Single gene for the large subunit of ribulose bisphosphate carboxylase in maize yields two differentially regulated mRNAs

(S1 nuclease mapping/chloroplast RNA polymerase/in vitro transcription)

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ABSTRACT A second mRNA coding for the large subunit of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] has been found in the plastids of maize leaves. The 5' terminus of this mRNA was shown by S1 nuclease analysis to be 238 nucleotides upstream of the previously described large subunit mRNA (McIntosh, L., Poulsen, C., & Bogorad, L., 1980 Nature (London) 288, 556-560). The same two mRNAs were produced in a homologous in vitro transcription system using cloned plastid DNA as a template. The ratio of the two mRNAs changes during light-induced plastid development and can be altered in the in vitro system by manipulation of transcription conditions.

The enzyme ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] is the most abundant protein in green plants (1) and catalyzes the first step in carbon fixation. The holoenzyme is comprised of eight nuclear-encoded (2) small subunit monomers and eight plastid-encoded (2, 3) large subunit (LS) monomers (4). Two levels of regulation of the LS mRNA in maize are known. (i) The abundance of LS mRNA increases during light-induced plastid development (unpublished data), and (ii) LS mRNA is abundant in the chloroplasts of bundle sheath cells from mature leaves but not in the chloroplasts of mesophyll cells (5). In addition, it is assumed that mechanisms exist for coordinating the expression of the plastid-encoded LS genes and the nuclear-encoded small subunit genes. The factors responsible for these regulatory events are unknown. An understanding of LS mRNA synthesis in vivo and development of accurate in vitro systems will be assets for characterizing those factors.

In the present study, we demonstrate that the single LS gene on the maize plastid chromosome (3, 6) encodes two distinct mRNAs that differ in the lengths of their 5'-untranslated regions. The relative abundance of these two mRNA species changes during light-induced development of the plastid. The 5' terminus of the larger LS mRNA is shown to correspond to the site of transcriptional initiation; the generation of the smaller mRNA is discussed.

MATERIALS AND METHODS

DNA. The plasmid pZmc460 consists of an insert of maize chloroplast (cp) DNA BamHI fragment 9 (7) in pBR322. LS-E was constructed by inserting the HincII to BglII subfragment of Bam 9 into pBR322 using G-C tailing. LS-J was derived from LS-E by Bal 31 digestion at the HincII site and ligation. mp8-pZ3 was constructed by inserting the 925-base-pair (bp) Sma I/Avo I fragment of Bam 9 into M13 mp8. Molecular cloning and plasmid DNA isolation were carried out essentially as described (8). Topoisomerase I reactions were carried out as described by the supplier (Bethesda Re-
from the translational start site in earlier work (11). The DNA probe used in those experiments extended only 196 nucleotides upstream from the translational start site. Subsequently, RNA blot hybridization experiments using total maize leaf RNA have shown that there are two RNA species, of 1.6 and 1.8 kilobases (kb), complementary to a LS gene probe (unpublished data). To elucidate the structure of the larger LS mRNA, S1 nuclease protection experiments were carried out using single-stranded DNA from mp8-pZ3, an M13 phage with an insert of cpDNA complementary to LS mRNA from 790 nucleotides upstream to 135 nucleotides downstream of the LS translational start site (Fig. 1). Plastid RNA was isolated from dark-grown plants illuminated for 15 hr and hybridized to excess mp8-pZ3 DNA. The RNA-DNA hybrids were digested with S1 nuclease, subjected to alkaline agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with 32P-labeled LS-E DNA. Two protected bands are seen in the left-hand lane of Fig. 2A. The band of ≈200 nucleotides results from hybridization to the previously described 1.6-kb LS mRNA (11). The band of ≈440 nucleotides, resulting from hybridization to the newly described 1.8-kb LS RNA, locates the 5' terminus of this mRNA ≈500 nucleotides upstream from the translational start site and accounts for the size difference between the 1.8- and 1.6-kb mRNA.

Developmental Regulation of Two LS mRNAs. It was of interest to determine whether the abundance of the two LS mRNAs changed coordinately after dark-grown maize seedlings were exposed to light. Total leaf RNA was isolated from dark-grown plants illuminated for 0, 2, 10, 20, 44, or 68 hr and analyzed by S1 nuclease protection as described in Fig. 2A. The resulting autoradiograph (Fig. 2B) shows that the relative abundance of the 1.8-kb RNA increases during the first 20 hr of light-induced development and then decreases to etioplast levels by 44 hr. Greenhouse-grown plants show the same LS mRNA pattern as dark-grown seedlings illuminated for 44 hr.

In Vitro Transcription Yields Both LS mRNAs. The recombinant plasmid pZmc460 contains the maize plastid DNA fragment Bam 9, which includes the entire LS gene and a portion of the divergently transcribed genes for the β and ε subunits of CF1 (3, 13), cloned in pBR322. pZmc460 was transcribed in vitro using partially purified maize plastid RNA polymerase and [α-32P]UTP plus unlabeled ribonucleotides. The resulting RNA was hybridized to mp8-pZ3, digested with S1 nuclease, and the RNA-DNA hybrids were subjected to gel electrophoresis and autoradiography. As the KCl concentration in the transcription reaction was increased to 100 mM, the specificity of initiation increased (Fig. 3). At concentrations >100 mM, KCl began to inhibit specific initiation. The predominant S1 nuclease protected band of ≈440 bp corresponds to the 5' terminus of the *in vivo* 1.8-kb mRNA.

Previous results have shown that template conformation affects transcriptional preference of the maize plastid RNA polymerase *in vitro* (14). To determine the effect of template conformation on the transcription initiation of the 1.8-kb LS mRNA, autoradiograms were exposed for different times after a 2 hr exposure, and the exposed areas were compared using a densitometer to determine the relative 1.8-kb and 1.6-kb mRNA levels. These results are shown in Fig. 2C. The relative area for the 1.8-kb mRNA increases as the exposure time is increased, indicating that the 1.8-kb mRNA is preferentially transcribed. Further experiments are needed to determine the effect of template conformation on the transcription initiation of the 1.6-kb LS mRNA.
mRNA, pZmc460 was linearized with BamHI, relaxed with DNA topoisomerase, or subjected to mock incubation without topoisomerase. Transcription of the modified template in vitro followed by S1 nuclease analysis of the product demonstrated that relaxation results in a decrease in transcription of LS mRNA by a factor of 5 to 10 (Fig. 3).

When pZmc460 was transcribed with an unpurified plastid extract in the absence of added MgCl₂ (not shown) or in the presence of 50 mM sodium citrate, total transcription decreased by a factor of 20. The 5' termini corresponding to both the 1.6- and 1.8-kb in vivo RNAs could be seen after S1 nuclease analysis of these transcription products (Fig. 4B).

5' Terminus of the 1.8-kb LS mRNA. To precisely map the 5' terminus of the 1.8-kb LS RNA, total leaf RNA isolated from dark-grown seedlings illuminated for 20 hr was hybridized to a 5'-end-labeled Dde I/EcoRI DNA fragment that extends from 415 to 227 nucleotides upstream from the LS translational start site (Fig. 1). After S1 nuclease digestion, the protected DNA fragment was electrophoresed alongside DNA sequencing reactions of the same Dde I/EcoRI fragment. The cluster of protected fragments observed (Fig. 5) locates the 5' terminus of the 1.8-kb LS mRNA at 302–303 nucleotides upstream from the translation start site. In vitro transcribed LS mRNA protected the same fragment as in vivo RNA, although the band intensity was lower. As in other plastid genes examined (16), sequences resembling the Escherichia coli consensus promoter are found just upstream from this 5' terminus (see Fig. 7).

The Upstream 5' Terminus Is a Transcriptional Start Site. To determine whether the 5' termini of either the 1.8- or 1.6-kb RNAs represent in vivo transcriptional start sites, RNA from greenhouse-grown plants was end-labeled in vitro using [α-32P]GTP and vaccinia virus guanylyltransferase (17, 18). The labeled RNA was hybridized to mp8-pZ3, and the RNA-DNA hybrids were digested with S1 nuclease and subjected to gel electrophoresis. Because the guanylyltransferase reaction is specific for di- or triphosphate 5' termini (19–21), only termini representing unmodified transcriptional start sites will be labeled. Several intensely labeled bands survive the S1 nuclease digestion, in the presence or absence of the hybridized probe mp8-pZ3 (Fig. 4A). These presumably represent end-labeled abundant RNAs with S1 nuclease-resistant secondary structures. The only probe-dependent band comigrates with the 1.8-kb RNA-protected band. Although RNA blot hybridization showed the 1.6-kb species to be the predominant LS RNA in the sample assayed (data not shown), no guanylyltransferase-labeled residue of the 1.6-kb RNA was seen.

Because only the 1.8-kb in vivo LS mRNA could be labeled by guanylyltransferase, an experiment was carried out to determine whether DNA sequences surrounding the 1.8-kb transcriptional start site are essential for production of the 1.6-kb RNA in vivo. Subfragments of cpDNA fragment BamHI-9 lacking the start site for the 1.8-kb RNA were inserted into pBR322 and used as templates for in vitro transcription and S1 nuclease analysis as described. The insert in plasmid LS-E was truncated at the Hinfl site between the
1.8- and 1.6-kb RNA 5' termini (Fig. 1) and thus contains the 194 bp upstream from the 1.6-kb RNA 5' terminus. Plasmid LS-J was derived from LS-E by Bal 31 nuclease digestion inward from the HincII site, and extends ≈80 bp upstream from the 1.6-kb RNA 5' terminus. Neither LS-E nor LS-J served as templates for the production of detectable 1.6-kb mRNA under transcription conditions where both RNAs were synthesized from pZmc460 (Fig. 4B).

The above results would be explained if the 1.6-kb RNA is derived from the 1.8-kb RNA by specific cleavage. Attempts were made to determine whether the crude plastid extract, shown to produce both LS mRNA species in vitro, could process the 1.8-kb RNA into the 1.6-kb RNA, RNA isolated from dark-grown seedlings illuminated for 20 hr (when the 1.8-kb mRNA is most abundant) was incubated in the extract under conditions used for transcription in Fig. 4 and was analyzed by S1 nuclease protection as described above. No shift in the relative abundance of the two LS mRNA species was evident after 30 min of incubation (Fig. 6). Continued incubation of in vitro synthesized LS mRNA in the plastid extract also failed to demonstrate a shift from 1.8 to 1.6 kb (data not shown).

DISCUSSION

Two mRNAs transcribed from the single maize plastid LS gene have been detected by S1 nuclease analysis of in vivo RNA, confirming previous RNA blot hybridization experiments and locating the additional sequences of the 1.8-kb RNA at its 5' end. The same two LS mRNAs were produced in a homologous in vitro transcription system using cloned maize DNA as a template. Subsequent high resolution S1 nuclease analysis has shown that the 1.8-kb mRNA begins ≈238 nucleotides upstream of the previously mapped 1.6-kb mRNA 5' terminus.

During light-induced development of maize plastids, the ratio of abundance of the 1.8- to the 1.6-kb LS mRNA increases, peaks after 20 hr of illumination of dark-grown seedlings, and then falls back to the ratio found in etioplasts by 44 hr. To understand the mechanism underlying the differential production of these two mRNAs during development, it is necessary to describe the production of each mRNA. Guanylyltransferase labeling and S1 nuclease experiments have demonstrated that the observed 5' terminus of the 1.8-kb mRNA is its in vivo transcription start site. Two mechanisms for production of the 1.6-kb mRNA are possible: a second transcriptional start site may exist or the 1.8-kb RNA may be cleaved 238 nucleotides from its 5' terminus. Sequences resembling prokaryotic promoter regions are found upstream from the 5' terminus of the 1.6-kb mRNA (22), although these sequences are 15 to 20 bp further from the 5' terminus than is usually found. Both the 1.8- and 1.6-kb mRNAs are produced in vitro (Fig. 4). However, the in vitro system does not appear to convert the 1.8- to the 1.6-kb species under the same incubation conditions (Fig. 6), suggesting that the 1.6-kb species may arise independently. However, the inability of guanylyltransferase to label the 1.6-kb mRNA suggests that its in vivo 5' terminus may result from removal of the initiating nucleotide (and possibly additional nucleotides) or removal of the second and third phosphates from the initiating nucleotide. In addition, DNA sequences extending only 80 or 195 bp upstream from the 1.6-kb 5' terminus do not serve as templates for the production of detectable 1.6-kb RNA in vitro, suggesting that sequences near the 1.8-kb start site may be necessary for production of the 1.6-kb mRNA. If the 1.6-kb mRNA results from an independent transcription start, its in vivo 5' terminus must be modified and its in vitro functional promoter must extend further than 195 bp upstream. If the 1.6-kb mRNA results from processing the 1.8-kb transcript, either the processing activity of this in vitro system is very low or processing occurs only as a cotranscriptional event.

Comparison of the DNA sequence upstream from the translational start site of the tobacco (23) and spinach (24) LS genes with that of maize (11, 13) reveals two regions of homology separated by a nonhomologous region of variable size (Fig. 7). Proceeding upstream from the initiating ATG codons, the spinach sequence is identical to maize until −18 bp, and tobacco is nearly identical to maize until −65 bp (the 5' terminus of the 1.6-kb mRNA of maize). A second region of homology centers around the transcriptional start site for the 1.8-kb maize mRNA at −302 bp. If the 5' termini of the sole LS mRNAs of tobacco (23) and spinach (24) are aligned...
with this maize start site, a region of nearly perfect homology extends for 19 bp downstream and 39 bp upstream. The sequences preceding the 1.6-kb mRNA 5' terminus are not homologous to sequences preceding the tobacco and spinach genes. We conclude that the maize 1.8-kb LS mRNA represents the homologue of the spinach and tobacco transcripts.

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   "TTGACA -330 TA TAAT 1.8kb"

   maize: GATTAGGGT TGGGTTGCGC TATATCTATC AAGAAAATGA TATGTTGTCG AGTTAACTTAC ATCGTATTG CATGGTAATG TGAAGAGATT ATTGCTCGCT ATGCTGTAAGA

tobacco: GAGTAAAGT TATATCTATC AAGAAAATGA TATGTTGTCG AGTTAACTTAC ATCGTATTG CATGGTAATG TGAAGAGATT ATTGCTCGCT ATGCTGTAAGA

   spinach: GTGGTGGCC CATAATATC AAGAAAATGA TATGTTGTCG AGTTAACTTAC ATCGTATTG CATGGTAATG TGAAGAGATT ATTGCTCGCT ATGCTGTAAGA

   CGTGTATTTA TCGGGTTGCA TATATTATG TATGTTGTCG AGTTAACTTAC ATCGTATTG CATGGTAATG TGAAGAGATT ATTGCTCGCT ATGCTGTAAGA

   FIG. 7. Comparison of LS promoter region sequences. The sequences upstream from the initiating ATG codon of the maize (11, 13), tobacco (23), and spinach (24) LS genes are compared. The numbers above each line denote base pairs upstream from the initiating ATG of maize. The 5' termini of the 1.6-kb maize mRNA (11) and 1.8-kb maize mRNA (aligned with the tobacco (23) and spinach (24) termini) are shown by arrowed lines. The tobacco and spinach sequences were arbitrarily interrupted to align the two major regions of homology.