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ORIGINAL PAPER

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Chloroplast rRNA transcription from structurally different tandem promoters: an additional novel-type promoter

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Abstract Identification of transcription initiation sites in the promoter region of the tobacco chloroplast rRNA operon has been carried out by ribonuclease protection of in vitro capped RNAs and primer extension experiments. A promoter with typical chloroplast -10 and -35 motifs (P1) drives initiation of transcription from position -116 relative to the mature 16*s* rRNA sequence. In addition, we have found that a second primary transcript starts at position -64. This proximal promoter (P2) lacks any elements similar to those reported so far in chloroplast promoter regions, and hence P2 represents a novel-type promoter. Both transcripts are present in chloroplasts from green leaves and in non-photosynthetic proplastids from heterotrophically cultured cells (BY2), but their relative amounts appear to differ. The steady state level of the P2 transcript, with respect to P1, is higher in BY2 proplastids than in leaf chloroplasts.

Key words Chloroplast transcripts · In vitro capping Plastid promoters · rRNA

Introduction

Chloroplast ribosomes of higher plants are 70*s* in size and have many prokaryotic features (Weil 1987; Sugiura 1989, 1992; Mache 1990; Subramanian et al. 1991). Whereas their protein components are encoded in both the chloroplast and the nuclear genomes, the rRNA genes are clustered in the plastid DNA in the order 16*s*-23*s*-4.5*s*-5*s*, resembling the prokaryotic arrangement (Sugiura 1989, 1992; Mache 1990). As in bacteria (Srivastava et al. 1990), chloroplast rRNA genes are transcribed as long primary transcripts encompassing the whole operon, which are processed to produce the different mature rRNAs (Delp and Kössel 1991). Therefore, rRNA primary transcripts must arise from promoters located in the region upstream of the 16*s* rRNA coding region. Transcription initiation sites in this region have been inferred through diverse techniques, including S1 mapping, primer extension, in vitro transcription, and homology to prokaryotic promoter consensus sequences (reviewed in Delp and Kössel 1991). By in vitro capping of the primary transcripts, the transcriptional start site of maize plastid rRNA was unambiguously assigned to position -117 (Strittmatter et al. 1985). The maize rRNA promoter region, and the tentative equivalents in other species, include typical prokaryotic -10 and -35 motifs situated at very similar distances. The consensus sequences do not differ from the promoters for protein-coding genes. This suggested that the same kind of RNA polymerase is involved in the transcription of both kinds of genes (Delp and Kössel 1991).

Here we report the existence of a new promoter (P2) for the tobacco chloroplast rRNA operon in addition to the upstream promoter P1 possessing -10 and -35 elements. P2 does not contain any apparent motif so far reported. This fact and the proximity to the coding region, suggest differences in transcriptional regulation for P2 and the later RNA processing of its transcript.

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Materials and methods

Plant growth and RNA extraction. Tobacco plants (*Nicotiana tabacum* var. Bright Yellow 4) were grown for 2 months at 24 °C under an 18-h photoperiod in a growth chamber. Culture of heterotrophic tobacco cells (BY2) was carried out for 5 days as described (Yasuda et al. 1988). Total leaf and BY2 RNAs were phenol-extracted as in De Vries et al. (1988).

Probe preparation. DNA probe A (608 bp), containing the first 203 bp of the 16*s* rRNA coding region and 405 bp of the upstream region (see Figs. 2 A and 3), was amplified from the pTB9 plasmid (Sugiura et al. 1986) by the polymerase chain reaction (PCR) during 30 cycles (each 94 °C for 1 min, 60 °C for 2 min, 72 °C for 1 min). The PCR primers were: 16M: 5′AAGAAGGCCCGGCTC-CAAGT3′ PR161: 5′CCCCTCCTCGGGCGGATTCCTC3′. The

PR16 plasmid (Vera et al. 1993) was cut with *Bam*HI and used as template for the synthesis of an antisense RNA probe (a-PR16) which includes the first 203 nucleotides (nt) of 16*s* rRNA and the upstream 77-nt region. In vitro transcription reactions (without ^{32}P-rUTP) were as described (Vera et al. 1992).

In vitro capping, ribonuclease protection assay and primer extension. Total leaf RNA was capped with [^{32}P]GTP and guanylyltransferase as described (Vera and Sugiura 1992). Ten micrograms of the capped RNA were annealed with 114 ng of the A probe, or 300 ng of the a-PR16 riboprobe, and digested with a mixture of ribonucleases A and T1. The ribonuclease protection assay has been reported previously (Vera and Sugiura 1992; Vera et al. 1992). For primer extension experiments, oligonucleotides PER16 (Vera et al. 1993) and PE16 (5′CCATGAGATTCATAG3′) were 5′end-labeled with ^{32}P and used to prime total leaf and BY2 RNA as in Vera et al. (1993). Oligonucleotides were prepared with a DNA synthesizer (Applied Biosystems, Type 380 A).

Results

Multiple 5′ ends for chloroplast pre-16*s* rRNAs

Primer extension experiments were conducted to detect the 5′ termini of tobacco plastid 16*s* rRNA. Oligonucleotides PER16 and PE16 were used as primers since they correspond to sequences at the junction site between the leader and the 16*s* rRNA coding region, and therefore they can only prime transcripts longer than the mature 16*s* rRNA (see Fig. 3). This allows better detection of low-abundance transcripts. When RNA from heterotrophic tobacco cell (BY2) cultures was analyzed, several bands of 126–124, 76, 55, 50 and 42 nt appeared (Fig. 1 A, lane P). The exact positions have been determined by running in parallel sequence ladders obtained by the dideoxy method using the same primer (Vera et al. 1993 and data not shown). Thus, the upper two bands have been mapped to positions -116 and -114 and probably correspond to the 16*s* rRNA primary transcript, as predicted from sequence alignment with other species and the presence of conserved promoter motifs upstream from that site (Delp and Kössel 1991; Vera et al. 1993 and see Fig. 3). A weak 76-nt band corresponds to position -64. This 5′ end was also detected by ribonuclease protection assays (Vera et al. 1993). The 55- and 50-nt bands do not appear in all experiments and they might be due to mispriming or false stops of reverse transcriptase. An extension product of 42 nt corresponds to position -30 and may be a major cleavage site in the 16*s* rRNA maturation pathway (Strittmatter et al. 1985; Vera et al. 1993). Fig. 1 B shows a parallel analysis of RNA from tobacco leaves and BY2 cells. Here the extended products are 4 nt shorter due to priming with the PE16 oligonucleotide (see Fig. 3). The two extension products reaching positions -114 and -116 are again detected in both leaf and BY2 cell RNAs, the signal for leaf RNA being much stronger. Though the experiments were not standardized to allow a quantitative comparison between the two RNA sources, we note that the transcript starting at -64 is relatively abundant in BY2 cells while it is barely detectable in leaf RNA.

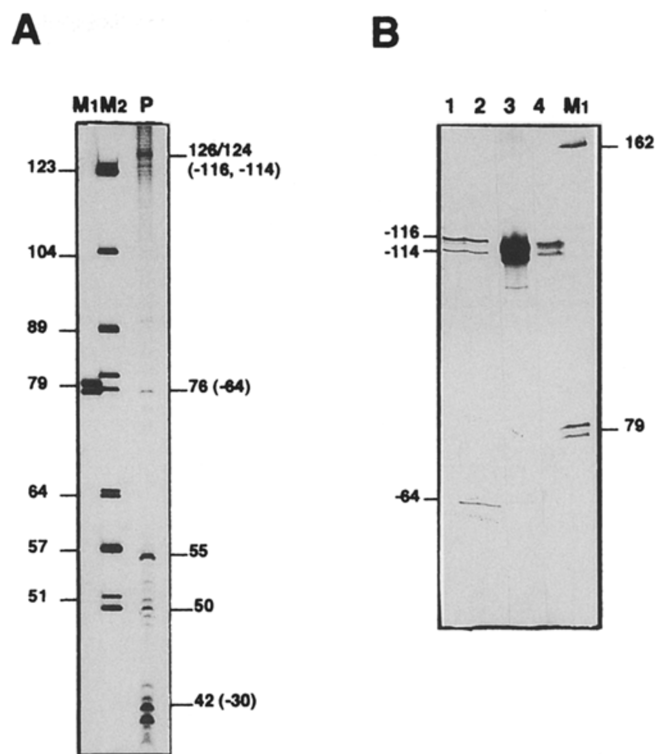

Fig. 1 A, B Detection of the 5′ends of pre-16*s* rRNA by primer extension. **A** 5′[^{32}P]-oligonucleotide PER16 was annealed to 30 µg of BY2 RNA and extended with reverse transcriptase (*lane P*). Corresponding positions with respect to the 5′end of 16*s* rRNA are in *brackets*. M_1 *Hin*cII digest of ϕx174 RF-DNA; M_2 *Hae*III digest of pBR322 DNA. **B** 5′[^{32}P]-oligonucleotide PE16 was annealed to 30 µg of BY2 RNA (*lanes 1 and 2*), and 30 µg (*lane 3*) or 10 µg of leaf RNA (*lane 4*) and extended as in A. M_1 *Hin*cII digest of ϕx174 RF-DNA. All sizes are in nt

Determination of the transcription initiation sites

To verify that the 5′ end at position -116 corresponds to the rRNA transcription initiation site, capped leaf RNA was annealed to DNA probe A, depicted in Fig. 2 A (see also Fig. 3), covering that position, and was digested with ribonucleases A and T1. As illustrated in Fig. 2 B, an intense band of 320 nt was protected which fits the expected size (319 nt) deduced from primer extension experiments (lanes 2 and 3). Therefore, it can be concluded that position -116 is a real site of transcription initiation for the rRNA operon of tobacco chloroplasts. The structure of this promoter is ″-35 TTGACG(18 nt)-11 TATATT″ (see Fig. 3). No cappable transcript of higher chain length could be detected. At moderate concentrations of ribonucleases, several smaller and less intense bands were still visible (Fig. 2 B, lane 2), and were first considered as partially digested products. However, one of these bands (about 270 nt) comigrates with an unexpected protected product in a control using the antisense RNA probe a-PR16 (Figs. 2 A, B, lane 1). Its size fits the primer extension product reaching position -64 (Fig. 1). This protected frag-

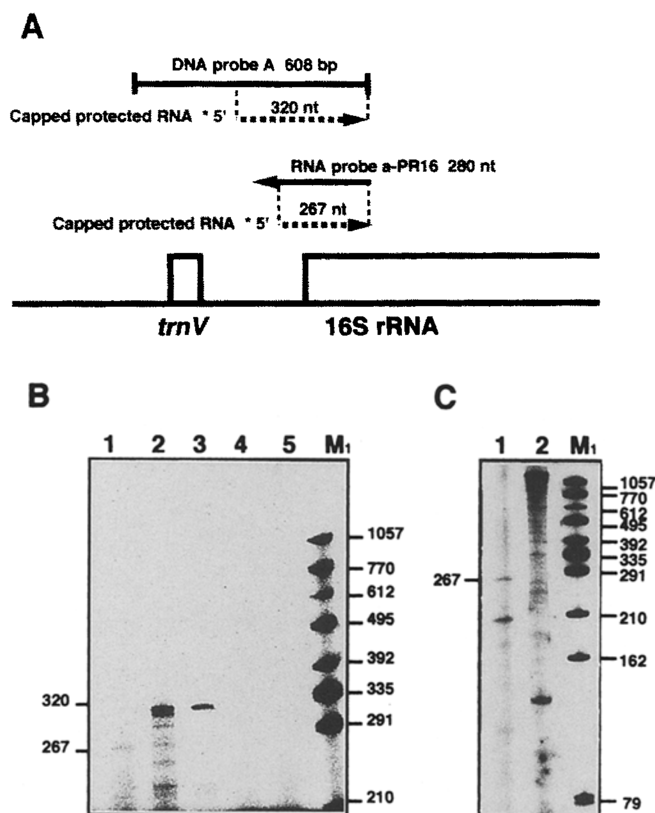

Fig. 2 A–C Determination of the transcription start sites of the rRNA operon by in vitro capping and ribonuclease protection assay. **A** experimental strategy. **B** capped leaf RNA was annealed with probe a-PR16 (*lane 1*) or probe A (*lanes 2 and 3*) or no probe (*lanes 4 and 5*), and digested with 20 units of RNase T1 and 0.1 units of RNase A (*lanes 1, 2 and 4*) or 40 units of RNase T1 and 0.2 units of RNase A (*lanes 3 and 5*). **C** capped leaf RNA was annealed with the probe a-PR16 and digested with 40 units of RNase T1 and 0.2 units of RNase A (*lane 1*). *Lane 2* shows one-hundredth of the total capping reaction without RNase digestion. M_1 represents *Hinc*II digest of ϕx174 RF-DNA as size markers. All sizes are in nt

ment was detected in BY2 cells in experiments using uncapped RNA and uniformly radiolabeled probes which can not discriminate between processing products and primary transcripts (Vera et al. 1993). Aside from a 200-nt non-specific band which also appeared without any riboprobe (Vera and Sugiura 1994), the same result was consistently reproduced using a very high concentration of ribonucleases (Fig. 2C, lane 1), thus excluding the possibility of incomplete digestion. Absence of this band after protection with the DNA probe A (Fig. 2B, lane 3) is explained by the low efficiency in annealing to the target RNA of dsDNA probes with respect to antisense RNA, which can preclude detection of primary transcripts present in low amounts (Vera and Sugiura 1992). We therefore concluded that there is a second transcription initiation site in the tobacco chloroplast rRNA operon located at position -64. We have designated the corresponding promoter as P2.

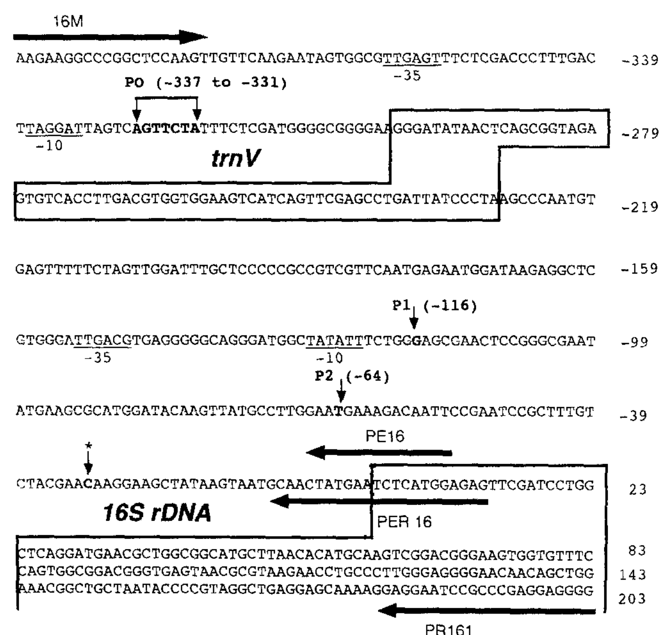

Fig. 3 The upstream region of tobacco chloroplast 16*s* rRNA. The sequence corresponds to probe A. Coding regions are *boxed*. The positions and orientations of the oligonucleotides used in PCR and primer extension are indicated by *bold arrows*. Initiation sites of transcription are indicated by *thin vertical arrows* with positions in brackets. *P1* and *P2* are in vivo initiation sites, and *PO* is used by *E. coli* RNA polymerase in vitro. The -35 and -10 motifs are *underlined*. The putative major processing site is indicated with an *asterisk* (Vera et al. 1993). The nucleotide sequence has been published (Shinozaki et al. 1986, accession No. Z00044)

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                                                          5'
                                                          ▼
Tobacco1    CCGGGCGAATATGAAGCGCATGGATACAAGTTATGCCTTGGAATGAAAGACAATTCCG
Spinach2    **A***************************************************C*GA
Epifagus3   *****A************T******C*************C****T**********ATG
Soybean4    **A*T**************C*************************G****G*******
Pea5        ***********GT****C*************C*A*TTA**TCT*CTC**TTC*G*AAC
Mustard6    **AT*******************************A**********************
Spirodela7  **A***T***************************************************
Maize8      GGCTAAT***C***********************-********C**************
Rice9       GGCTAAT***C***********************-********C**************
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Fig. 4 Comparison of the P2 region from tobacco with homologous regions from monocot and dicot plants. The transcription initiation site in tobacco is indicated by a *triangle*. *Asterisks* indicate nucleotide identity. *Gaps* have been introduced to maximize homology. [1] Shinozaki et al. 1986; [2] Briat et al. 1982; [3] Wolfe et al. 1992; [4] Von Allmen and Stutz 1988; [5] Shapiro and Tewari 1986; [6] Przybyl et al. 1984; [7] Keus et al. 1983; [8] Strittmatter et al. 1985; [9] Hiratsuka et al. 1989

Discussion

By using ribonuclease protection assay of in vitro capped RNA, we have confirmed the site of transcription initiation (P1) for the tobacco chloroplast rRNA operon. Typical Pribnow box and -35-like elements appear in front of this site (Fig. 3) showing a high conservation with other plant species (Delp and Kössel 1991). Maize chloroplast rRNA transcription has been shown to start at position -117 (Strittmatter et al. 1985), in accordance with the -116

site determined in this study. We also found a second initiation site (-64) located downstream from P1. This promoter, P2, lacks any recognizable elements in its sequence which suggests that it may be differentially regulated. Recognition of P2 might require a distinct RNA polymerase or the same core enzyme with different sigma-like factor(s).

Alignment of the P2 region of tobacco chloroplast DNA with the equivalent regions of different vascular plants, shows a high level of conservation with both dicots and monocots (Fig. 4). Pea is an exception since several substitutions occur around the putative initiation site of transcription. However, at present no correlation can be established between these changes and promoter activity since transcription data from this position are not available for plant species other than tobacco.

The existence of a third promoter upstream from P1, including the preceding gene *trnV* (Schwarz et al. 1981), is suspected on the basis of primer extension experiments (data not shown). Similar results were obtained by using *E. coli* RNA polymerase in an in vitro system (Tohdoh et al. 1981, see Fig. 3). Very fast processing by an RNaseP-like enzyme might be responsible for the low level of detection of that transcript. In fact, very short products (about 100 nt) appearing after in vitro capping and ribonuclease protection with probe A (see Figs. 2 and 3) could account for the existence of a cappable tRNA-Val transcript. Further experiments with more specific probes are required to clarify this point.

In spite of the strong post-transcriptional control (Rochaix 1992; Gruissem and Tonkyn 1993) diverse lines of evidence point towards a specific role of transcriptional regulation in plastid gene expression (Igloi and Kössel 1992). Thus, multiple promoters have been observed in front of several chloroplast genes and gene clusters (Yao et al. 1989; Berends-Sexton et al. 1990; Haley and Bogorad 1990; Meng et al. 1991; Kapoor et al. 1994). A light-inducible promoter which lacks apparent -10 and -35 elements was demontrated for the *psbD-psbC* operon (Berends-Sexton et al. 1990; Christopher et al. 1992). In addition, specific transcriptional regulation of chloroplast genes lacking the -35 motif has been suggested (Neuhaus et al. 1989; Vera et al. 1992). In this regard it is noteworthy that spinach chloroplast 16*s* rRNA might be transcribed from a promoter located upstream, rather than from P1, in a region where no -10 and -35 motifs are found at the proper distance (Baeza et al. 1991).

Tiller and Link (1993) have shown that the degree of phosphorylation of plastid sigma-like factors confers different DNA-binding properties to the RNA polymerase and that this is related to the plastid type. Evidence supporting the existence of an additional nuclear-encoded plastid RNA polymerase has also been reported (Hess et al. 1993; Lerbs-Mache 1993). Regulation of these factors may lead to differential usage of diverse groups of promoters depending on physiological and/or developmental conditions. If this is so, the tobacco plastid rRNA operon might be an ideal model to study the mechanisms involved. We have shown that in comparison with that of the P1 transcript, the steady state level of the P2 transcript (lacking -10 and -35 motifs), is much higher in proplastid-containing BY2 cells than in chloroplasts from green leaves. The difference is conspicuous, and likely to be due to changes in transcriptional acitivity, although variations in pre-rRNA stability can not be excluded.

The question also arises as to how the P2 transcript is processed. According to the bacterial model (Gegenheimer and Apirion 1981; Srivastava and Schlessinger 1990), plastid pre-16*s* rRNA is predicted to be cleaved by a ribonuclease III-like enzyme which recognizes a putative secondary structure formed by the flanking regions (Tohdoh and Sugiura 1982; Strittmatter et al. 1985). In the parasitic plant *Epifagus*, deletions in the 16*s* rRNA flanking regions preclude the formation of this structure, thereby suggesting a different pathway of processing (Wolfe et al. 1992). Ribonuclease III is a dispensable enzyme in bacteria as mutants lacking its activity accumulate normal levels of mature RNA (Srivastava and Schlessinger 1990). This may reflect the existence of alternative maturation pathways. The occurrence of transcription from P2 in the tobacco plastid rRNA operon is consistent with this hypothesis.

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