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Differential expression of the partially duplicated chloroplast S10 ribosomal protein operon

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Abstract. The chloroplast S10 ribosomal protein operon is partially duplicated in many plants because it initiates within the inverted repeat of the circular chloroplast genome. In spinach, the complete S10 operon ($S10_B$) spans the junction between inverted repeat B (IR_B) and the large single-copy (LSC) region. The S10 operon is partially duplicated in the inverted repeat A (IR_A), but the sequence of $S10_A$ completely diverges from $S10_B$ at the junction of $S10_A$ and the LSC region. The DNA sequence shared by $S10_A$ and $S10_B$ includes *trnI1*, the *rpl23* pseudogene (*rpl23ψ*), the intron-containing *rpl2* and *rps19*, which is truncated in $S10_A$ at the $S10_A$/LSC junction (*rps19′*). Transcription of *rps19′* from the promoter region of $S10_A$ could result in the synthesis of a mutant S19 protein. Analysis of RNA accumulation and run-on transcription from $S10_A$ and $S10_B$ using unique probes from the $S10_A$/LSC and $S10_B$/LSC junctions reveals that expression of $S10_A$ is reduced. The difference in $S10_A$ and $S10_B$ expression appears to be the result of reduced transcription from $S10_A$, rather than differences in RNA stability. Transcription of $S10_B$ can initiate at three distinct promoter regions, P1, P2 and P3, which map closely to transcripts detected by S1 nuclease analysis. P1 is located upstream of *trnI1* and has the highest transcription initiation frequency in vitro of the three promoter regions. The DNA sequence of P1 is most similar to the chloroplast promoter consensus DNA sequence. Interference by the highly and convergently transcribed *psbA-trnH1* operon is considered as a mechanism to explain the reduced activity of the $S10_A$ promoters.

Key words: Chloroplast – Transcription – Ribosomal protein – Promoter – Gene regulation

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Introduction

The circular plastid genome contains genes encoding proteins for photosynthetic functions as well as genes whose products are required for organelle transcription and translation (i.e., putative RNA polymerase subunits, tRNAs, rRNAs and ribosomal proteins). While the expression of genes for photosynthetic proteins has been widely studied in chloroplasts and other plastid types, there is little information on the expression of genes for proteins with genetic functions (reviewed in Gruissem 1989; Mullet 1988). It has been suggested that mRNAs encoding proteins involved in plastid transcription and translation are preferentially translated in non-photosynthetic plastids (Deng and Gruissem 1988) or under conditions of reduced protein synthesis (Liu et al. 1989) in order to maintain housekeeping functions (Deng and Gruissem 1988). In addition, unlike mRNAs for photosynthetic proteins, the accumulation of mRNAs for ribosomal proteins is not highly induced during greening (Posno et al. 1986; Russell and Bogorad 1987; Barkan 1988; Bisanz-Seyer et al. 1989). Therefore, the expression of such constitutive genes may be regulated by mechanisms that differ from those controlling the expression of genes for photosynthetic proteins. To approach this problem, we have analyzed the transcription and mRNA accumulation of the S10 ribosomal protein operon in spinach chloroplasts.

Like prokaryotic cells, plastids also synthesize 70S ribosomes. While the rRNAs are encoded by the plastid genome, only one-third of the estimated 58–62 ribosomal proteins are plastid encoded (Schmidt et al. 1983; Posno et al. 1984; Mache et al. 1985). The S10 operon is the largest ribosomal protein operon in the plastid genome. The plastid S10 operon has homology in both gene order and nucleotide sequence to three ribosomal protein operons in *Escherichia coli*: S10, spc and α (Tanaka et al. 1986). We selected this particular operon because it contains genes for both small *(rps)* and large *(rpl)* ribosomal subunits, which are organized into a polycistronic transcription unit that is conserved in plastid genomes

from different plant species, cyanelles and *Euglena* (Sugiura 1992; Michalowski et al. 1990; Christopher and Hallick 1990). The conserved gene order in plant plastid DNA is *trnI1, rpl23, rpl2, rps19, rpl22, rps3, rpl16, rpl14, rps8, infA, secX, rps11,* and *rpoA*. In spinach, *rpl23* is disrupted by a deletion relative to the tobacco homolog (Zurawski et al. 1984) leading to a premature termination codon. Therefore, *rpl23* is most likely a pseudogene in spinach (*rpl23ψ*).

The location of the S10 ribosomal protein operon in the chloroplast genome varies between different plant species. In *Marchantia* (Ohyama et al. 1988), the S10 operon is located in the large single-copy (LSC) region near the junction with inverted repeat B (IR_B). In spinach (Zurawski et al. 1984), tobacco (Shinozaki et al. 1986) and rice (Hiratsuka et al. 1989), the 5′ end of the operon is located within the inverted repeat and continues into the LSC region only from within IR_B (see Fig. 1). Therefore, in these higher plant plastid genomes, the S10 operon is partially duplicated. The exact location of the junction between the inverted repeat and the LSC region within the S10 operon differs between plant species. In tobacco, the junction is between the *rpl2* and *rps19* genes, such that the first three genes in the operon, *trnI1, rpl23* and *rpl2,* are present in two copies per genome and *rps19* and the remaining downstream genes are present in only one copy (Sugita et al. 1984). In rice (Moon and Wu 1988) and maize (McLaughlin and Larrinua 1987), the junction is between *rps19* and *rpl22,* while in spinach and *Nicotiana debneyi* (Zurawski et al. 1984), soybean (Spielmann et al. 1988; Spielmann and Stutz 1983), petunia (Aldrich et al. 1988) and mustard (Nickelsen and Link 1990), the junction disrupts the *rps19* gene. Consequently, there are two different *rps19* genes in these species: from within IR_B, the complete *rps19* gene continues into the LSC region (based on nucleotide homology to the comparable gene in *E. coli*), while in the inverted repeat A (IR_A), the *rps19′* gene is identical to *rps19* only up to the junction of the LSC (Fig. 2). In spinach, the homology between *rps19′* and *rps19* encompasses only 48 of 92 codons present in *rps19*. An open reading frame that is completely unrelated to the *rps19* DNA sequence and has no homology to any known genes continues from *rps19′* into the LSC for an additional 66 codons. In addition, the *psbA-trnH1* genes are located on the opposite strand in the LSC region adjacent to IR_A. Therefore, the S10 operons located in IR_A ($S10_A$) and IR_B ($S10_B$) are identical only for 1.6 kb of coding sequence and for approximately 21 kb upstream (Crouse et al. 1978).

Because the upstream regulatory sequences are identical, these two operons might be expected to be expressed at the same level. In this case, transcripts originating in $S10_A$ would encode a non-functional S19 ribosomal protein that could potentially disrupt the structure of the ribosome. We report here that despite identical promoter sequences, $S10_A$ and $S10_B$ are apparently not expressed at identical levels. We attribute the differences in expression to reduced transcription initiation at the $S10_A$ promoter regions, rather than differences in the stability of transcripts. Transcription of the $S10_B$ operon can initiate at multiple promoters in vitro. We suggest that the activity of the homologous promoters in $S10_A$ could be repressed by transcriptional interference from the convergently transcribed *psbA-trnH1* genes.

Materials and methods

Plants. Spinach plants (*Spinacea oleracea* L. cv. Marathon Hybrid) were grown hydroponically in 0.5× Hoagland solution under greenhouse conditions. Medium-sized leaves (4–10 cm) were harvested before 9 a.m. and plastids were isolated and used immediately for either RNA isolation, run-on transcription assays or preparation of the in vitro transcription extract.

Plasmid constructs. Spinach plastid DNA fragments consisting of LSC DNA immediately adjacent to both inverted repeats were subcloned from either the *Pst*I-7 fragment spanning the IR_B/LSC junction or from a 343 bp *Xba*I-*Hin*dIII fragment spanning the IR_A/LSC junction (Zurawski et al. 1984). The clone adjacent to IR_B is a 277 bp *Taq*I-*Hae*III fragment and the clone adjacent to IR_A is a 264 bp *Xba*I-*Hae*III fragment (these constructs contain 7 bp of identical IR sequences). The vectors utilized include pUC118/119 for the isolation of single-strand DNA and Bluescript KS^+ (Stratagene) for the generation of radioactive RNA probes with T3 and T7 RNA polymerase.

Deletion constructs. The region surrounding the 5′ end of the $S10_B$ operon was cloned as a 1.4 kb *Xba*I-*Sma*I fragment containing 1 kb of upstream sequence, *trnI1* and *rpl23ψ*. Various deletion clones of this construct were made starting from upstream of *trnI1,* using either restriction enzymes or Bal31 exonuclease. The sequences remaining after deletion were cloned into pUC19 and a reporter construct, the T7 bacteriophage early terminator (T7Te; Neff and Chamberlin 1980) was cloned into the *Sma*I site 12 bp downstream of *rpl23ψ*. Transcription by chloroplast RNA polymerase terminates at T7Te and generates a stable RNA with a discrete set of 3′ ends (Stern and Gruissem 1987).

RNA isolation. Spinach leaves were homogenized in a Waring Blendor (modified with razor blades) with homogenization buffer [0.33 M sorbitol, 50 mM HEPES, pH 6.8, 10 mM dithiothreitol (DTT), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM Na_4PO_7] at a ratio of 60 g of leaves to 150 ml of ice-cold buffer. The homogenate was filtered through four layers of Miracloth (Calbiochem), pelleted at 4000 *g* for 30 s at 4° C and resuspended in 6 ml of homogenization buffer per 60 g starting material. Intact chloroplasts were then isolated from Percoll gradients (Gruissem et al. 1986b), phenol-extracted and nucleic acids were ethanol-precipitated. After resuspension of the pellet in 10 mM TRIS-HCl, pH 7.5, 1 mM EDTA, RNA was precipitated overnight at 4° C by the addition of 0.25 vol. 10 M LiCl. RNA was pelleted and resuspended in RNAse-free (diethyl pyrocarbonate-treated) H_2O and stored at −70° C until use.

RNA blot hybridization and S1 nuclease analysis. Chloroplast RNA was electrophoresed in 2.2 M formaldehyde 1% agarose gels and blot hybridization was carried out according to standard protocols (Sambrook et al. 1989). The probe for S1 nuclease protection analysis was a 721 bp *Sca*I-*Sma*I fragment containing 331 bp of upstream DNA, *trnI1* and *rpl23ψ*. After digestion with *Sma*I (downstream of *rpl23ψ*) the DNA was dephosphorylated and labeled at the 5′ end with [γ-^{32}P] ATP, followed by digestion with *Sca*I and purification of the fragment from a denaturing polyacrylamide gel. The probe (1.5×10^5 cpm) was ethanol precipitated with 30 μg of either chloroplast RNA or tRNA. After resuspension in 10 μl of hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA), the sample was heated to 60° C for 10 min and then placed at 42° C overnight. S1 nuclease (150 U) was added in 100 μl of digestion buffer (250 mM NaCl, 30 mM sodium acetate, pH 4.6, 1 mM $ZnSO_4$, 10 μg/ml denatured salmon sperm DNA) and incubated at 37° C for 45 min. After addition of 10 μg of carrier tRNA, nucleic acids were precipitated and electrophoresed on 7 M urea, 5% polyacrylamide gels along with 500 cpm of probe.

Run-on transcription. Plastid run-on transcription reactions were performed as published (Deng et al. 1987). Percoll-gradient purified plastids were suspended in 20 ml of IC buffer (0.33 M sorbitol, 50 mM HEPES, pH 7.9, 10 mM DTT, 1 mM Na_4PO_7), pelleted at 4000 *g* for 30 s at 4° C and resuspended in a small volume of the same buffer. Reactions were carried out in 100 μl containing 66 mM sorbitol, 22 mM HEPES pH 7.9, 200 μM Na_4PO_7, 10 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 500 μM ATP, CTP and GTP, 50 mM UTP, 100 μCi [α-^{32}P] UTP. The reaction was started by adding plastids (20 μl) and pipetting up and down five times to disrupt membranes. After 8 min at 25° C, the reaction was terminated by the addition of 20 μl of stop buffer (5% sodium sarcosinate, 50 mM TRIS-HCl, pH 8.0, 25 mM EDTA). Labeled RNAs were purified by phenol extraction and ethanol precipitation and hybridized to filter-bound single-strand DNAs representing either strand of the LSC adjacent to each inverted repeat.

In vitro transcription. Chloroplast transcription extract was prepared and reactions carried out as published (Gruissem et al. 1986b). Briefly, a crude transcription extract was prepared by high-salt precipitation and DEAE-cellulose chromatography of chloroplast proteins. Equimolar amounts of plasmid DNA from each promoter construct were mixed with the transcription extract in a final reaction volume of 25 μl containing 10 mM KCl, 3.75 mM $MgCl_2$ 2 mM DTT, 0.5 mM ATP, GTP and CTP, 25 μM UTP and 15 μCi of [α-^{32}P]UTP. Transcription reactions were carried out for 1 h at 25° C. Radioactive RNAs were purified by phenol extraction and ethanol precipitation and electrophoresed in 7 M urea 6% polyacrylamide gels. Radioactive RNAs were excised from the dried gels and quantitated by scintillation counting. After subtracting the background from the control lane, the amount of radioactivity (cpm) in a particular RNA was regarded as a measure of transcription activity from the corresponding promoter.

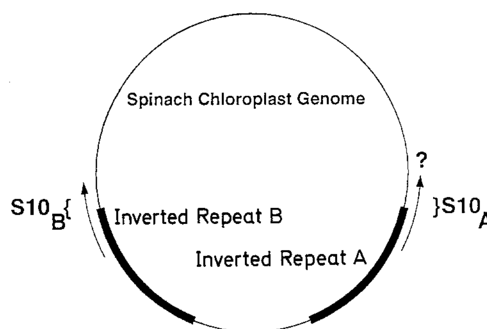

Fig. 1. Schematic diagram of the plastid genome of spinach. The circular molecule is approximately 145 kb in size and consists of two 23 kb inverted repeats (shown as *dark bars*) separated by large and small single-copy regions. For simplicity, only the $S10_A$ and $S10_B$ operons are shown and their orientation is indicated by the *arrows*. The *question mark* indicates the uncertainty regarding the expression of the $S10_A$ operon

Results

An intact rpl23 *gene is absent from spinach*

In spinach, *rpl23* is interrupted by a 14 bp deletion at amino acid position 42 compared with the homologous gene from tobacco (Zurawski et al. 1984), which creates a premature termination codon for a truncated protein of 46 amino acids. Based on the interrupted open reading frame, we believe that *rpl23* is most likely to be a pseudogene in spinach (*rpl23ψ*). In support of this notion, no polypeptide corresponding to the two overlapping open reading frames encoded by the spinach chloroplast *rpl23* gene was detected in either a coupled transcription-translation system or in 50S chloroplast ribosomes (Thomas et al. 1988a). Because we have been unable to identify by hybridization a sequence homologous to *rpl23* in nuclear DNA or elsewhere in the plastid genome (data not shown), it is unlikely that this gene has been transferred to the nucleus during evolution, as have a majority of plastid ribosomal protein genes (Gantt 1988). In contrast, *rpl23* encodes a functional ribosomal protein in tobacco (Yokoi et al. 1991). Because *rpl23* is one of the least conserved ribosomal protein genes (Christopher et al. 1988; Tanaka et al. 1986), we believe that either the ribosomes of spinach and tobacco differ in their subunit composition, or *rpl23* function has been transferred to another locus and the DNA sequence has evolved to such a degree that it is not detectable by hybridization using the spinach *rpl23* DNA as a probe.

S10 mRNA transcribed from IR_A does not accumulate

Figure 1 shows the location of the S10 ribosomal protein operons relative to the inverted repeat and the LSC region in the spinach chloroplast genome. The $S10_A$ operon starts in IR_A and terminates at the junction with the LSC

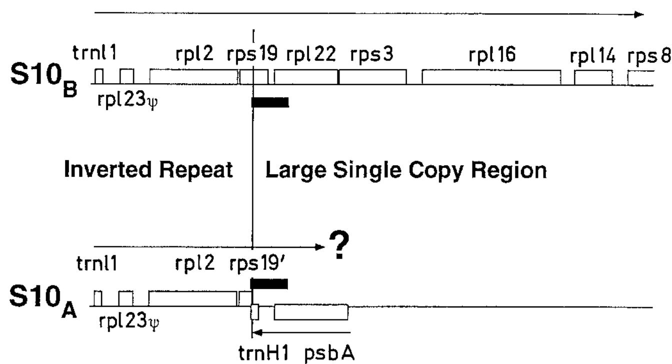

Fig. 2. Gene map of the region (from Fig. 1) surrounding the boundary between each inverted repeat and the large single-copy region (separated by the *vertical line*). The *arrows* indicate the direction of transcription and the *dark bars* represent the probes used to assess RNA accumulation and transcription. These probes overlap the inverted repeats for 7 bp

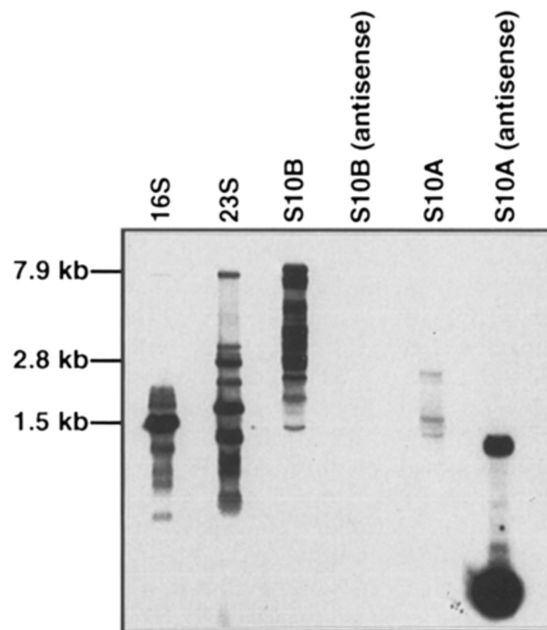

Fig. 3. RNA gel blot analysis of the $S10_A$ and $S10_B$ operons. Each lane (containing 10 µg of chloroplast RNA) was hybridized separately with either nick-translated DNA probes for the 16S and 23S rRNAs or to RNA probes corresponding to each strand of the large single-copy region adjacent to each inverted repeat. Lanes labeled "antisense" detect RNA that is transcribed from the opposite strand of each operon. The sizes of rRNAs are indicated to the left in kilobases

region, whereas the $S10_B$ operon starts at the identical position in IR_B and continues into the LSC where it terminates with the *rpoA* gene. As depicted in the diagram in Fig. 2, the S10 ribosomal protein operon is only partially duplicated because it spans the border between IR_B and the LSC. In addition to *trnI1*, *rpl23ψ* and *rpl2*, there are two genes for *rps19*: a complete gene at the border of IR_B/LSC and a truncated gene *(rps19′)* in IR_A, which is homologous to *rps19* for 48 amino acids, but completely diverges from *rps19* in the LSC. We investigated whether or not mRNA encoding a truncated ribosomal protein *(rps19′)* accumulates in spinach chloroplasts. Transcription might be expected to initiate at equal frequencies from promoters for the S10 operons within IR_A and IR_B because the DNA sequences upstream of $S10_A$ and $S10_B$ are identical for approximately 21 kb.

In order to differentiate the mRNAs from each operon it was necessary to use hybridization probes from the unique LSC region (see Fig. 2 for their location). Therefore, RNA blot hybridization with the LSC-specific probes would detect only those stable RNAs that extend from $S10_A$ and $S10_B$ into the LSC. Figure 3 shows the results of hybridization of chloroplast RNA to RNA probes specific for each strand of the IR/LSC border as well as to probes for rRNA. The RNA probe for $S10_B$ mRNA detects approximately 15 RNAs, the largest of which is 8 kb in size. The 8 kb RNA corresponds to the size of the $S10_B$ operon in spinach (Zurawski et al. 1984; Sijben-Müller et al. 1986; Zhou et al. 1989) and probably represents a full-length polycistronic transcript with *trnI1* and continuing through *rpoA*. Most of the smaller transcripts most probably represent processing products of the primary transcripts, although a subpopulation of transcripts may be derived from initiation at promoters internal to the $S10_B$ operon. The $S10_B$ RNA probe would not detect transcripts or processed RNAs that do not contain sequences complementary to the probe, and therefore it is possible that the RNA pattern from the $S10_B$ operon may be more complex than shown in Fig. 3. The sizes and complexity of RNAs detected from the spinach $S10_B$ operon are similar to those of RNAs from the homologous operon in *Euglena* (Christopher and Hallick 1990) and cyanelles (Michalowski et al. 1990). No hybridization is detected using an RNA probe specific for the opposite strand ($S10_B$-antisense), indicating that there is no accumulation of RNA in the antisense orientation. In contrast to the hybridization signal obtained with the $S10_B$ RNA probe, there is only a weak hybridization signal to the RNA probe specific for $S10_A$ RNA. The sizes of the three RNAs detected by the probe for $S10_A$ range from 1.4 to 2.6 kb and are distinctly different from those detected for $S10_B$. The 1.4 kb RNA is not long enough to contain sequences from the $S10_A$ promoter (1.6 kb from the IR_A/LSC junction) and may represent a processed form of a primary transcript or result from transcription initiation closer to the IR_A/LSC junction. If one assumes that the 1.6 and 2.6 kb RNAs initiate at sites in the $S10_A$ promoter region that correspond to transcription initiation sites in $S10_B$, they would be expected to terminate at specific sites in the LSC approximately at the junction of IR_A/LSC and approximately 1 kb from this junction. The $S10_A$ hybridization signal cannot be accounted for by cross-reaction to rRNA. We conclude, therefore, that a very low amount of $S10_A$ RNA accumulates (compared with $S10_B$), potentially encoding a truncated ribosomal protein S19 and that there is some transcriptional read-through into *psbA-trnH1*. Figure 3 also shows that, as expected, the RNA probe specific to the opposite strand ($S10_A$-antisense) hybridizes strongly to RNA encoding *psbA* and *trnH1*.

Different levels of transcription from $S10_A$ *and* $S10_B$

The differences in accumulation of RNAs from the $S10_A$ and $S10_B$ operons could result from a difference in transcription initiation from promoters within the inverted repeat. It should be noted, however, that DNA sequences in the inverted repeat are identical in all plants for which the complete plastid DNA nucleotide sequence is known: tobacco (Shinozaki et al. 1986), rice (Hiratsuka et al. 1989) and liverwort (Ohyama et al. 1986). In addition, it has been reported that in spinach, *trnI1* (Kashdan and Dudock 1982) as well as *rpl23, rpl2* and *rps19* (Zurawski et al. 1984; J.T. and W.G., unpublished results) have identical DNA sequences in the inverted repeat. Therefore, based on DNA sequence alone, it is expected that transcription initiation frequency should be similar at the identical promoters of the S10 operons. In this case, the reduced accumulation of RNA from $S10_A$ could be explained by transcription termination at or near the IR_A/LSC junction, although no DNA sequences are detectable in this region that have significant similarity to known transcription terminators (Stern and Gruissem 1987). Alternatively, transcription from $S10_A$ could continue into the LSC region at IR_A, but the resulting transcript is rapidly degraded into specific, but unstable RNAs shown in Fig. 3. In both cases, we would not detect accumulation of $S10_A$ transcripts. In addition, $S10_A$ transcripts that continue into the LSC could potentially act as antisense RNA to form double-stranded RNAs with the RNAs transcribed from the *psbA* and *trnH1* genes in the LSC region adjacent to IR_A (see Fig. 3). It is unlikely that such $S10_A$ RNA would be available for translation, as antisense RNA is known to inhibit expression (e.g. Simons and Kleckner 1988; Oeller et al. 1991).

In order to distinguish among these possibilities, we used a chloroplast run-on transcription assay to determine the level of transcription from $S10_A$ and $S10_B$ into the LSC. Figure 4 shows the results of hybridization of run-on transcripts to immobilized single-stranded phagemid DNA containing 264 or 277 bases of DNA corresponding to each DNA strand of the LSC directly adjacent to IR_A and IR_B, respectively. A high level of labeled RNA transcripts was detected with the antisense DNA strand adjacent to $S10_A$ (corresponding to the high levels of RNA detected for *psbA* and *trnH1*) representing transcription from the *psbA* and *trnH1* genes. A sixfold lower level of hybridization was detected by the DNA probe for $S10_B$ mRNA than was found for *psbA*/*trnH1*, which represents transcripts initiated at the promoters for the $S10_B$ operon. A very low level of hybridization was detected by the probe for $S10_A$ mRNA, which extends into the LSC (visible on a long exposure). The level of labeled transcripts detected from $S10_A$ is at least 20-fold lower compared with the level of labeled transcripts from the opposite strand (encoding *psbA*/*trnH1*). No labeled transcripts were detected with the probe for the antisense strand of $S10_B$, which corresponds to the absence of RNA from this DNA strand in the RNA blot (Fig. 3).

Together, these results suggest that the rate of transcription of $S10_A$ from within IR_A into the LSC is ex-

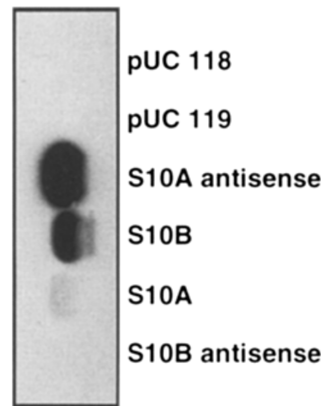

Fig. 4. Run-on transcription analysis of the $S10_A$ and $S10_B$ operons. Radioactive run-on transcripts were hybridized to filter-bound single-strand DNA representing each strand of the large single-copy region adjacent to each inverted repeat. Lanes labeled "antisense" detect RNA that is transcribed from the opposite strand of each operon. pUC 118 and pUC 119 are vector controls

tremely low or, alternatively, termination of transcription in the opposite DNA strand at or distal to the *trnH1* 3′ end is very efficient. Due to the limitations of the experimental approach, the results do not allow us to distinguish between these two possibilities. It has been reported, however, that chloroplast RNA polymerase can terminate transcription in vitro with high efficiency in regions 3′ to certain tRNA genes (Stern and Gruissem 1987). Although no individual tRNA genes have been tested in the antisense orientation for transcription termination, it has been shown that sequences between *psbA* and *trnH1* that coincide with an inverted repeat sequence 3′ to *psbA* can terminate transcription in vitro at a low frequency in the antisense orientation (Stern and Gruissem 1987). However, even if transcription termination occurs, the observed transcription termination frequency at the *psbA-trnH1* intergenic region cannot account for the low level of labeled $S10_A$ transcripts shown in Fig. 3. There is a formal possibility that during the 8 min run-on transcription reaction, there was a rapid turnover of $S10_A$ RNA. Previous results have demonstrated, however, that there is not a significant amount of RNA turnover for the bulk of run-on transcripts synthesized in the run-on assay system (Deng et al. 1987).

In the following control experiment, we investigated the possibility that the large amount of antisense RNA to $S10_A$ already present in chloroplasts (in the form of *psbA*/*trnH1* RNA) could interfere with the run-on transcription assay by competing with the filter-bound DNA for hybridization to run-on transcripts from $S10_A$ (data not shown). Radioactive RNA complementary to the 3′ end of *psbA* and *trnH1* was synthesized in vitro and hybridized to filter-bound single-strand DNA in the presence or absence of non-radioactive run-on RNA. We detected a decrease in filter-bound hybridization of 20%–30% in the presence of competing run-on RNA over a wide range of probe concentrations (from 100–100,000 cpm), indicating that there is a low level of competition for $S10_A$ RNA hybridization in solution. The low level of hybridization in solution between $S10_A$

RNA and the antisense *psbA/trnH1* RNA, however, is not sufficient to explain the observation that transcription of the $S10_A$ operon is reduced 80%–90% (Fig. 4) relative to $S10_B$ and/or does not continue into the LSC to a significant level (Fig. 3).

The 5′ ends of different S10 RNAs map to putative promoter sequences

We have used S1 nuclease protection assays to define the 5′ ends of RNAs in the region of the S10 operon containing *trnI1* and *rpl23ψ*. A control RNA blot hybridization with a DNA probe containing 975 bp of sequences 5′ upstream to *trnI1* failed to detect hybridization to chloroplast RNA (data not shown). Therefore, S1 nuclease analysis was performed using a 721 nucleotide DNA probe that extends from 331 bp 5′ of *trnI1* to 12 bp 3′ of *rpl23ψ*. Figure 5B shows the results of an S1 nuclease protection assay that detects four major protected DNA fragments (A-D) of approximately 415, 318, 206 and 75 nucleotides, respectively, as well as several minor bands. The most abundant RNAs have 5′ ends indicated as A and B, while C and D correspond to 5′ ends previously reported (Thomas et al. 1988a). RNA A contains the unprocessed $tRNA^{Ile}$. Processing of tRNA from longer polycistronic transcripts has been reported previously (Gruissem et al. 1983; Greenberg et al. 1984). The 5′ end of RNA B is located immediately 3′ to *trnI1* and most probably represents a transcript from which $tRNA^{Ile}$ has been processed. Based on the results from the RNA blot analysis and transcription run-on assay shown in Figs. 3 and 4, respectively, it is most likely that the four major RNAs detected in the S1 nuclease assay are produced by transcription from the $S10_B$ operon, rather than transcription from $S10_A$. It is possible, however, that some of the minor labeled RNAs in Fig. 5B represent transcripts from $S10_A$ or, less likely that all RNAs are a mixture of transcripts derived from transcription initiation at both $S10_B$ and $S10_A$.

The relative location of the 5′ ends of the RNAs A, C, and D, shown in the diagram of Fig. 5A, suggests that the corresponding 5′ ends of the RNAs could result either from transcription initiation or processing of a longer transcript. DNA sequence analysis of the $S10_B$ operon region represented by the DNA probe used in the S1 nuclease protection assay revealed three putative promoter regions with DNA sequences similar to the consensus chloroplast promoter DNA sequence (Gruissem and Zurawski 1985a, b; Kung and Lin 1985; Hanley-Bowdoin and Chua 1987). The plastid promoter consensus DNA sequence consists of two conserved hexanucleotide sequences ctp1 (5′TTGACA) and ctp2 (5′TATAAT), which are similar to the consensus DNA sequences of the *E. coli* promoter -35 and -10 boxes, and which are also separated by an optimal 16–18 bp. The diagram in Fig. 5A shows the location of the three putative promoter regions, P1, P2, and P3, relative to the protected RNAs A-D. P1 is located 39 bp 5′ to *trnI1*, P2 is located 74 bp 5′ to *rpl23ψ*, and P3 is located within the *rpl23ψ* gene 40 bp 3′ from the *rpl23ψ* 5′ end. Therefore,

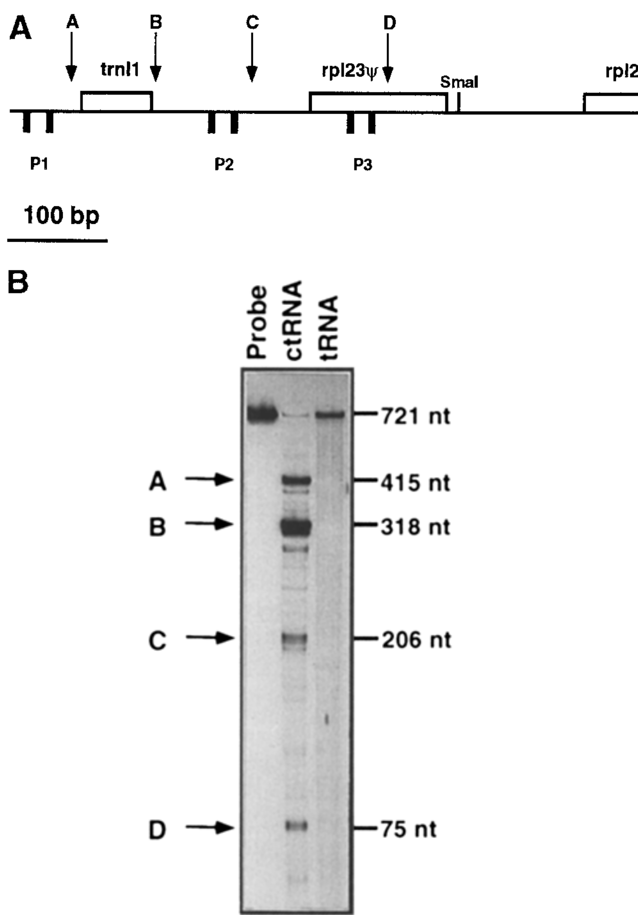

Fig. 5A. Gene map showing the location of 5′ ends detected by S1 analysis (A-D) relative to the *trnI* and *rpl23ψ* genes as well as the putative promoters P1, P2 and P3. The ctp1 and ctp2 elements of each promoter are indicated by the *thick vertical lines*. **B** S1 nuclease protection analysis of the 5′ end of the $S10_B$ operon. The major protected bands are indicated to the left (A-D) and their sizes are shown to the right in nucleotides

P1, P2, and P3 are located approximately 10 bp upstream of the 5′ end of RNAs A, C, and D, respectively. We were unable to cap RNA from the S10 region using guanylyl transferase and [α-^{32}P]GTP, which would have allowed us to differentiate between primary transcripts and processed RNAs (the latter are not substrates for guanylyl transferase). Therefore, we employed in vitro transcription analysis to determine whether P1, P2, and/or P3 can serve as promoters for the $S10_B$ operon.

Transcription of $S10_B$ can initiate from three promoter-like sequences in vitro

A set of $S10_B$ DNA constructs shown in Fig. 6A with progressively larger deletions of DNA sequences corresponding to P1, P2, and P3 were tested using an in vitro transcription assay to determine whether P1, P2 and/or P3 can initiate transcription. To generate transcripts with defined 3′ ends in the in vitro transcription assay using a crude chloroplast protein extract, the DNA sequence for the T7 phage early terminator was cloned into the

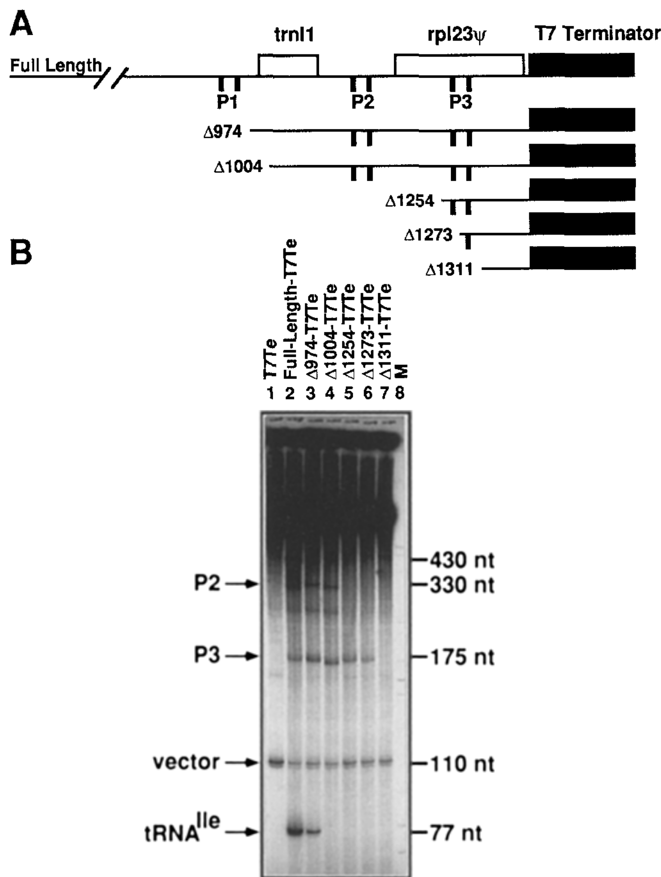

Fig. 6A, B. In vitro transcription analysis of the $S10_B$ operon. **A** Diagram of deletion constructs transcribed in vitro. *trnI* and *rpl23ψ* are indicated as *open boxes* and putative promoters P1, P2 and P3 are shown as pairs of *vertical lines* representing the ctp1 and ctp2 elements. Each deletion clone is followed by the T7 terminator as a reporter *(shaded)*. The full-length construct contains 974 bp upstream of the *Bam*HI site and the number of base pairs deleted in each construct is indicated after the Δ symbol. **B** In vitro transcription products were electrophoresed in a 6% denaturing polyacrylamide gel. The lane labeled T7Te utilized a plasmid containing the T7 terminator alone as a control. The lane labeled M contains a set of size markers. Transcripts that initiated at P2 and P3 as well as within the vector are indicated to the left. Also indicated is the mature $tRNA^{Ile}$. The sizes of each transcript are indicated on the right in nucleotides

*Sma*I restriction enzyme site 3′ to the *rpl23ψ* gene. Previous experiments have demonstrated that the T7 terminator can be used as a reporter for transcription in the in vitro system, because chloroplast RNA polymerase terminates transcription with a high frequency at the T7 sequence (which can form a stem/loop structure; Neff and Chamberlin 1980) producing RNAs with a stable and discrete set of 3′ ends (Stern and Gruissem 1987). Therefore, a promoter located 5′ to the T7 reporter sequence will produce a stable RNA of a size dependent on the site of transcription initiation and any 5′ processing reactions.

The in vitro transcription assays using the DNA deletion constructs shown in Fig. 6 produce a discrete set of RNAs of sizes expected if transcription initiates at P1, P2 and P3. Transcription of the full-length DNA clone (lane 2), which includes DNA sequences approximately 1 kb upstream of *trnI1*, generates an RNA that is the expected size for the mature $tRNA^{Ile}$ (77 nucleotides). The RNA of approximately 430 nucleotides corresponds in size to an RNA extending from the 3′ end of *trnI1* to the end of the T7 terminator (414 nucleotides), most probably resulting from processing of $tRNA^{Ile}$ from a larger RNA initiated at P1 upstream of *trnI1*. The 430 nucleotide transcript is present only in those lanes (2, 3) in which an intact *trnI* gene was present. It has been demonstrated previously that the chloroplast extract is efficient in processing tRNAs from longer precursors (Greenberg et al. 1984; Gruissem and Zurawski 1985b). Therefore, we would not expect to detect a significant level of primary transcript from the P1 promoter. Transcription initiation at P2 would result in an RNA of approximately 330 nucleotides, and a discrete labeled RNA of this size was detected only in those reactions in which P2 DNA sequences were intact (lanes 2–4). In addition to the tRNA-sized transcript, a labeled RNA was detected of a size corresponding to transcription initiation at P3 (170–180 nucleotides).

Transcription of the DNA deletion constructs together with quantitation of the labeled RNAs by scintillation counting demonstrates that P1, P2, and P3 can function as independent promoters for the $S10_B$ operon in vitro. Deletion Δ974 (representing the number of base pairs removed from the 5′ end of the full-length construct) removes all P1 DNA sequences (leaving 13 bp 5′ to *trnI1*). A transcript of the size of the mature $tRNA^{Ile}$ is still produced, but the amount is reduced to 30% of the level from the full-length DNA construct (lane 3). Further deletion to +17 within *trnI1* results in the disappearance of the full-length or a truncated $tRNA^{Ile}$ transcript, although a labeled RNA corresponding to transcription initiation at P2 is still detectable (lane 4). Therefore, we conclude that P1 is the primary promoter for *trnI1*, but deletion of P1 does not completely eliminate transcription of *trnI1*, indicating a possible internal promoter (see Discussion). Deletion of DNA sequences for P2 (Δ1254), which leaves P3 intact, did not change the level of the approximately 175 nucleotide RNA that corresponds to initiation at P3 (lane 5). Deletion of an additional 19 bp (Δ1273), which removed DNA sequences corresponding to ctpl from P3, does not affect the size of the 175 nucleotide RNA, but reduces its level as determined by quantitation of the labeled transcript (lane 6). Removal of DNA sequences corresponding to ctp2 from P3 resulted in the disappearance of the 175 nucleotide RNA (lane 7), indicating that P3 DNA sequences can function in transcription initiation in vitro. The labeled RNA of ~110 nucleotides (labeled vector) detectable in all lanes initiates and terminates within the pUC19 vector DNA and serves as a convenient internal standard for ensuring equal molar amounts of plasmid DNA in the transcription reaction. The molar ratio of RNAs corresponding to $tRNA^{Ile}$ and RNAs initiating at P2, and P3 indicates that the relative transcription initiation frequencies of chloroplast RNA polymerase in vitro at P1, P2, and P3 are approximately 8:1:2, respectively. This ratio is similar to the ratio of RNAs A/B to RNA C and RNA D detected by S1 nuclease analysis (Fig. 5B).

Together, we conclude that P1, P2 and P3 are each recognized as a promoter in vitro. Thus, the RNAs A, C and D detected by S1 nuclease analysis of chloroplast RNA (Fig. 5) most probably represent transcripts that start at P1, P2, and P3, respectively, suggesting that transcription of the $S10_B$ operon can be initiated from multiple promoters.

Discussion

Does convergent $S10_A$ and psbA-trnH1 *transcription result in transcriptional interference?*

We have analyzed the expression of the complex S10 ribosomal protein operon, which is partially duplicated at the 5′ end as a result of its unique location spanning the IR/LSC junctions. The duplicated versions of the operon are termed $S10_A$ and $S10_B$. This partial duplication raises the possibility that two polycistronic S10 mRNAs exist, which would be initiated at identical promoters in $S10_A$ and $S10_B$, contain identical sequences for 1.6 kb, but diverge within the coding region of *rps19*. Transcription from within IR_A and IR_B would give rise to two different *rps19* mRNAs, which are identical for 48 codons and then diverge into functional and nonfunctional coding sequences. Alternatively, premature transcription termination at the IRA_A/LSC junction would result in a truncated *rps19′* mRNA. In either case, translation of such a truncated or mutant *rps19′* mRNA originating from IR_A could result in the synthesis of a nonfunctional S19 protein. In addition, because all ribosomal proteins (except S7 and S12) are required in equimolar amounts during ribosome assembly (Nomura et al. 1984), translation of an $S10_A$ RNA containing L2 coding sequences could result in an imbalance in the ratio of L2 protein relative to the other ribosomal proteins encoded in the S10 operon (*rpl2* is identical in both IRs). The mechanisms by which synthesis and accumulation of ribosomal proteins and ribosome assembly are regulated in plastids are currently not well understood. Within the constraints of the experimental approach, the RNA blot analysis indicates that RNAs that span the $S10_A$/LSC junction do not accumulate to high levels. We believe, therefore, that most of the translation of L2 is from the *rpl2* mRNA that originates from the $S10_B$ operon. In addition, if a nonfunctional *rps19′* mRNA accumulates, its abundance appears to be very low relative to that of the RNAs detected for the $S10_B$ operon, which include the functional *rps19* RNA. Therefore, we favor the conclusion that a significant amount of a truncated or otherwise mutant S19 protein does not accumulate. Our conclusion is in agreement with the conclusion reached by Thomas et al. (1988b) who also failed to detect significant levels of *rps19′* RNA. Our results differ from their data, however, in that while we only detected the monocistronic *psbA* mRNA by RNA blot analysis, these authors detected a longer transcript that, based on its size, would overlap *rps19′* in IR_A (although they detected monocistronic *psbA* transcript by S1 nuclease analysis). This would represent antisense RNA to $S10_A$ transcripts, and was suggested as the reason for the lack of detectable $S10_A$ RNA (Thomas et al. 1988b).

Our conclusion that expression of the $S10_A$ operon is repressed relative to $S10_B$ is further supported by the finding that transcription run-on assays do not detect significant levels of transcripts at the $S10_A$/LSC junction. It has been demonstrated previously that transcription run-on assays accurately reflect the transcriptional activity of plastid genes in different plants (Deng and Gruissem 1987; Mullet and Klein 1987). The transcription run-on assay has been optimized to avoid rapid turnover of RNA during the brief labeling pulse (Deng et al. 1987; Mullet and Klein 1987). It is unlikely, therefore, that the failure to detect transcription at the $S10_A$/LSC junction (compared with transcription from the $S10_B$ operon) is due to the greatly decreased stability of transcripts from this region. We cannot, however, exclude the possibility that transcription of $S10_A$ is efficiently terminated proximal to the $S10_A$/LSC junction. Analysis of the $S10_A$ DNA sequence 3′ of *rpl2* and at the IR_A/LSC junction has not revealed any specific DNA sequence motif or secondary structure that could be implicated in efficient transcriptional termination at the $S10_A$/LSC junction. Together with the results from the RNA blot analysis shown in Fig. 3, we suggest that $S10_A$ and $S10_B$ differ in their transcriptional activity in vivo.

The reduced transcriptional activity of $S10_A$ could be explained by "transcriptional interference" resulting from high levels of transcription of the *psbA* and *trnH1* genes on the opposite strand directly adjacent to the IR_A/LSC junction. The *psbA* and *trnH1* sequences are likely to be cotranscribed (Gruissem and Zurawski 1985c), and this operon is one of the most highly transcribed regions of the plastid DNA as measured by run-on transcription (Deng and Gruissem 1987) and analysis of the *psbA* promoter in vitro (Gruissem and Zurawski 1985a). Compared with transcription from $S10_B$ in the run-on assay (Fig. 4), transcription of the *psbA-trnH1* region is at least five-fold to ten-fold more active. Transcriptional interference could therefore result from the large number of RNA polymerase molecules transcribing *psbA-trnH1,* thus excluding those progressing along a weakly transcribed $S10_A$ operon. Interference by convergent transcription has been reported in λ phage and *E. coli* (Ward and Murray 1979), and the effect observed in these studies was over a distance larger than the approximately 1.6 kb separating the 3′ end of *psbA-trnH1* and the putative $S10_A$ promoters. Alternatively, transcriptional interference could result from changes in local superhelical conformation in the $S10_A$ promoter region caused by high levels of transcription initiating at *psbA*. It has been reported that transcription in prokaryotes generates positive supercoiling in front and negative supercoiling behind the transcribing complex (Liu and Wang 1987; Wu et al. 1988; Pruss and Drlica 1989). Positive supercoiling preceding *psbA-trnH1* RNA polymerase transcription complexes would negate the negative superhelicity of plastid DNA and could result in a relaxation of the DNA template in the $S10_A$ promoter region. Based on the distance that separates $S10_B$ from $S10_A$, such relaxation of negative superhelicity is less

likely to occur in the $S10_B$ promoter region. This model is consistent with the results obtained from the RNA blot analysis and transcription run-on assays shown in Figs. 3 and 4. Also, it has been shown that transcription of the plastid genes *rbcL* (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase), *atpB* (encoding the β-subunit of ATP synthetase), *rps4* (encoding S4), and an unidentified plastid gene from *Chlamydomonas* is affected by relaxation of negative superhelicity of the DNA templates in vitro or in vivo (Stirdivant et al. 1985; Lam and Chua 1987; Russell and Bogorad 1987; Thompson and Mosig 1987). It is not known in chloroplasts over what distance transcription can affect supercoiling of DNA, and further studies of convergently or divergently transcribed plastid genes will be necessary to determine whether or how changes in local superhelicity can affect the activity of their promoters.

Transcription of the $S10_B$ operon can be initiated at multiple promoter regions

DNA sequences of several putative chloroplast promoter regions have been reported. They were identified either by homology of chloroplast DNA sequences to the *E. coli* consensus promoter DNA sequences (Kung and Lin 1985), or more stringently, by functional identification in either homologous or heterologous in vitro transcription systems (for review see Hanley-Bowdoin and Chua 1987; Gruissem 1989). The genes for which a detailed promoter analysis has been reported are all highly expressed: photosynthetic genes such as *psbA, rbcL* and *atpB* (Link 1984; Bradley and Gatenby 1985; Gruissem and Zurawski 1985a; Hanley-Bowdoin et al. 1985; Eisermann et al. 1990), tRNA genes such as *trnM2, trnS1* and *trnR1* (Gruissem and Zurawski 1985b; Gruissem et al. 1986a), and the 16S rRNA gene (Sun et al. 1989). Essentially, plastid promoter sequences that have been functionally defined share significant sequence homology with the *E. coli* consensus promoter DNA sequence, and single base mutations that result in chloroplast promoter up- or down-regulation correspond to changes in the DNA sequence that increase or decrease the homology to the consensus DNA sequence, respectively. In addition, a subpopulation of chloroplast tRNA genes (*trnS* and *trnR*) has been identified that in vitro do not require upstream promoters for their transcription (Gruissem et al. 1986a). These tRNA genes, as well as *trnI1* located in the S10 operon, contain conserved sequences homologous to the A and B DNA sequence motifs of eukaryotic tRNA genes with internal promoters (Galli et al. 1981), although the significance of this homology remains unknown. The results we have obtained from the promoter analysis of the $S10_B$ operon in vitro are consistent with a model in which transcription of *trnI1* may be initiated at two promoters, one upstream and one internal to *trnI1*. We do not know how efficiently DNA sequences internal to *trnI1* are utilized for transcription initiation, but the longest RNA detected by S1 analysis is consistent with transcription initiation of $S10_B$ at the P1 promoter 5′ to *trnI1*. It is possible that weakly protected RNAs detectable between the major RNAs A and B in Fig. 5B could represent transcripts that initiate internal to *trnI1*.

From the S1 analysis and promoter studies it appears that P1 represents the major promoter for transcription of the $S10_B$ operon. Although promoters P2 and P3 are clearly functional in vitro and could give rise to the RNAs C and D detected by S1 nuclease analysis, they are less efficient in transcription initiation in vitro. The high frequency of transcription initiation at the P1 promoter relative to P2 and P3 is likely to be of functional significance for the following reason. In addition to the duplicate *trnI1* located in the S10 operons, a second $tRNA^{Ile}$ gene (*trnI2*) is cotranscribed with the 16S and 23S RNAs in many plant species. The $tRNA^{Ile1}$ (CAU) has a methionine anticodon (Kashdan and Dudock 1982), but contains a modified base in the anticodon loop and is charged with isoleucine, not methionine in vitro (Francis and Dudock 1982). It was suggested by Christopher and Hallick (1990) that the codon usage for the $tRNA^{Ile}$ encoded by *trnI1* is higher in genes encoding proteins for transcriptional and translational functions as compared with genes for photosynthetic proteins. Therefore, the cotranscription of *trnI1* with the S10 ribosomal protein operon from the P1 promoter may be necessary to maintain adequate levels of this tRNA for the translation of mRNAs from genes for housekeeping functions (Christopher and Hallick 1990). In addition, transcription initiation at P1 could also be part of a mechanism to regulate the expression of the $S10_B$ operon, because we have observed that *trnI1* can also terminate transcription in vitro (data not shown). Thus, it is likely that transcription of *trnI1* in fact exceeds that of the remainder of the S10 operon when transcription initiation occurs at P1. Transcription termination at *trnI1* could be modulated to regulate transcription into the remainder of the S10 operon. Transcription initiation at P2 and P3 could bypass the regulation by transcription initiation at P1 or termination after *trnI*.

The $S10_B$ operon is an example of chloroplast genes or polycistronic transcription units for which transcription can be initiated at multiple promoters. Other examples include the *psbD-psbC* operon (32–33 and 43 kDa proteins of photosystem II; Yao et al. 1989; Sexton et al. 1990), *atpB* (Chen et al. 1990) and the *psbE-psbF* and *petG* operons (encoding photosystem II and electron transport proteins, respectively; Haley and Bogorad 1990). The significance of transcription initiation of chloroplast genes at multiple promoters is currently not well understood. In the case of the *psbD-psbC* operon in barley chloroplast, Sexton et al. (1990) have suggested that transcription initiation at different promoters may provide a regulatory mechanism to control the accumulation of specific transcripts in response to environmental signals. Further studies are necessary to determine whether transcription initiation of the $S10_B$ operon in spinach at the P1, P2 and P3 promoters can be regulated as part of the developmental program or in response to fluctuating levels of $tRNA^{Ile}$(CAU).

	ctp1	spacing	ctp2
Consensus	TTGACA	17-18 bp	TATAAT
P1	TTGATT	18 bp	TCAATT
P2	TCCATA	18 bp	TATATT
P3	TTCAGT	17 bp	TATACT
*psb*A	TTGACA	18 bp	TATACT

Fig. 7. Promoter sequences from P1, P2, P3 and *psbA* are compared with the plastid consensus promoter sequence. The *overlined* bases in the consensus sequence are the "invariant" bases. Those bases identical to the consensus are *underlined*

Base changes at invariant positions correlate with P1, P2 and P3 promoter activity

The mutational analysis of several chloroplast promoter DNA sequences has shown that certain bases at invariant positions are critical for promoter activity and that changes in transcription initiation frequencies at specific promoters can be correlated with base substitutions at these invariant positions (for review see Gruissem and Zurawski 1985c; Hanley-Bowdoin and Chua 1987; Gruissem 1989). Figure 7 shows a comparison of the DNA sequences for the $S10_B$ promoter regions P1, P2, P3 with the DNA sequence for the *psbA* promoter and the chloroplast promoter consensus DNA sequence. The DNA sequence of the *psbA* promoter, which among several chloroplast promoter regions has been identified as the strongest promoter in vitro (Gruissem and Zurawski 1985a), is identical to the consensus promoter sequence for ctp1 (TTGaca) and ctp2 (TAtaaT) at the invariant positions (capital letters) and differs from the consensus at only one position (Kung and Lin 1985). Of the three $S10_B$ promoters, only P1 contains the invariant TTG in ctp1. This could explain why transcription of the $S10_B$ operon initiates with a higher frequency at P1 than at P2 or P3. It is interesting to note that the activity of P2 is reduced relative to P3 in vitro and that the DNA sequence in ctp1 of P2 (TCCATA) differs from the consensus plastid ctp1 promoter sequence at two of the invariant bases, while ctp1 of P3 differs at only one invariant base (TTCAGT). The ctp2 DNA sequences of both P2 and P3 (TATATT and TATACT, respectively) are identical at the invariant bases of the chloroplast consensus DNA promoter sequence. It is likely therefore, that P2 is less active than P3 in vitro as a result of the base substitutions in ctp1, but confirmation of this observation requires a detailed mutational analysis of the DNA sequences. The results from the deletion analysis of the P3 promoter region reinforce the notion that ctp2 is critical for positioning of the RNA polymerase relative to the transcription start site as well as for promoter activity. Transcription analysis of DNA constructs in which both ctp1 and ctp2 of P3 are retained (Fig. 6, lanes 2–5) shows a similar level of the 175 nucleotide RNA. Deletion of ctp1 from the P3 promoter reduces the level, but not the length, of the 175 nucleotide RNA (lane 6). Together, the results from our comprehensive analysis confirm that transcription of the plastid $S10_A$ and $S10_B$ ribosomal protein operons is complex and that the activities of their promoter regions are most likely to be differentially affected by their location relative to other highly transcribed genes at the respective IR/LSC junctions.

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