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Transcription analysis of the maize chloroplast gene for the ribosomal protein S4

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ABSTRACT

Maize seedlings contain several RNA species complementary to the rpS4 coding strand of the maize chloroplast ribosomal protein gene rpS4. All of these have the same 5' end about 182 bp upstream of the translation start codon for the protein S4. Northern and S1 nuclease analyses of RNA isolated from seedlings at different stages of greening show that the size of the pool of rpS4 transcripts does not change significantly upon illumination of dark-grown seedlings. The rpS4 gene has also been analyzed by in vitro transcription using maize chloroplast RNA polymerase preparations. The site of initiation in vitro has been mapped by S1 nuclease analysis to the same location as the 5' terminus of in vivo transcripts. A sequence resembling other plastid promoters occurs just upstream of this initiation site. The sensitivity of in vitro transcription to DNA template superhelicity has been assessed for the rpS4 gene promoter; its negative superhelicity-transcription rate profile resembles that of rbcL.

INTRODUCTION

The plastid genome has been shown to contain genes for a wide variety of proteins utilized in the plastid (1). Although many genes have been identified, regulation of their expression is still not well understood. To begin to understand the mechanisms for the control of expression of these genes during plastid differentiation, we have been examining the features of plastid transcription and the expression of different classes of maize chloroplast genes.

One crucial step towards understanding chloroplast transcription is the availability of an accurate and efficient, preferably homologous, in vitro transcription system. Maize chloroplast RNA polymerase has been isolated and has been shown to faithfully transcribe from chloroplast promoters (2-5). Recently, in vitro transcription systems have also been developed in several other higher plants (6-8). Using these in vitro transcription systems the general structure of the chloroplast promoter is becoming clearer. Based on comparisons of DNA sequences as well as deletion mutation analysis, each chloroplast promoter recognized to date has a "prokaryotic-like" structure

with two regions homologous to bacterial "-35" and "-10" promoter sequences upstream of the site of transcription initiation (7, 9, 10, and L.D. Crossland and L. Bogorad, unpublished). While the general structure of plastid promoters is known, only a little information is available about differential gene expression (5-11). To learn more about differential transcription, we have begun to characterize promoters for genes whose RNAs show different patterns of accumulation *in vivo*.

In this paper we present an analysis of *in vivo* transcript levels and *in vitro* transcription of the maize chloroplast gene *rp*S4. The *rp*S4 gene codes for chloroplast ribosomal protein S4, that, by analogy with *E. coli*, is thought to be involved in the early stages of ribosome assembly (12). Since it is likely that the product of this gene is needed throughout plastid development, the gene is probably expressed at very early, if not all, stages of plastid differentiation. The abundance of *rp*S4 transcripts has been followed during greening of dark-grown seedlings and 5' ends of these transcripts have been mapped. We have identified the site of initiation of *rp*S4 transcription in an *in vitro* homologous transcription system. We have assessed the sensitivity of *in vitro* transcription to the level of DNA template superhelicity. This is only the third plastid gene to be analyzed in this way; the two previously studied genes--maize genes *rbc*L and *atp*BE--responded quite differently from one another in this assay.

METHODS

DNA constructions and topoisomer preparation.

The following plasmids were constructed by inserting the indicated maize plastid DNA fragment into pBR322: pZmc541, BamHI fragment 5; pZmc748, EcoRI O (subclone of pZmc541); pZmcS4-320, 320 bp TaqI *rp*S4 promoter fragment; and pZmcS4-252, a 252 bp HinfI *rp*S4 promoter fragment (13 and D.R. Russell, unpublished). M13 mp9-*rp*S4 was constructed by inserting a 834 bp BglII-HindIII fragment containing the *rp*S4 promoter region into M13 mp9. In this orientation the DNA will hybridize to RNA synthesized from the *rp*S4 promoter. pSP64-320A and -320B consist of the 320 bp TaqI fragment inserted in both orientations into pSP64. Topoisomers of plasmid DNA containing the *rp*S4 promoter were prepared as described by Stirdivant et al. (5).

RNA isolation and S1 nuclease mapping.

Total leaf RNA was isolated as previously described and the concentration was estimated spectrophotometrically (14). Northern blot and slot blot

analyses were performed as described elsewhere (15), except that slot blots were performed using a slot blot manifold from Schleicher and Schuell. Total leaf RNA was used as described (16) for S1 nuclease mapping of the 5' ends of RNAs. S1 nuclease mapping of the 3' end of RNA was by a similar procedure except that the DNA fragment used as a hybridization probe was labeled at its 3' termini with Klenow fragment. SP6 RNA polymerase probes were generated by the procedure of Melton et al. (17).

Maize chloroplast transcription.

Maize plastid RNA polymerase was purified as described (5). A typical transcription reaction consisted of: 40 mM Tris-HCL pH 8, 10 mM $MgCl_2$, 75 mM KCl, 10 % glycerol (v/v), 2.5 mM dithiothreitol, 200 uM each of ATP, GTP, and CTP, 15 uM UTP (2 uM alpha ^{32}P-UTP), 20 ug/ml DNA, and chloroplast RNA polymerase in a 20 ul final volume. Except where noted in the text, all transcription reactions were performed at 37°. S1 nuclease analysis was as described (4) except that the products were separated on both native and denaturing polyacrylamide gels.

RESULTS

Analysis of in vivo rpS4 RNA.

RNA was isolated from maize seedlings to study the transcription of the rpS4 gene in vivo. The RNA was analyzed by the Northern blot procedure to assess the complexity and approximate sizes of the RNAs complementary to the gene sequence. S1 nuclease mapping was then used to precisely locate what was found to be the common 5' end and a presumptive 3' end of the rpS4 transcripts. In addition, the relative abundance of rpS4 RNA was assessed at a number of stages during the greening of the maize seedlings.

The ribosomal protein S4 may be involved in ribosome assembly, and would be required at early stages of plastid development. Therefore one would predict that rpS4 RNA should be detected at early stages of plastid development, i.e. during germination and growth of the etiolated plant in darkness as well as during greening. Total leaf RNA was isolated from maize seedlings grown in darkness for 7 days and then illuminated for various periods up to 44 hours. Equal amounts of RNA were then applied to an agarose gel, separated electrophoretically, transferred to Gene Screen filters and hybridized to different plastid genome probes.

Initially, pZmc748, a plasmid containing the entire rpS4 gene region, was nick-translated and hybridized to the RNA blots. This probe detected multiple RNAs. Smaller, strand-specific probes were generated using the SP6

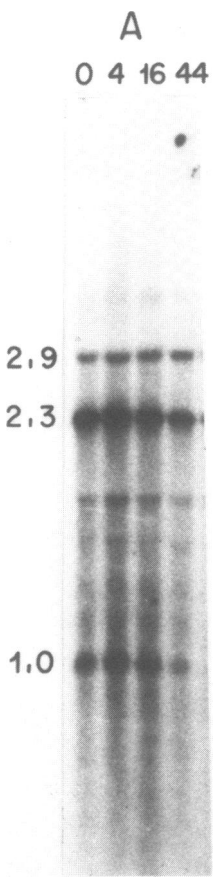

Figure 1. Northern analysis of rpS4 RNA.
Total leaf RNA was isolated from dark-grown maize seedlings illuminated for 0 to 44 hours. The blots were probed with a SP6 RNA transcribed from SP64-320A which hybridizes about 320 bp of the 5' rpS4 region. RNAs of 2.9, 2.3, and 1.0 kb were detected (several minor bands from 1.7 to 1.1 kb in length are also detected upon longer exposure).

polymerase system (17) and subcloned fragments of the rpS4 gene region. The smallest of these probes (designated SP64-320A) hybridized to the first 12 bp of the coding region of the rpS4 gene and 307 bp upstream. Figure 1 shows that the coding region complementary to the rpS4 RNA hybridized predominantly to three RNAs: 2.9 kb, 2.3 kb, and 1.0 kb. The opposite strand hybridizes to a single RNA of approximately 3.0 kb (data not shown). The latter RNA appears to be less abundant than the complementary rpS4 RNAs and the level of

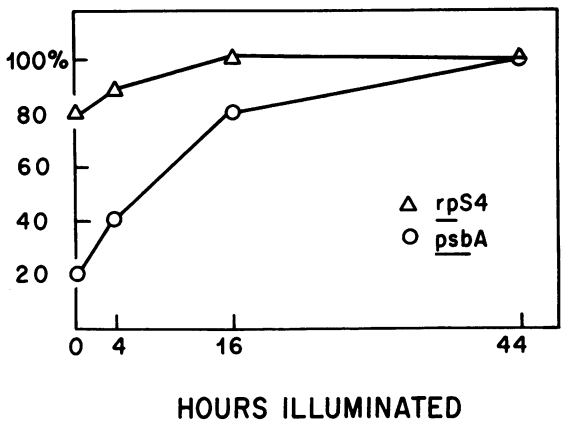

Figure 2. Slot blot analysis.
Equal amounts of total leaf RNA were loaded onto slot blot nitrocellulose filters and hybridized to the probes described above. Hybridization to the RNA was then quantitated by densitometry and the results plotted as a percentage of the maximum amount detected. S4 mRNA increases slightly upon illumination (etioplast S4 mRNA is about 80% of the maximum S4 mRNA detected at 16 hours, (A)). For comparison, the Q_B protein mRNA in the same sample increased about 5-fold over the same greening period (B).

this RNA does not change significantly during greening. This 3.0 kb RNA may be a precursor containing several tRNAs coded for on the opposite strand of the rpS4 gene.

Figure 1 also suggests that the in vivo steady-state levels of the rpS4 RNAs change only slightly, if at all, over the greening period tested. Earlier work has shown that under these greening conditions psbA RNA increases 5 to 10 fold while 16S rRNA does not change significantly (15). To better quantitate the levels of rpS4 RNA, RNA abundancies were measured by slot blot hybridization. Figure 2 shows that rpS4 RNA increased only slightly over the first 16 hours of illumination. Over this same greening period, the 16S rRNA also shows little change (data not shown) while the psbA RNA levels increased at least 5 fold in the same samples.

S1 mapping of the 5' end.

To localize the 5' ends of the RNAs complementary to rpS4, a hybridization probe was used which covers the region from 455 to 1133 bp upstream of the rpS4 gene. In Northern experiments, this probe did not reveal any RNAs hybridizing to the rpS4 gene region (data not shown). However, a probe containing the region 455 bp upstream of the coding region to 379 bp in the coding region did detect RNAs complementary to rpS4. Therefore it is likely

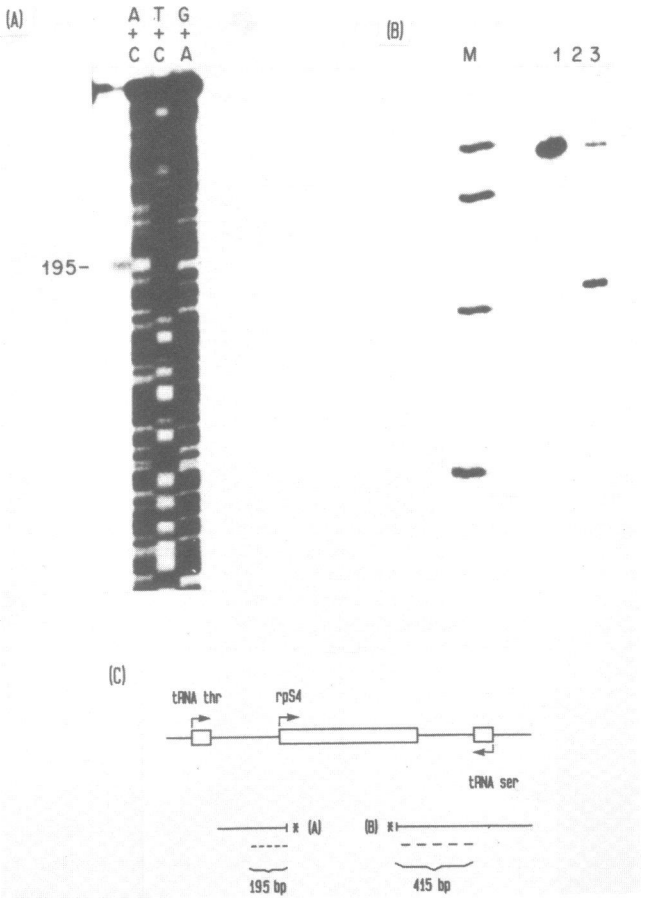

Figure 3. S1 nuclease mapping of 5' and 3' ends of rpS4 RNA.

(A). A 320 bp TaqI fragment was 5' end-labeled and the DNA strands were separated to produce a single-end labeled DNA probe. The strand which hybridizes to rpS4 RNA was hybridized to total leaf RNA, digested with S1 nuclease, and separated on a denaturating polyacrylamide gel. Sequencing reactions were run in adjacent lanes. A band was detected at about 195 bp which coincides with a 5' end about 182 bp upstream of the rpS4 start codon. The end may be at one of two or three adjacent A residues. (B). A 590 bp MspI-EcoRI fragment was 3' end-labeled at the MspI site, hybridized to total leaf RNA, digested with S1 nuclease, and the protected fragment was separated on a denaturing polyacrylamide gel. The reactions contained the labeled DNA probe plus the following: lane 1, maize RNA but no S1 nuclease; lane 2, S1 nuclease but no maize RNA; and lane 3, S1 nuclease and maize RNA; note that in lane 3 only one band of about 415 bp was detected along with some full-length probe. The size standards shown in the lane designated "M" are 622, 527, 404, and 309 bp. (C). The figure shows a schematic diagram of the gene structure around the rpS4 gene. The tRNA ^{thr} is transcribed from the same strand as rpS4; the tRNA ^{ser} is transcribed from the opposite strand. The positions of the DNA probes used in mapping the 5' (A) and 3' (B) ends of the RNAs are shown.

that the 5' end of each of the complementary RNAs lies within 455 bp of the coding region of the rpS4 gene.

S1 nuclease mapping was used to further define the 5' end and the possible site of transcription initiation of the longest complementary RNA. A 320 bp TaqI fragment was isolated from pZmc748, 5' end-labeled with gamma ^{32}-P ATP, and strands were separated to produce a single-end-labeled single-stranded DNA probe spanning the 5' region from +12 to -307 with respect to the translation start codon (see Figure 3 C). This probe was hybridized to total-leaf RNA, digested with S1 nuclease, and the products separated on a denaturing polyacrylamide gel (see Figure 3 A). Only one protected band of about 195 bp was detected. This places the 5' end of the RNA about 182 bp upstream of the translation start codon. The start is at one of two or three adjacent A nucleotides in the center of a region of 6 A nucleotides. A small amount of full-length 320 TaqI fragment was also detected on the autoradiograph. The latter could represent incompletely denatured probe isolated from the strand-separation gel or RNA(s) that extend 5' to the end of the probe fragment and therefore protect the full length of the probe. To test these possibilities, a single-stranded M13 clone (containing the 834 bp region from the middle of the rpS4 gene at the HindIII site at +379 to the 5' BglII site at -455) was hybridized to total-leaf RNA, digested with S1 nuclease, separated on an alkaline agarose gel, blotted and probed with an rpS4 DNA probe (data not shown). Again, only one protected band was detected (about 560 nucleotides) which marked the 5' end at the same site shown using the smaller probe.

Since only one 5' end was detected within 455 bp upstream of the 5' translation codon and all three RNAs detected hybridize to a probe of this region (none hybridize to probes upstream of position -455), all three RNAs must share a common 5' end 182 bp upstream of the rpS4 gene. As shown below, this 5' end coincides with the 5' end of the in vitro transcript of rpS4.

S1 mapping of a 3' end.

Knowing that the three most abundant RNAs complementary to rpS4 have common 5' ends, the 3' ends of these RNAs could be estimated to lie about 200, 1500, and 2100 bp downstream of the coding region. The latter two would probably fall within the adjacent, BamHI fragment (BamHI-14). Since the sequence of Bam HI fragment 14 is not known, no attempts were made to locate the 3' ends of the two larger RNAs. However the smallest RNA (about 1 kb) would lie within BamHI-5 and would be long enough to code for the protein. To locate the 3' end of this RNA, a 590 bp MspI 3' end-labeled EcoRI fragment

was isolated (see Figure 3 C) and used in S1 nuclease mapping as described above. A strong protected band of about 415 nucleotides was found and a smaller amount of full-length probe was detected (Figure 3 B, lane 3). Considering the location of the 5' end mapped above, the RNA is about 1070 nucleotides long and corresponds in size to the 1 kb RNA observed by Northern analysis. The 3' end abuts the 3' end of the tRNA ^{ser} coded for on the other strand.

Analysis of in vitro rpS4 promoter transcription.

The 5' end of the transcript of rpS4 produced by maize chloroplast RNA polymerase in vitro was located by hybridization of radioactive RNA to unlabeled single-stranded DNA spanning the presumptive promoter region, S1 nuclease digeston and gel electrophoresis (4). The unpaired parts of the RNA-DNA duplex were digested away with S1 nuclease and the remaining trimmed, duplex fragments were subjected to gel electrophoresis and measured by autoradiography. The single-stranded hybridization probe used to detect the rpS4 promoter was an 834 nucleotide fragment (spanning 380 bp of the rpS4 coding region and the adjacent 455 bp upstream of the start codon) cloned into m13 mp9.

Transcription from several increasingly smaller DNA templates was used to localize the 5' end of the rpS4 transcript (data not shown). The largest DNA template used was pZmc541, containing BamHI fragment 5; it is about 6000 bp and includes the entire rpS4 gene plus about 5000 bp upstream of the gene. In addition two shorter templates were transcribed: pZmcS4-320, containing a subclone which spans the region from -124 to +195 with respect to the 5' end of the RNA mapped in vivo, and pZmcS4-252, containing a subclone spanning the region from -76 to +176. For each transcription, the same single-stranded DNA probe described above was used to locate the 5' end of the in vitro synthesized RNA. The position of transcription initiation, can be deduced from nuclease resistant RNA-DNA duplexes. Protected duplexes of 565 bp, 195 bp, and 176 bp all indicate a single in vitro transcription start site 182 bp upstream of the rpS4 translation start codon.

To define the transcription start site more precisely, the S1 nuclease protected RNA from a transcription of pZmcS4-320 was separated on a denaturing polyacrylamide gel with sequencing reactions for size standards. Figure 4 shows a single band centered around 195 bp, placing the start site around 182 bp upstream of the start codon of the rpS4 gene. The start site may be at one of two adjacent A residues.

Previous tests of maize chloroplast RNA polymerase preparations of the

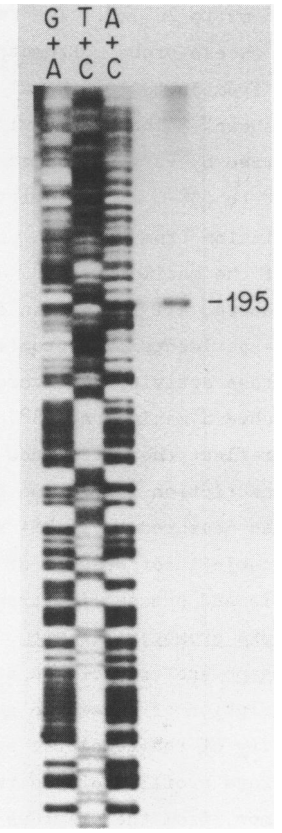

Figure 4. In vitro transcription to locate the 5' end of the rpS4 transcript.

The S1 nuclease-protected RNA from a transcription of pZmcS4-320 was run out on a denaturing polyacrylamide gel alongside DNA sequencing reaction ladders to more closely locate the position of *in vitro* transcription initiation. A single band of about 195 bp was detected. Transcription is initiated from one of two adjacent residues.

type used here showed them to be devoid of nucleases that act on transcripts of *rbc*L (4). Crude extracts of chloroplasts are reported to contain enzymes that process primary transcripts of some tRNAs (6) but we have used more highly purified preparations (5).

Effects of ionic strength and temperature on transcription.

Conditions for optimal *in vitro* transcription were assessed by varying the ionic strength and the temperature of transcription incubation (data not shown). Transcription sensitivity to ionic strength was tested by varying

the KCl concentration from 25 mM to 200 mM. The resultant RNA was analyzed by S1 nuclease mapping using excess probe. Promoter-specific transcription of rpS4 reached a maximum in from 50 to 100 mM KCl and declined to less than 50% by 150 mM KCL (data not shown). The sensitivity of in vitro transcription to temperature was assessed by varying the temperature of incubation during transcription from 10° to 43°. Transcription was almost undetectable below 25°. Transcription from the rpS4 promoter peaks at 37° and then declines to about 50% of the maximum by 43°. [We have also tested three other maize chloroplast promoters, all reached an optimum at 30° to 37°, (data not shown).] Earlier experiments by Bottomley et al. (18) measured maize chloroplast RNA polymerase activity on heterologous DNA templates and found that RNA synthesis reached a maximum at 48°. The differences in temperature optima probably reflect the difference in assays; the S1 nuclease assay used here measures transcription initiation from a specific chloroplast promoter while earlier studies measured total RNA synthesis assayed by trichloroacetic acid precipitable incorporation of ^{3}H-UTP, which would include both promoter specific and nonspecific transcription.

Effects of supercoiling on rpS4 promoter activity.

The maize genes rbcL and atpB are transcribed at different rates in vitro (11) and the relative transcription of these two genes has been shown to vary with the level of superhelicity of the template; surprisingly, negative superhelicity-transcription rate profiles of the two genes are strikingly different (5). We did not know, from the previous work, how other plastid genes would behave, whether either the rbcL or the atpBE profile is characteristic of most chloroplast genes, etc. Therefore, we have examined the effect of DNA conformation on transcription from the rpS4 promoter. Plasmid DNA containing the rpS4 gene was incubated with topoisomerase and increasing concentrations of ethidium bromide (19) to produce a series of topoisomers ranging from relaxed DNA to topoisomers with sigma values up to about -0.145 [plasmid DNA isolated from E. coli is usually about -0.05 (20)]. The topoisomers were transcribed with maize chloroplast RNA polymerase and the resultant RNA analyzed by S1 nuclease digestion (see Figure 5). Transcription from the rpS4 promoter is almost undetectable from relaxed or linear DNA templates. Transcription increases sharply as the supercoiling level increases to a maximum, approximately 15 times greater, near -0.06 and then decreases slowly at higher levels of supercoiling.

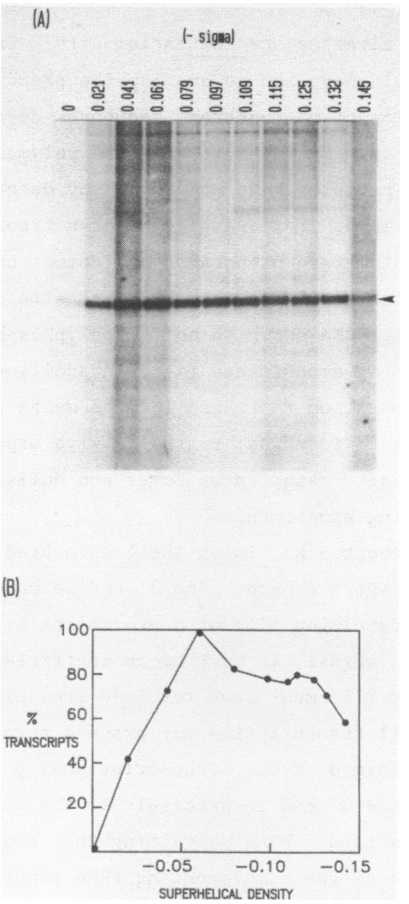

Figure 5. The effects of negative supercoiling on transcription from the rpS4 promoter.

A series of topoisomers of pZmcS4-320 plasmid DNA were provided as templates for maize chloroplast RNA polymerase. The RNA produced was hybridized to single-stranded probe DNA and the resultant ^{32}P-labeled RNA-DNA duplex was subjected to S1 nuclease digestion. (A) shows a autoradiogram of a polyacrylamide gel used to separate the duplexes containing the transcription products (the protected rpS4 RNA is about 195 nucleotides). The relative frequency of transcription initiation was quantitated by densitometry of autoradiograms and the results are plotted in (B) as a percentage of the maximum transcription detected.

DISCUSSION

Mapping with different probes has shown that the product of transcription of rpS4 in vitro as well as the three predominant leaf RNAs complementary to rpS4 have a common 5' end, i.e. they originate from a single promoter, presumably the rpS4 promoter. We feel this site represents a transcription

initiation site and not a processed end: We have deleted upstream sequences to position -76 without altering the initiation site. If processing occurred an upstream promoter would have had to overlap the promoter sequence described here but an examination of the upstream sequences does not suggest a stronger promoter sequence. Furthermore, the RNA polymerase preparations used to transcribe this promoter have not shown any detectable processing activity of mRNA transcripts. For example, we have transcribed the rbcL promoter with the RNA polymerase preparaton and detect only the expected site of initiation (data not shown). The in vitro rbcL site is identical to the 5' end of the in vivo rbcL RNA shown to be the polyphosphorylated 5' terminus in earlier in vitro capping experiments (4). In addition, we have used the same RNA polymerase preparation to transcribe the maize psbA gene and again observed an initiation site identical to the in vivo mapped site [which, in turn corresponds to the site reported by Boyer and Mullet (21) for pea psbA RNA using in vitro capping experiments].

The shortest RNA, about 1 kb, is of the length predicted by the 5' and 3' S1 nuclease mapping reported here. The 3' end we have mapped may represent a transcription termination site or a processing site. If the 3' end represents a termination signal, it must be an inefficient terminator as the longer two RNAs (2.9 and 2.3) must have resulted from elongation through this site. Alternatively, all transcription may proceed through this region. The 1 kb RNA would then be formed by post transcriptional processing. It is important to note that the 3' end is precisely at the end of the $tRNA^{ser}$ coded for on the other strand. RNAs containing this region may form strong secondary structures due to the complementing tRNA sequences. If this structure forms in vivo, the stem and loop structures may act as a RNA processing signal, generating the 1 kb RNA observed. At present, we do not know which RNA(s) are translationally active. Since the 1 kb RNA would contain only the rpS4 coding sequence and none of the adjacent tRNA sequences, it is possible that this RNA may be an adequate template for the synthesis of the S4 protein. As indicated in Figure 3C, rpS4 is flanked by two tRNA genes. The upstream $tRNA^{thr}$ is transcribed from the same strand as the rpS4 mRNA. No transcripts spanning both the tRNA ^{thr} and rpS4 genes have been detected by Northern blotting.

The abundance of rpS4 transcripts, relative to total RNA, does not change significantly when dark-grown maize seedlings are illuminated for up to 44 hours. Under these same greening conditions 16S rRNA also showed little change while psbA mRNA, for example, increased about 5-fold (15). In other experiments we have shown that chloroplast DNA/gram fresh weight of leaf

tissue increases linearly and only about 2-fold during the first 16 hours of greening (D.R. Russell, unpublished). Therefore, at least at the level of steady-state pools of RNA, rpS4 appears to be constitutively expressed independently of the gene copy number.

Sensitivity of transcription of rpS4 by the homologous RNA polymerase to negative superhelicity of the template has also been examined. Transcription of rpS4 reaches a maximum at sigma = -0.06 and then decreases to about 50% of the maximum activity at sigma = -0.145. The point of maximal transcription sensitivity is similar to that observed for transcription from the rbcL gene promoter (5). Both rpS4 and rbcL gene promoters are optimally transcribed *in vitro* from templates at a superhelical density of about -0.06 and then transcription from both decline at higher levels of negative supercoiling. However, transcription from the rpS4 promoter declines more slowly than for rbcL transcription; at sigma = -0.1 rbcL transcription is less than 50% of maximal, while rpS4 transcription does not decline to 50% until much higher levels of negative supercoiling. This pattern is markedly different than that observed for the atpBE gene which does not reach maximal transcription until higher levels of negative supercoiling and then remains at the transcription maximum (5). However, whereas transcription of rbcL was at least 50 times higher at optimal negative superhelicity than from the relaxed template, transcription of rpS4 was only about 15-fold greater.

In Figure 6, the sequence of the probable rpS4 promoter region is compared with three other maize chloroplast promoters from which transcription has been studied *in vitro*. All four promoters show some sequence homologies and appear to contain the general features of a "prokaryotic-like"

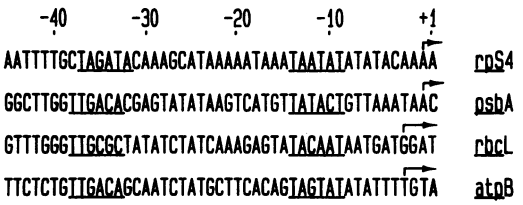

Figure 6. Maize chloroplast promoter sequences.
Sequences are listed for four promoters which have been transcribed in the maize plastid RNA polymerase *in vitro* system. The sites of initiation from these promoters have been confirmed both *in vivo* and *in vitro* (rbcL, Crossland et al., 1984; atpBE, Krebbers et al., 1982, Stirdivant et al., 1985; psbA, L.D. Crossland and L. Bogorad, unpublished; rpS4, this paper). The regions of partial sequence homology with prokaryotic promoters are underlined.

promoter as noted for other plastid genes (9). This is consistent with promoter deletion experiments for the psbA genes of mustard (7) and maize (L. Crossland and L. Bogorad, unpublished) and the $tRNA^{met}$ gene of spinach (10). The four maize chloroplast promoters have been aligned to maximize the conservation of nucleotides and spacing shown to be common in prokaryotic promoters (the first TA and last T in the -10 region, TTG in the -35 region, and a spacing of 16 to 18 bp between the -35 and -10 regions). When aligned in this manner, all four promoters have TG variations just upstream of the -35 region. One difference among the four promoters is the high dA-dT content of the rpS4 promoter (85% for rpS4 as compared to 65% for the other three promoters).

At present we do not feel we can correlate DNA sequence with in vitro promoter strength and differential expression. In preliminary experiments we have observed that the relative promoter strength is dependent on in vitro transcription parameters such as ionic strength, temperature, and promoter DNA template superhelicity. In general, we have found that the in vitro promoter strengths of the rpS4, rbcL, and atpB gene promoters are all within the same order of magnitude while the psbA gene promoter is consistently 3- to 5-fold stronger (data not shown). A direct correlation of promoter sequence and strength will require further experiments.

ACKNOWLEDGEMENTS

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