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Transcription from Heterologous rRNA Operon Promoters in Chloroplasts Reveals Requirement for Specific Activating Factors[1]

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The plastid rRNA (*rrn*) operon in chloroplasts of tobacco (*Nicotiana tabacum*), maize, and pea is transcribed by the plastid-encoded plastid RNA polymerase from a σ^{70}-type promoter (P1). In contrast, the *rrn* operon in spinach (*Spinacia oleracea*) and mustard chloroplasts is transcribed from the distinct Pc promoter, probably also by the plastid-encoded plastid RNA polymerase. Primer-extension analysis reported here indicates that in Arabidopsis both promoters may be active. To understand promoter selection in the plastid *rrn* operon in the different species, we have tested transcription from the spinach *rrn* promoter in transplastomic tobacco and from the tobacco *rrn* promoter in transplastomic Arabidopsis. Our data suggest that transcription of the *rrn* operon depends on species-specific factors that facilitate transcription initiation by the general transcription machinery.

In higher plants the genes for the plastid 16S, 23S, and 5S rRNA are encoded in the plastid genome and are transcribed as a large precursor RNA, which is subsequently processed into the various mature rRNA species. The plastid *rrn* operon in maize, pea, and tobacco (*Nicotiana tabacum*) is transcribed from a promoter with two conserved blocks ("-10" and "-35") of hexameric sequences reminiscent of the *Escherichia coli* σ^{70}-type promoters (P1 or Nt-Prrn-114; Strittmatter et al., 1985; Sun et al., 1989; Vera and Sugiura, 1995; Allison et al., 1996). The P1 promoter is recognized by the PEP. In addition to P1, the *rrn* operon in tobacco has a second promoter (P2 or Nt-Prrn-62), which is inactive in chloroplasts and functions only in BY2 tissue culture cells (Vera and Sugiura, 1995) and in plants lacking PEP (Allison et al., 1996). This second promoter is transcribed by the nuclear-encoded plastid RNA polymerase (Allison et al., 1996).

In contrast to maize, tobacco, and pea, the *rrn* operon in spinach (*Spinacia oleracea*) and mustard is transcribed from promoters lacking properly spaced -10/-35 elements in vivo (Baeza et al., 1991; Iratni et al., 1997; Pfannschmidt and Link, 1997). In spinach the strong Pc promoter is active in chloroplasts but not in roots. The Pc site appears to be faithfully recognized by the mustard PEP in vitro (Pfannschmidt and Link, 1997). A second weak promoter transcribing the spinach *rrn* operon is upstream of tRNA-(GAC)^{Val} (Iratni et al., 1997). It is not known which of the plastid RNA polymerases is recognizing this second promoter.

Since the *rrn* operon in the chloroplasts of higher plants is either transcribed from P1 or Pc, promoters that have transcription initiation sites 26 nucleotides apart, it was uncertain whether both promoters may function in the same plastid. Given the potential overlap between Pc and P1, promoter exclusion was proposed as the mechanism to explain transcription from Pc but not from the P1 promoter in spinach (Iratni et al., 1994). Mapping of RNA 5′ ends upstream of *rrn* in the present study led to the identification of transcripts characteristic of both P1 and Pc in Arabidopsis. Given the confidence that both promoters may be active in the same plastid, we designed transgenic experiments to understand promoter selection in the plastid *rrn* operon in higher plants. Specifically, we have tested transcription from the spinach *rrn* promoter in transplastomic tobacco and from the tobacco *rrn* promoter in transplastomic Arabidopsis. We conclude from this study that tobacco plastids lack the factor required for transcription from Pc, whereas spinach has an intact P1 promoter but lacks the cognate P1 activator. These findings suggest that P1 and Pc activity depends on promoter-specific factors that are essential for the recognition of these promoters by the general transcription machinery.

MATERIALS AND METHODS

Construction of Vector pPS105

pPS102 is a pBSKS+ plasmid (Stratagene) derivative that contains a chimeric *uidA* gene as a *Sac*I-*Hin*dIII fragment. The chimeric *uidA* gene consists of the following: between the *Sac*I and *Nco*I sites, the spinach (*Spinacia oleracea*) promoter fragment and a ribosome-binding site; between the *Nco*I and *Xba*I sites, the *uidA*-coding region with an N-terminal c-*myc* tag; and between the *Xba*I and *Hin*dIII sites, the 3′ untranslated region of the *rps16* ribosomal

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Abbreviation: PEP, plastid-encoded plastid RNA polymerase.

protein gene (Tr*ps16;* Staub and Maliga, 1994). The *Sac*I-*Nco*I fragment was obtained by PCR amplification of spinach plastid DNA using the following oligonucleotide primers: O1, ccgagctCCCAACGTCAGTTTTTCT; and O2, ccgctagccatggat*ccctccc*tacaacGTAGACAAAGCGGATTC.

In the primer sequences, anchor sequences derived from the plastid genome are designated in uppercase letters; nucleotides added during vector construction are designated in lowercase letters; restriction sites are underlined; and the ribosome-binding site is in italic font. The *uidA*-coding region was tagged by translationally fusing it with a PCR approach at its N terminus with amino acids 410 to 419 (EQKLISEEDL) of the human c-myc protein (Kolodziej and Young, 1991; L.A. Allison, unpublished data).

Plasmid pPS105 was obtained by cloning the *uidA* gene as a *Sac*I-*Hin*dIII fragment into *Sac*I-*Hin*dIII-digested pPRV111A plastid-transformation vector, as described by Zoubenko et al., 1994.

Tobacco Plastid Transformation

For plastid transformation, tungsten particles were coated with DNA and introduced into the leaves of tobacco (*Nicotiana tabacum)* plants using the Dupont PDS1000He Biolistic gun at 1100 p.s.i. Transgenic shoots were selected aseptically on RMOP medium containing 500 mg/L spectinomycin dihydrochloride (Svab and Maliga, 1993). Transgenic cuttings were rooted and maintained on agar-solidified Murashige-Skoog salts containing 3% Suc (Murashige and Skoog, 1962).

Primer-Extension Analysis

For RNA isolation, wild-type Arabidopsis (RLD ecotype) seeds were germinated and grown in vitro on a medium containing Murashige-Skoog salts (Murashige and Skoog, 1962) and 2% Suc. Cotyledon samples were collected from 10-d-old seedlings. Leaves were harvested from 3-week-old plants. Root tissue was obtained from 2-week-old Arabidopsis cultures grown in liquid ARM medium (Marton and Browse, 1991). The leaves of transgenic pGS31A-16 Arabidopsis plants (Sikdar et al., 1998) were collected from plants maintained on agar-solidified ARM medium. Wild-type and transgenic tobacco leaves were taken from plants grown aseptically on a medium containing Murashige and Skoog salts and 3% Suc (Murashige and Skoog., 1962). Spinach leaves were derived from seedlings grown aseptically on a medium containing Murashige-Skoog salts and 2% Suc.

Total cellular RNA was prepared according to the method of Stiekema et al. (1988). Primer-extension reactions were carried out using 3 μg (wild-type Arabidopsis, tobacco, and spinach leaves and cotyledons) or 10 μg (transgenic tobacco and Arabidopsis leaves; wild-type Arabidopsis roots) of total RNA, as described by Allison and Maliga (1995). The following oligonucleotide primers were used: 16S rRNA: O3, 5′-TTCATAGTTGCATTACTTAT-AGCTTC-3′; *uidA*: O4, 5′-GGCCGTCGAGTTTTTTGATT-TCACGGGTTGGGG-3′; *aadA*: O5, 5′-CGCTCGATGACGC-CAACTACC-3′.

Sequence ladders generated with the same primers using the Sequenase II kit (United States Biochemical) were used as molecular size markers.

RESULTS

In Arabidopsis Chloroplasts Both P1 and Pc Are Active

Primer-extension analysis was carried out to identify the plastid *rrn* operon promoter in Arabidopsis. Two RNA species were identified in leaves with 5′ ends mapping to 111 and 139 nucleotides upstream of 16S rRNA, the first gene of the *rrn* operon (Fig. 1A). The position of the 5′ end of these transcripts suggests transcription initiation from P1 and Pc promoter homologs (Fig. 2). The ratio of the two transcripts was approximately 10:1.

Both P1 and Pc are active in roots and in leaves (Fig. 1B). However, no −139-transcript was detectable in cotyledons, suggesting organ-specific utilization of Pc (Fig. 1B).

Testing Transcription from the Spinach *rrn* Promoter in Tobacco Chloroplasts

To test whether sequence differences between the spinach and tobacco *rrn* operon promoters are responsible for promoter choice, transcription from the spinach promoter was tested in transgenic tobacco plants. For this, the spinach *rrn* operon promoter was PCR amplified as a 292-bp DNA fragment containing the region between *trnV* and the *rrn* operon-processing site (Fig. 2). This promoter fragment was cloned upstream of a *uidA* reporter gene, which was subsequently linked to a spectinomycin-resistance (*aadA*) gene in plastid vector pPRV111A (Fig. 3A). The resulting

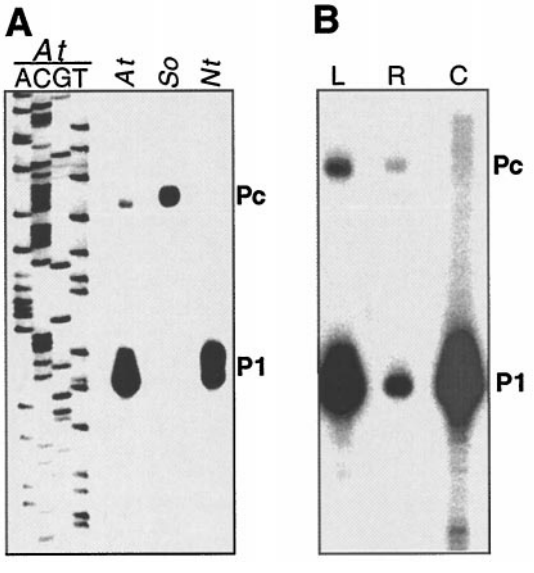

Figure 1. Identification of promoters for the plastid *rrn* operon in Arabidopsis. A, Primer-extension analysis to identify RNA 5′ ends upstream of the *rrn* operon in Arabidopsis (*At*), spinach (*So*), and tobacco (*Nt*) leaves. The Arabidopsis *rrn* promoter sequence obtained with the same primer is shown for comparison. Nucleotides at which P1 and Pc transcription initiates are marked in Figure 2. B, Pc is active in Arabidopsis leaves (L) and roots (R) but not in cotyledons (C).

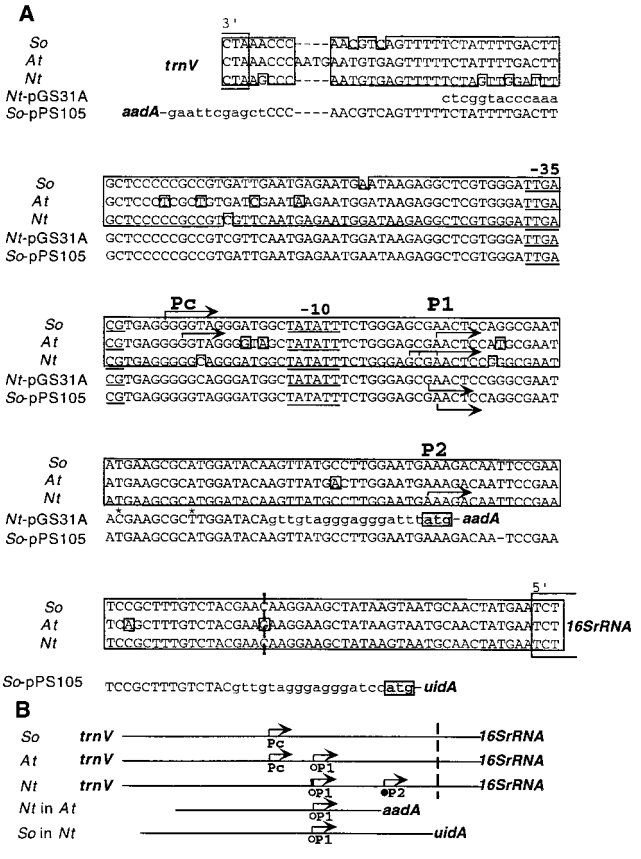

Figure 2. Promoters for the *rrn* operon in spinach (*So*), Arabidopsis (*At*), and tobacco (*Nt*). A, Alignment of DNA sequences between *trnV* and *16SrDNA*. DNA sequences of the tobacco *rrn* promoter fragment in transplastomic Arabidopsis from plasmid pGS31A and of the spinach *rrn* promoter fragment in transplastomic tobacco from plasmid pPS105 are also shown. Nucleotides derived from the plastid genome are shown in uppercase letters, whereas those added during vector construction are shown in lowercase letters. Conserved sequences in the plastid genomes are boxed. Transcription-initiation sites are marked with horizontal arrows. The RNA-processing site is marked with a dashed vertical line. Stars in pGS31A denote mutations introduced to eliminate translation-initiation codons. B, Schematic representation of transcription-initiation sites in A. PEP and nuclear-encoded plastid RNA polymerase promoters are marked with open and filled circles, respectively.

plasmid, pPS105, was introduced into tobacco chloroplasts by particle bombardment, where the linked *aadA* and *uidA* genes integrated into the plastid genome via the plastid-targeting sequences (Fig. 3A). Plastid transformants were selected by spectinomycin resistance.

Promoter utilization in the *uidA* gene was tested in the leaves of transgenic Nt-pPS105–1 tobacco plants. Primer-extension analysis identified a single 5′ end mapping to the P1 site within the spinach promoter fragment (Fig. 3B). This finding indicates that all sequence information required for transcription from the P1 promoter is present in spinach. Since there is no transcript initiating from the Pc site, even when the blots are overexposed, tobacco chloroplasts cannot recognize Pc sequences.

Testing Transcription from the Tobacco *rrn* Promoter in Arabidopsis Chloroplasts

Because tobacco chloroplasts lack the ability to initiate transcription from Pc, we were interested in determining whether sequences for Pc function are present. Spinach plastid transformation was not available; therefore, we could not test transcription initiation from the tobacco *rrn* operon promoter in spinach. However, an Arabidopsis plant expressing *aadA* from the tobacco Prrn promoter was available (Sikdar et al., 1998; Fig. 2). Therefore, we could test transcription from the tobacco *rrn* promoter in the At-pGS31A transplastomic plants in which both Pc and P1 are active upstream of the native *rrn* operon. In the transplastomic plants, primer-extension analysis identified only one RNA 5′ end upstream of *aadA*. This 5′ end maps to the P1 site (Figs. 2 and 4B). Since in the transgenic Arabidopsis plant no transcript initiates at Pc within the tobacco *rrn* promoter fragment, tobacco plastids apparently lack functional Pc promoter sequences.

DISCUSSION

We conclude from our studies that transcription initiation from the P1 and Pc promoters depends on promoter-

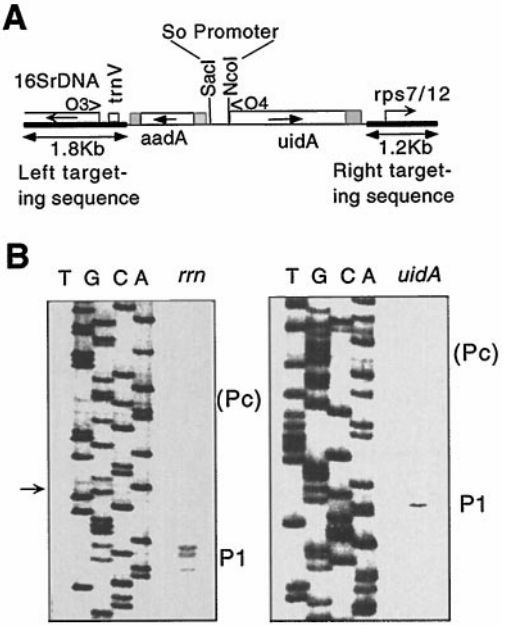

Figure 3. Transcription initiation in the spinach *rrn* promoter fragment in tobacco chloroplasts. A, Plastid-targeting region of plasmid pPS105. Shown are: *aadA*-selectable marker gene, *uidA* reporter gene, and *Sac*I and *Nco*I sites for insertion of spinach *rrn* promoter. Direction of transcription is shown by horizontal arrows. O3 and O4 mark the position of oligonucleotides used for primer extension. B, Primer-extension analysis to map RNA 5′ ends in transplastomic Nt-pPS105 tobacco plants. Data are shown for the native *rrn* operon using primer O3 and for the *uidA* transgene driven by the spinach *rrn* promoter region using primer O4. No signal was detected at the Pc position, even when the film was overexposed. DNA sequences obtained with the same primers are shown alongside primer extension. Sequences above the mark for *rrn* were derived from cloning vector.

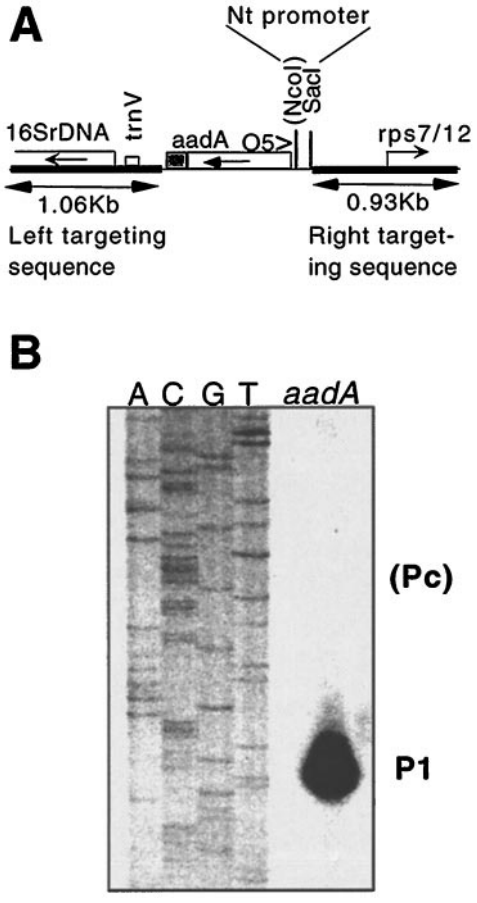

Figure 4. Transcription initiation in the tobacco *rrn* promoter fragment in Arabidopsis chloroplasts. A, Plastid-targeting region of plasmid pGS31A with the spectinomcyin-resistance (*aadA*) gene. The position of primer O5 is shown. Direction of transcription is marked by horizontal arrows. B, Primer-extension analysis to map RNA 5′ ends. The primer-extension product is run alongside the DNA sequence obtained with the same primer.

specific transcription factors that are necessary for transcription initiation by the general transcription machinery. Spinach apparently has the P1 promoter sequence, as evidenced by transcription initiation from the spinach *rrn* promoter in tobacco at the P1 site. Although other genes, e.g. *rbcL*, *atpB*, and *psbB*, are transcribed by PEP from σ^{70}-type promoters (Mullet et al., 1985; Westhoff, 1985), the P1-PEP promoter is silent in spinach. Therefore, the lack of P1 activity in spinach is probably due to the lack of a functional P1-specific activating factor. This conclusion is strengthened by the observation that P1 is silent in spinach roots where Pc is inactive, indicating that transcription from Pc is not the reason for the lack of P1 activity in chloroplasts (Iratni et al., 1997). Tobacco chloroplasts apparently lack the factor required for Pc recognition, otherwise transcription from the spinach *rrn* promoter region in tobacco would initiate at both the P1 and the Pc site. Since tobacco does not have a Pc factor, it is not surprising that it does not have a functional Pc promoter sequence, as suggested by the lack of transcription initiation at Pc from the tobacco *rrn* promoter in Arabidopsis plastids. Relevant in this regard could be point mutations in tobacco compared with spinach in the *rrn* upstream region (Fig. 2A).

Our data can be best explained by the requirement for a positive regulatory factor(s) for *rrn* transcription. A good candidate for the Pc-activating factor is the protein CDF2, the binding of which is correlated with transcription from Pc in spinach (Iratni et al., 1997). The target site of the *rrn* P1 activator in pea seems to be sequences upstream of the −35 hexamer required for specific transcription initiation (Sun et al., 1989). This site coincides with the CDF2-binding site (Baeza et al., 1991). The proposed plastid transcriptional activators would have a function analogous to the Fis protein, for which there are multiple binding sites upstream of the P1 ribosomal operon promoter in *E. coli* (Ross et al., 1990, 1993; Condon et al., 1992).

Selective activation of plastid housekeeping genes early during chloroplast development is an important regulatory step (Baumgartner et al., 1993; DuBell and Mullet, 1995). In the case of the rRNA operon, this may be accomplished by a gene-specific transcriptional activator that allows selective control of plastid gene transcription by the nucleus.

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