) by in vitro transcripts synthesized in reactions containing pZmc830 (probe I, slow-moving strand [lane 1]), pZmc802 (probe II, fast- [lane 2] or slow-moving [lane 3] strand), no exogenous template (probe I, slow-moving strand [lane 4]), or no exogenous template (probe II, fast- [lane 5] or slow-moving [lane 6] strand). Lanes 7 to 9: Primer extension assays using the single-stranded, *Ban*I-*Pst*I primer to detect in vitro transcripts synthesized in reactions containing pZmc830 (lane 7), pZmc802 (lane 8), or no exogenous template (lane 9). The relative mobilities of the S1 probes are indicated (I and II). The assay products corresponding to the −300 *rbcL* transcripts are indicated. The primer was not retained on the gel. The sizes in nucleotides of ϕX174-*Hae*III DNA fragments are shown on the left. (B) Plasmids pZmc830 and pZmc802 (Fig. 1), their *Ban*I S1 probes (I and II, respectively), and the *Ban*I-*Pst*I reverse primer are depicted. The position of the ^{32}P-labeled 5′ end of each S1 probe is identified with an asterisk. The primer was also 5′-end labeled at the *Ban*I site and used to assay RNAs transcribed from both pZmc830 and pZmc802. The S1-protected and primer-extended products observed for −300 *rbcL* RNA are indicated by solid lines; predicted products for the −105 and −63 RNAs are indicated by broken lines. The sizes in nucleotides of the S1 nuclease probes, primer, and assay products are shown on the right.

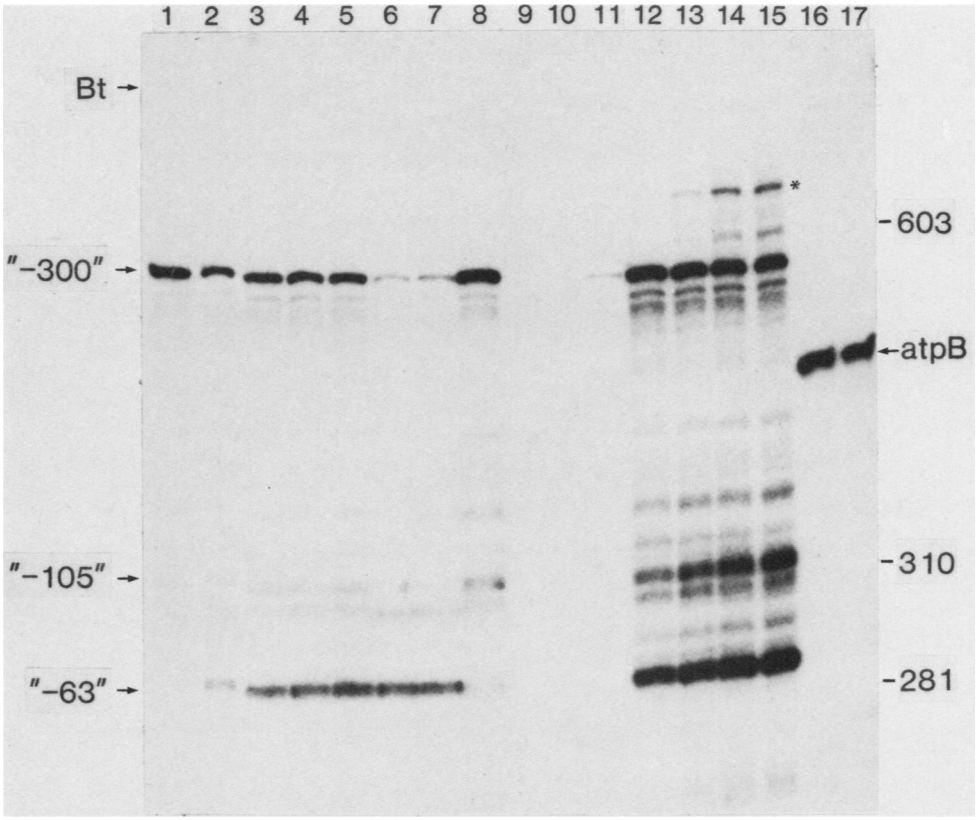


FIG. 5. Time course of in vitro processing of the maize *rbcL* −300 RNA and transcription of the maize *rbcL* gene. In vitro *rbcL* −300 RNA, which was synthesized preparatively using pea high-salt extract, was added to standard maize in vitro transcription reactions in the absence of exogenous template and incubated at 30°C for the times indicated below. Parallel transcription reactions containing maize high-salt extract and pZmc800 as template, but no exogenous in vitro RNA, were also incubated at 30°C. In vitro *rbcL* RNAs were analyzed by S1 nuclease protection by using the slow-moving strand of a 1,116-nt *Bst*NI probe (Bt; Fig. 3C). In vitro *atpB* RNAs were analyzed by using the complementary fast-moving strand. Lanes 1 and 16: S1 nuclease assays of in vitro RNAs synthesized in a preparative pea high-salt transcription reaction and assayed for *rbcL* (lane 1) and *atpB* (lane 16) transcripts. Lanes 2 to 8 and 17: S1 assays of in vitro processing products resulting from the incubation of *rbcL* −300 RNA in the presence of maize high-salt extract for 0 (lane 2), 5 (lane 3), 15 (lane 4), 30 (lane 5), 45 (lane 6), and 60 min (lane 7) and the incubation of *atpB* −300 RNA with maize high-salt extract for 60 min (lane 17). Lane 8: S1 assay of in vitro *rbcL* −300 RNA incubated for 60 min under standard in vitro transcription conditions in the absence of high-salt extract. Lane 9: S1 nuclease assay of in vitro *rbcL* transcripts synthesized by maize high-salt extract during 60 min in the absence of exogenous RNA and template. Lanes 10 to 15: S1 assays of in vitro *rbcL* transcription products synthesized by maize high-salt extract using pZmc800 at 0 (lane 10), 5 (lane 11), 15 (lane 12), 30 (lane 13), 45 (lane 14), and 60 min (lane 15). The S1 nuclease-protected products corresponding to *rbcL* transcripts (−300, −105, and −63) are identified. Transcripts which originated from the *atpB* promoter region and resulted from incorrect transcription of the *rbcL* coding strand are indicated with an asterisk. The sizes in nucleotides of ϕX174-*Hae*III DNA fragments are shown on the right.

artifact owing to the high AT content of the region. However, the intensity of this signal cannot account fully for the strong −105 signals in lanes 12 to 15. The processing activity is also specific for *rbcL* mRNA. The *atpB* −300 RNA was stable at 30°C for 60 min in the presence of maize high-salt extract (Fig. 5, cf. lanes 16 and 17). In addition to the specific *rbcL* 5′-end-processing activity, there was general nuclease activity in the maize high-salt extract, as indicated by a decrease in total *rbcL* RNA with time (cf. lanes 7 and 8), which prevented quantitation of this experiment. We were unable to show directly that the −105 RNA is a processed form of the −300 *rbcL* transcript.

Mutagenesis of sequences upstream of the −300 *rbcL* initiation site depresses transcription in vitro. In bacterial systems, mutations in 5′ upstream regions can alter gene expression (29, 54). Because of the sequence similarities between *E. coli* and plastid DNA 5′ flanking regions (10), we decided to examine the effect of mutation immediately upstream of the maize −300 *rbcL* site on chloroplast gene transcription in vitro. Plasmid pZmc830, which contains only the −300 transcription initiation site (Fig. 1), was restricted at a unique *Acc*I site. The 3′ ends were repaired with DNA polymerase I (large fragment), and the resulting blunt ends were religated. The mutant clone, pZmc831, has an AT dinucleotide inserted 11 bp before the −300 site (see Fig. 7B). The pZmc831 insertion was confirmed by sequencing both strands of a 150-bp fragment derived from *Eco*RI and *Hin*dIII digestion of adjacent vector polylinker DNA.

Plasmids pZmc830 and pZmc831 were transcribed in vitro by maize and pea chloroplast RNA polymerases (Fig. 6A) and assayed by using S1 nuclease protection and an in vitro-specific probe (I, Fig. 4B). When pZmc830 was the template for the homologous and heterologous plastid RNA polymerases (Fig. 6A, lanes 1, 3, and 6), transcription was initiated accurately at position −300. In contrast, when pZmc831 was the template in the maize high-salt extract

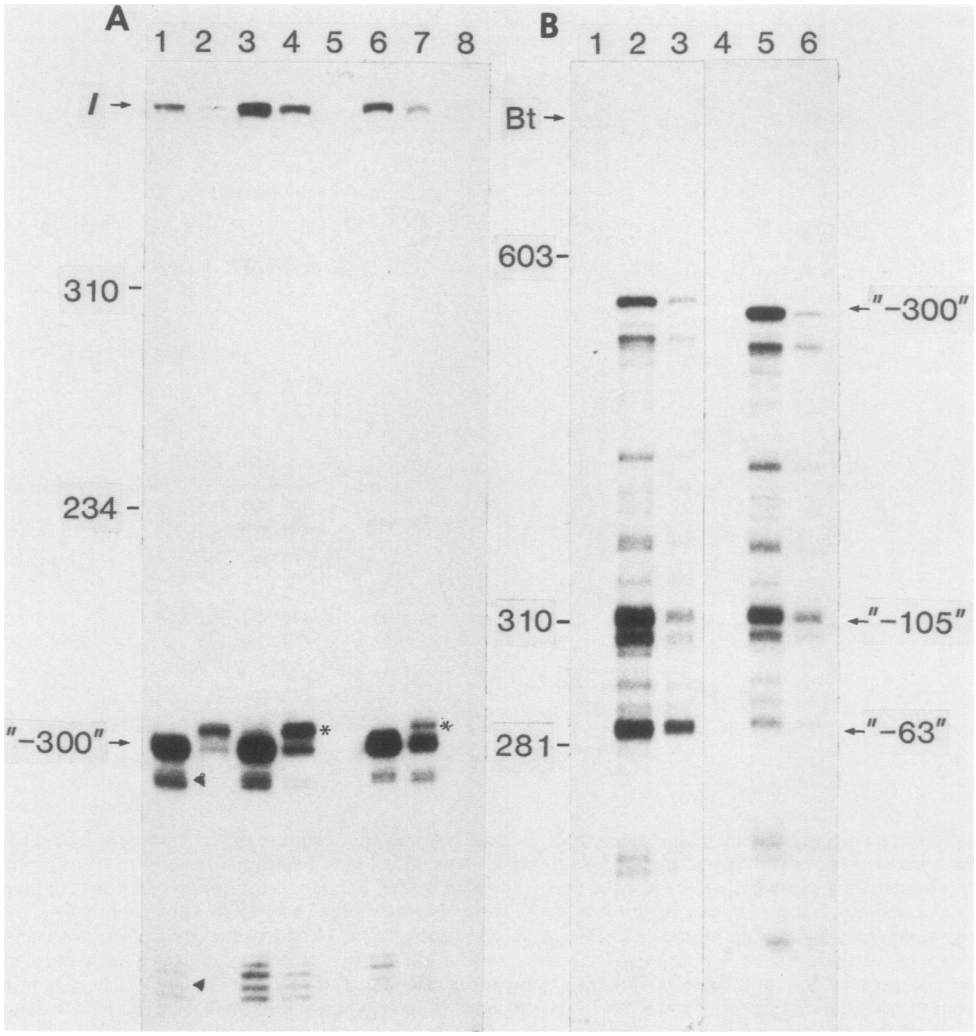


FIG. 6. In vitro transcription of modified −300 maize *rbcL* templates. (A) Plasmid pZmc830 and modified plasmid pZmc831 (see Fig. 7B) were used as templates in transcription reactions, and the in vitro RNAs were analyzed by S1 nuclease protection using a single-stranded, 428-nt *Ban*I probe (I; Fig. 4B). Lanes 1 to 5: S1 assays of in vitro RNAs transcribed by maize high-salt extract at 20°C for 60 min from pZmc830 (lane 1) and pZmc831 (lane 2) and at 30°C for 30 min using pZmc830 (lane 3), pZmc831 (lane 4), and no exogenous template (lane 5). Lanes 6 to 8: S1 assays of in vitro RNAs transcribed by pea high-salt extract at 30°C for 30 min from pZmc830 (lane 6), pZmc831 (lane 7), and no exogenous template (lane 8). Products which reflect S1 nuclease digestion artifacts are indicated (◀). (B) Plasmid pZmc810 and modified plasmid pZmc821 were used as templates in transcription reactions (30°C, 30 min), and the in vitro RNAs were analyzed by S1 nuclease protection using a single-stranded, 1,116-nt *Bst*NI probe (Bt; Fig. 3C). Lanes 1 to 3: S1 assays of in vitro RNAs synthesized by maize high-salt extract using no exogenous template (lane 1), pZmc810 (lane 2), and pZmc821 (lane 3). Lanes 4 to 6: S1 assays of in vitro RNAs synthesized by pea high-salt extract using no exogenous template (lane 4), pZmc810 (lane 5), and pZmc821 (lane 6). The S1 nuclease-protected products corresponding to *rbcL* transcripts (−300, −105, and −63) are identified. The −302 RNA, which is transcribed from the modified *rbcL* template pZmc831, is indicated by asterisks. The sizes in nucleotides of ϕX174-*Hae*III DNA fragments are shown to the left of each panel.

(lanes 2 and 4), there was little correct transcription initiation, and a new, slightly larger RNA was the predominant product. When pZmc831 was transcribed in the pea high-salt extract (lane 7), the new transcript was also observed, but it was a minor product, and the authentic −300 RNA was the major *rbcL* transcript. In both maize and pea chloroplast transcription systems, pZmc831 was a less efficient template than pZmc830. Transcription of pZmc831 demonstrated that maize and pea chloroplast in vitro systems do not have completely identical promoter recognition requirements; the homologous system exhibited greater sensitivity to template alterations. The new pZmc831 transcript (−302) is 2 nt longer than the wild-type −300 RNA at its 5′ end (Fig. 7B).

The reduced template efficiency of pZmc831 described above was more pronounced at 20°C (cf. lanes 2 and 4, Fig. 6A) and reflected, in part, a greater stringency for transcription initiation at 20 than at 30°C. The effect of temperature on transcription was also shown by the increased full-length protection of the S1 nuclease probe (I) at 30°C because of incorrect initiation within upstream vector sequences (cf. lanes 1 and 3, Fig. 6A). Primer extension analysis has established that the incorrectly initiated products correspond to discrete positions in both chloroplast and vector sequences (unpublished observations). These sites may be analogous to tight binding sites described for *E. coli* RNA polymerase (32, 39).

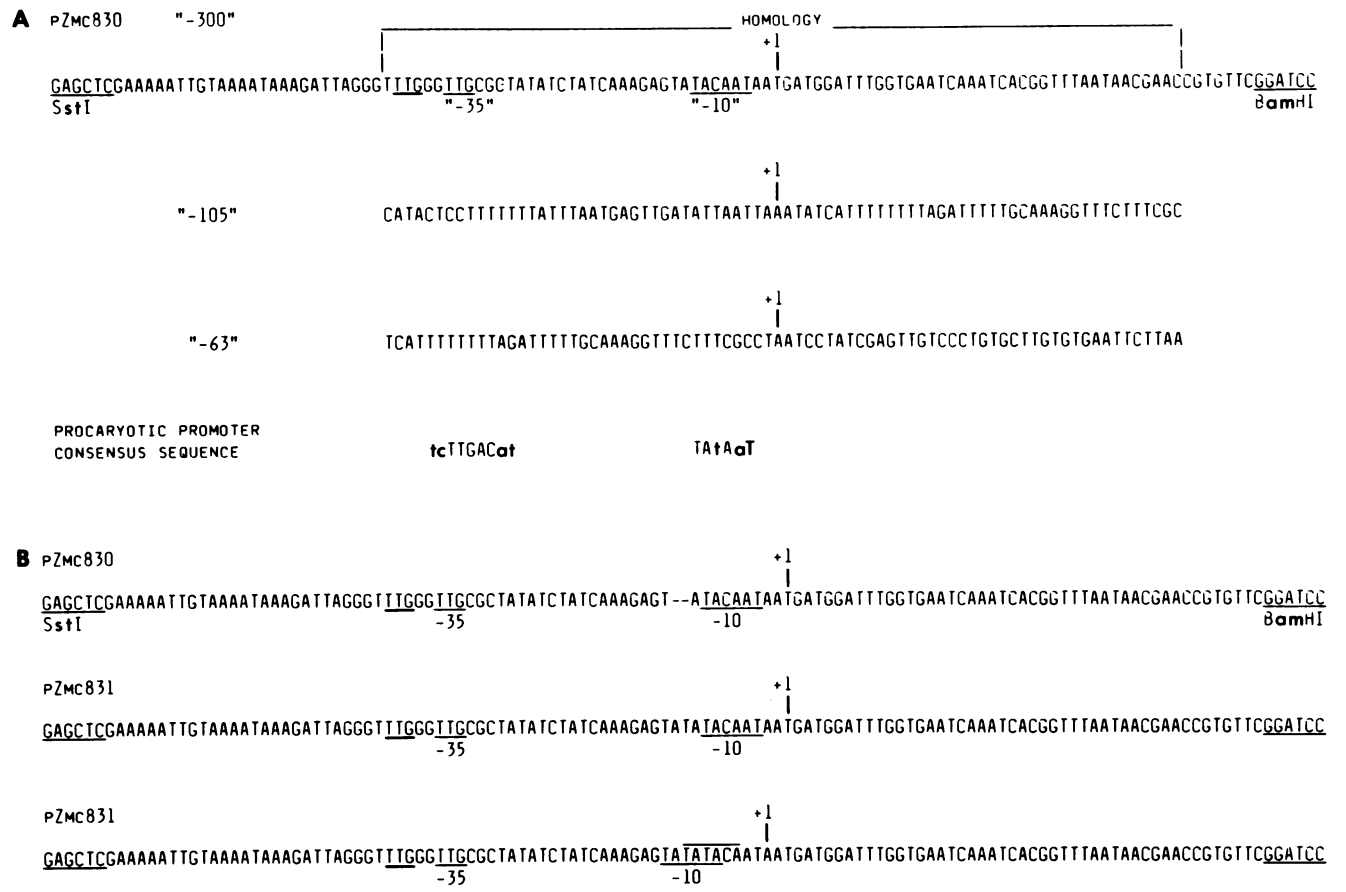


FIG. 7. DNA sequences surrounding the 5′ termini of *rbcL* transcripts. (A) The sequences of maize *rbcL* −300 (pZmc830), −105, and −63 regions (33, 38) are compared with the *E. coli* promoter consensus sequence (29). Sequences homologous to *E. coli* −10 and −35 promoter elements are underlined. The nucleotide positions of the 5′ termini of −300 and −63 *rbcL* transcripts are indicated (41). The location of the 5′ end of the −105 RNA was determined by size comparison with ϕX174-*Hae*III DNA fragments and is approximate. (B) The −300 regions of plasmids pZmc830 and pZmc831 are compared. The AT insertion in pZmc831, which was confirmed by Maxam and Gilbert sequencing techniques (37), is designated by dashes in the pZmc830 sequence. The nucleotide positions of the 5′ termini of the −300 and −302 *rbcL* transcripts are shown. The ends of the two RNAs were mapped by primer extension by using the *Pst*I-*Ban*I primer and coelectrophoresis with the products of a Maxam and Gilbert sequencing ladder generated from the slow-moving strand of the 428-nt *Ban*I probe (I; Fig. 4B). Sequences homologous to *E. coli* −10 and −35 promoter elements are underlined. Two alternative −10 regions are indicated for pZmc831.

The modified pZmc831 chloroplast DNA sequence was inserted into pZmc820 at unique *Sst*I and *Bam*HI restriction sites to give pZmc821. Plasmids pZmc821 and pZmc810, which differ only by the AT dinucleotide insertion, were transcribed by using maize (Fig. 6B, lanes 1 to 3) and pea (lanes 4 to 6) chloroplast RNA polymerases and assayed by S1 nuclease protection with the in vitro-specific *Bst*NI probe (Fig. 3C). Similar to the results obtained with pZmc831, *rbcL* transcription was depressed in vitro when pZmc821 was the template (cf. lanes 2 and 3 and lanes 5 and 6, Fig. 6B). However, unlike pZmc820, which does not contain the −300 region or direct any *rbcL* RNA synthesis in vitro (Fig. 3A, lane 3, and Fig. 3B, lane 5), there was some *rbcL* transcription with pZmc821. The electrophoretic analysis in Fig. 6B did not resolve the −300 and −302 transcripts seen in Fig. 6A, and consequently it is not known whether pZmc821 directed transcription initiation from position −302, analogous to pZmc831. The limited template modification upstream of the −300 site in pZmc821 reduced the levels of the −105 and −63 RNAs, as well as the level of −300 RNA. This observation supports the conclusion that synthesis of the smaller *rbcL* RNAs is dependent on the −300 region.

DISCUSSION

The molecular mechanisms underlying the synthesis of chloroplast mRNAs are not well understood. Transcription in procaryotes has been used as a model to describe chloroplast RNA synthesis because of similarities between plastid and bacterial genomes. However, evidence supporting this hypothesis is indirect, based on DNA sequence homology (60) and expression of chloroplast genes in bacterial systems (20, 22, 24, 52). Only a few studies have examined directly the synthesis of chloroplast RNAs at the molecular level by using plastid enzymes in vitro (16, 27, 28, 34, 45). In the work reported here, we used homologous and heterologous chloroplast enzymes in vitro to characterize two aspects of maize plastid RNA synthesis, i.e., the initiation of *rbcL* gene transcription and 5′-end processing of the primary transcript.

Three maize *rbcL* transcripts, with 5′ ends corresponding to positions −300, −105, and −63, are present in vivo. The −105 RNA, which has not been described previously, is a minor species. A minor *rbcL* RNA, with its 5′ end located at approximately the same position, has also been observed in

barley by primer extension analysis (C. Poulsen, personal communication). In vitro synthesis of maize −105 *rbcL* RNA was affected by temperature and time, but the detection of this transcript was not altered by varying S1 nuclease protection conditions. In earlier studies, the −105 RNA may not have been detected, especially in fully greened plant tissue, or may have been viewed as an S1 nuclease artifact because of the high AT content of surrounding sequences. However, the similarities between the in vivo *rbcL* transcripts of maize and barley and our in vitro transcription studies of the maize *rbcL* gene indicate that the −105 RNA is an authentic transcript.

Three maize *rbcL* transcripts were synthesized by pea and maize chloroplast RNA polymerases in vitro. The 5′ termini of the RNAs synthesized by pea high-salt extract were identical to the 5′ ends of the in vivo maize *rbcL* transcripts. By indirect comparison, the 5′ ends of *rbcL* RNAs synthesized by maize chloroplast polymerase in vivo and in vitro were also identical. The relative amounts of the three in vitro transcripts only partially correlated to observed in vivo ratios and could be altered by reaction temperature. The proportion of −300 *rbcL* RNA was greater in vitro at 20 than at 30°C, suggesting that chloroplast RNA polymerase is relatively more active at lower reaction temperatures than the RNA processing enzymes and nucleases also present in the extracts. When the homologous system was used for in vitro RNA synthesis at 30°C, the relative amounts of the −300 and −63 RNAs paralleled in vivo levels. However, at 30°C, the pea high-salt extract synthesized very little −63 maize *rbcL* RNA. In peas, −178 and −65 *rbcL* transcripts have been identified in vivo (41). The −65 pea transcript was present at very low levels, which may reflect reduced activity of an RNA processing enzyme required for the synthesis of pea −65 RNA in vivo and maize −63 RNA in vitro. The large amount of −105 *rbcL* RNA synthesis in both in vitro transcription systems did not correlate with the low abundance of this transcript in vivo. This discrepancy may be due to the failure in vitro of a control mechanism which normally represses −105 RNA synthesis in vivo or the stabilization of the −105 transcript in vitro.

The 5′-end heterogeneity of maize *rbcL* RNA could have resulted from multiple transcription initiation sites. Presumably in this case, each 5′ upstream region would contain sequences able to bind RNA polymerase and initiate transcription. To examine this possibility, we analyzed the transcriptional capacities of maize chloroplast DNA templates which included either the −300 (pZmc830) or the −105/−63 (pZmc802) region of the *rbcL* gene. In these clones, chloroplast sequences were oriented such that *rbcL* transcription is convergent with vector *lacZ* transcription. This eliminated potential artifacts arising from promoter occlusion by the upstream *lacZ* promoter (1, 36). The maize chloroplast DNA template containing the −300 region directed accurate transcription initiation in vitro. No in vitro transcripts could be detected for the template which includes only the −105/−63 plastid DNA region. This was confirmed by S1 nuclease protection and primer extension analyses to verify that the negative result for the −105/−63 template was not an artifact of the assay system. These results established that a promoter for maize *rbcL* gene transcription is located within the 109-bp sequence surrounding the −300 site. Comparison of the −300 region of monocot *rbcL* genes and the analogous −180 region of dicot *rbcL* genes uncovered an 80-bp sequence flanking the transcription initiation site which is at least 85% conserved between higher plant species (21, 41; Fig. 7A). DNA sequences surrounding the −105 and −63 sites are less well conserved. The sequence upstream of the −300 5′ terminus contains procaryotelike promoter elements (Fig. 7A). The putative *rbcL* Pribnow box diverged by only 1 nt from the canonical procaryotic sequence. Two potential −35 *rbcL* promoter elements, which are identical to the *E. coli* consensus sequence at the highly conserved TTG nucleotides, were present. The second TTG has a procaryotelike spacing of 18 bp from the putative *rbcL* −10 region. In contrast to the −300 region, no sequences resembling procaryotic promoters could be detected 5′ to the −63 *rbcL* terminus. Although an AT-rich region is located immediately upstream of the −105 site, there is no homology with *E. coli* −10 or −35 consensus sequences. *E. coli* RNA polymerase holoenzyme was unable to initiate transcription within the −105/−63 region, although it transcribed the −300 template accurately (data not shown). These observations, coupled with the capacity of the −300 region to support transcription initiation in vitro by chloroplast RNA polymerase, suggest that chloroplast and procaryotic promoters share sequence homology.

The above conclusion is further supported by the effect of template modification on the transcription of the −300 region. An AT dinucleotide was inserted 11 bp upstream of the maize −300 *rbcL* site (pZmc831). This modification did not change the sequences of the putative −10 and −35 promoter elements, but it altered the distance between them from 18 to 20 bp. In *E. coli*, the optimal distance for transcription between these two regions is 17 bp, and deviations from this distance usually reduce the transcriptional efficiency of the promoter (3, 56). Transcription of the modified −300 template by either maize or pea chloroplast RNA polymerase was depressed in vitro relative to wild-type levels. Furthermore, in the mutant clone, transcription was initiated at position −302 as well as at −300. With the homologous chloroplast RNA polymerase, the −302 RNA was the major in vitro species transcribed from the modified template. The −302 *rbcL* transcript was also synthesized by pea chloroplast RNA polymerase, but at a much lower level. *E. coli* RNA polymerase transcribed the mutant template similarly to the pea enzyme (data not shown). In *E. coli*, insertions and deletions within promoter regions, but outside −10 and −35 sequences, can shift transcription initiation sites. The new sites are associated with the utilization of alternative promoter elements that allow the maintenance of optimal −10 to −35 spacing (42) or favorable distances between −10 elements and upstream regulatory regions (57). Our transcription results did not establish that similar adjustments in promoter recognition occur between chloroplast templates and RNA polymerase. However, examination of the insertion mutant revealed that alternative sequences, which might function as −10 promoter elements, are present (Fig. 7B). The greater sensitivity of the homologous transcription system to changes in the −300 template may reflect the presence of a regulatory factor that is strongly affected by the mutation and absent from the heterologous transcription systems. In maize, *rbcL* mRNA synthesis is cell-type specific (11, 35) and may be subject to control by additional transcription factors.

The transcriptional properties of the maize *rbcL* subclones suggest that the −300 RNA is the primary transcript and that the −105 and −63 RNAs are processed products. This hypothesis is supported by the transcriptional studies of −300 *rbcL* deletion (pZmc820) and insertion (pZmc821) mutants derived from the original 963-bp maize *rbcL-atpB* plasmid (pZmc800). None of the three *rbcL* RNAs was

synthesized when the deletion clone served as template for in vitro transcription. However, the 109-bp deletion of pZmc820 was a major template modification and could have disrupted the function of distal sequences. Therefore, it is significant that the deletion did not interfere with *atpB* gene transcription, even though the 5′ border of its promoter is within 65 bp of the deletion site (unpublished observations). The insertion mutant pZmc821, which differs from pZmc810 only by 2 additional bp upstream of the −300 5′ *rbcL* terminus, does not direct efficient synthesis of any of the *rbcL* RNAs. It is unlikely that this modification would have had artifactual effects on distal sequences. The transcriptional characteristics of pZmc821 also ruled out the existence of promoter elements required for −105 or −63 RNA synthesis which are located outside the 191-bp subclone (pZmc802) and which are not associated with the −300 region. These results established that the presence and integrity of the −300 *rbcL* promoter region are required for −105 and −63 RNA synthesis.

A precursor-product relationship was confirmed between the maize −300 and −63 *rbcL* RNAs. Presumably because of low levels of processing activity in the pea high-salt extract, transcription of the maize *rbcL* gene by the heterologous chloroplast system at 20°C produced exclusively the −300 RNA. This transcript was purified and subsequently incubated with the maize high-salt extract at 30°C in the absence of exogenous *rbcL* template. Analysis of the in vitro RNAs from these reactions showed that the −300 transcript was processed to the −63 RNA in an extract- and time-dependent manner. Crossland et al. (16) reported that the −300 maize *rbcL* RNA was not converted to the −63 species in vitro, but their reaction conditions were different from those used in the experiment described above. We were not successful in resolving *rbcL* transcription and −105 RNA synthesis into separate reactions in vitro. Purified −300 RNA was stable when incubated with pea high-salt extract for 60 min at 30°C, suggesting that if the −300 RNA is processed to the −105 RNA, the processing reaction may involve a complex which cannot be reconstituted in vitro with purified precursor RNA.

Processing of chloroplast transcripts, as reported here for maize *rbcL* RNA, may be a common event in vivo. Multiple, overlapping transcripts have been found to correspond to many regions of the plastid genome (48, 50). Chloroplast tRNA processing has been characterized in vivo (44) and in vitro (26). Gruissem et al. (27) described efficient and correct tRNA processing by a spinach chloroplast extract which is similar to the extracts used in this study. Site-specific 5′-end cleavage of monocistronic mRNA has not been observed in other sytems and may be unique to chloroplasts. In nuclei, mRNA is capped at the transcription initiation site (43). In mitochondria (12, 61) and procaryotes (9, 25), 5′-end mRNA processing separates polycistronic transcripts into individual RNAs.

Multiple *rbcL* transcripts have been observed in several higher plant species, but their physiological importance is unknown. In maize, the synthesis of all three *rbcL* RNAs is dependent on transcription initiation at the −300 promoter. As a consequence, the relative amounts of the three *rbcL* transcripts may be regulated at the level of RNA processing. Processing has been implicated in developmental regulation of *rbcL* transcript levels in maize and barley, because the amount of −300 RNA relative to −63 RNA increases transiently during greening (49, 50). In *E. coli*, processed mRNAs can be translated with different efficiencies and possess distinct turnover rates relative to their corresponding primary transcript (6, 47). Similar mechanisms for the control of *rbcL* gene expression may operate in chloroplasts of higher plants.

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