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Sequence of a putative promoter region for the rRNA genes of tobacco chloroplast DNA

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ABSTRACT

The nucleotide sequence of the segment of tobacco chloroplast DNA adjacent to and including the start of the 16S rRNA gene has been determined. The region just preceding this gene was found to contain a $tRNA^{Val}$ gene and promoter-type sequences similar to those which occur in E. coli were found before this tRNA gene. E. coli RNA polymerase can recognize these sequences and in vitro co-transcribes the tRNA and rRNA genes.

INTRODUCTION

Tobacco chloroplast DNA (ctDNA) contains two clusters of rRNA genes (1, 2, 3). The 16S, 23S, 4.5S and 5S rRNA genes in each cluster are arranged sequentially on the same DNA strand and transcription proceeds from the 16S to 5S rRNA gene (4, 5). In order to understand the regulatory mechanisms which affect rRNA synthesis in chloroplasts, we have sequenced the promoter region of a tobacco chloroplast rRNA operon which was cloned in the E. coli plasmid pMB9. This DNA sequence, which is presented here, encodes chloroplast $tRNA^{Val}$ as well as the start of the 16S gene and contains sequences similar to the "Pribnow box" and the "-35 region" found in E. coli promoters (6, 7). In vitro transcription of the DNA fragment containing this region reveals that E. coli RNA polymerase co-transcribes the tRNA and rRNA genes.

MATERIALS AND METHODS

Recombinant plasmid pTC1 containing an EcoRI fragment of Nicotiana tabacum (var. Bright Yellow 4) ctDNA had been constructed as described (1) and the plasmid DNA was prepared by

the procedure of Katz et al. (8). The pTC1 DNA was digested with EcoRI and the 3 kbp fragment (formerly designated as the 1.9×10^6 dalton fragment, Ref. 1) was separated from vector pMB9 by electrophoresis in a 0.7% low-melting agarose gel. The 3 kbp fragment was eluted from this gel as suggested by Bethesda Research Laboratories, Inc. A cleavage map of the fragment was constructed as described previously (4, 5). DNA sequence analysis was carried out by the method of Maxam and Gilbert (9). Partial cleavage products were analyzed by electrophoresis in 12% polyacrylamide slab gels (52 × 28 × 0.05 cm). Hybridization to Southern blots was done as described (1). *In vitro* transcription was performed essentially as described by Gilbert et al. (10). RNA polymerase was prepared from *E. coli* A19 ($RNase\ I^-$) as described (11). S_1 nuclease mapping was carried out according to the method of Berk and Sharp (12).

RESULTS AND DISCUSSION

DNA sequence. We had previously cloned a 3 kbp EcoRI fragment of tobacco ctDNA which contains most of the 16S rRNA gene together with its 5' flanking region (1). The location of the 16S coding sequence within this fragment had been determined by electron microscopic analysis of R-loops (4). A detailed physical map of restriction sites was constructed by cleaving it with nine restriction endonucleases (Fig. 1) and the nucleotide sequence of the 766 bp Bst EII - Pvu II sub-fragment from within this 3 kbp fragment was determined using the sequencing strategy shown in Fig. 1. The sequence of non-coding strand (RNA-like sequence) of the DNA fragment is shown in Fig. 2. The 5' end of the 16S rRNA coding region was located by the S_1 nuclease mapping (an unpublished result) and is shown as position 1 in Fig. 2. The sequence thus covers the first 139 bp of the 16S rRNA coding region and the 627 bp which preceed this. Although the coding regions for the chloroplast and *E. coli* (13) 16S rRNAs have a high degree of homology, as was reported for maize chloroplast DNA (14), the regions preceding these genes are dissimilar (6, 7).

$tRNA^{Val}$ sequence. A tRNA gene was found between positions -229 and -300 in the DNA upstream from the 16S rRNA coding region.

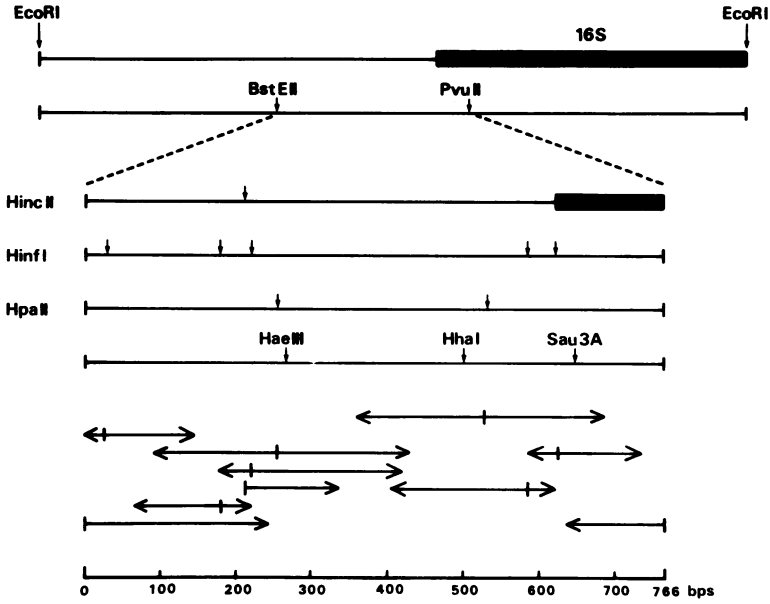


Fig. 1 Physical map of the cloned 3 kbp fragment from tobacco chloroplast DNA and the strategy for sequencing part of it. The region coding for the 16S rRNA is shown by thickened lines and sites of cleavage by restriction endonucleases are denoted by arrows. The region of the DNA which was sequenced is shown on the expanded map. Horizontal arrows indicate the direction in which and extent to which DNA segments were sequenced.

The presence of a tRNA gene in this region was confirmed by hybridization of total chloroplast tRNA to a Southern blot of the 1.8 kbp Bst EII - EcoRI sub-fragment (Fig. 3). Judging by the anticodon sequence, it is a $tRNA^{Val}$ gene. The CCA sequence found at the 3' end of all tRNAs is not coded for by this chloroplast DNA in contrast to E. coli tRNA genes which are known to code for this CCA sequence (15, 16). In this respect, this chloroplast tRNA gene resembles a nuclear eukaryotic tRNA gene, rather than prokaryotic one. Fig. 4 shows the sequence and cloverleaf structure that can be deduced for the $tRNA^{Val}$ based on the DNA sequence which codes for it. It is identical to the maize chloroplast $tRNA^{Val}$ (17) and similar to E. coli $tRNA^{Val}_{2B}$ (65% homology, 18).

In vitro transcription. Using the 766 bp Bst EII - Pvu II

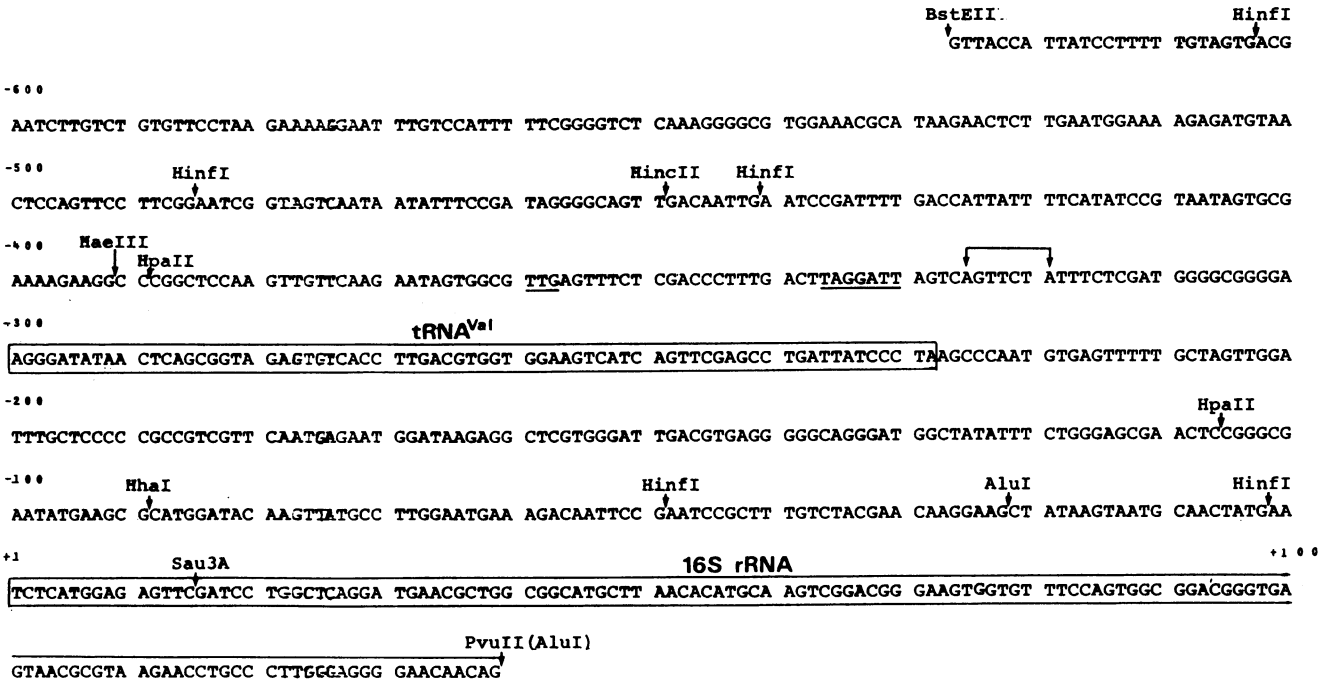
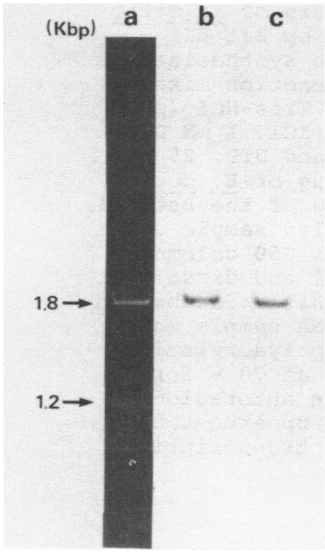


Fig. 2 DNA sequence in the region at the start of the 16S rRNA gene in cloned tobacco chloroplast DNA. Arrows indicate restriction enzyme sites and a possible "Pribnow box" and "-35 region" are underlined. Possible sites for the start of transcription are indicated by arrows ( ).



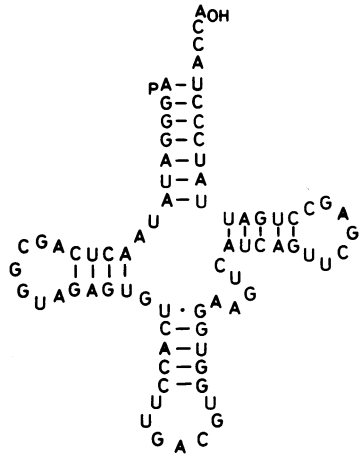


Fig. 3 (left) Hybridization of tobacco chloroplast tRNA with Bst EII sub-fragments of the 3 kbp EcoRI fragment. The Bst EII digest was electrophoresed in a 1.2% agarose gel (a), transferred to a Millipore filter and hybridized with tobacco chloroplast (b) total tRNA, and (c) 16S rRNA. The RNAs were labeled at their 5' ends using [γ^{32}P]ATP and polynucleotide kinase.

Fig. 4 (right) Sequence of unmodified bases and cloverleaf secondary structure of tobacco chloroplast $tRNA^{Val}$ which can be predicted from the sequence of the DNA which codes for it.

fragment as a template, we examined RNA synthesis by purified E. coli RNA polymerase. The reaction conditions were identical to those used for the in vitro transcription of E. coli rRNA operons (10). An autoradiogram in Fig. 5 shows that the 766 bp fragment yields two major RNA products containing about 460 and 240 bases respectively. Our preliminary experiment using [γ^{32}P]ATP as substrate shows that the RNA synthesis starts predominantly with ATP.

The starting points of the in vitro RNA products were determined by a S_1 nuclease mapping procedure. The coding DNA strand of the 392 bp Hinf I fragment (between positions -50 and -441) was 5' end-labeled, then hybridized to the unlabeled in vitro product and was finally digested with S_1 nuclease. The

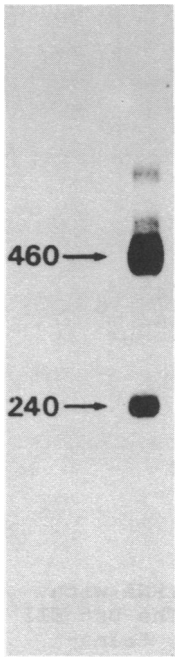


Fig. 5 Gel electrophoresis of *in vitro* transcripts from the 766 bp Bst EII - Pvu II fragment. RNA was synthesized at 37°C for 10 min in the reaction mixture (20 µl) containing 18 mM Tris-HCl (pH 7.8), 12 mM $MgCl_2$, 70 mM KCl, 1 mM DTT, 350 µM each of ATP, CTP and UTP, 25 µM [α^{32}P]GTP (10 µCi), 0.3 µg of *E. coli* RNA polymerase and 0.1 µg of the Bst EII - Pvu II fragment. Labeled sample was passed through a Sephadex G50 column, precipitated with ethanol and dissolved in 10 µl of formamide. After incubation at 37°C for 5 min, the RNA sample was electrophoresed in a 5% polyacrylamide slab gel. Gels were run at 70 V for 6 hr at 5°C, dried and then autoradiographed. The RNA in the uppermost band represents an end-to-end transcription product of the template.

protected DNA fraction was sized by polyacrylamide gel electrophoresis. As shown in Fig. 6, the protected fraction shows a rather wide size distribution of between 270-277 nucleotides (corresponding to positions -319 to -326 on the template DNA). Before these positions, there is the sequence TAGGATT (between positions -337 and -331) which is similar to the "Pribnow box" (TATRATR) proposed as the signal for RNA polymerase recognition (19). A TTG sequence (between positions -360 and -358) was also found before this sequence. Such a TTG sequence occurs in the "-35 region" of all rRNA operons in *E. coli* so far examined (6, 7). Therefore the *E. coli* RNA polymerase recognizes these sequences in the ctDNA and the large transcripts (about 460 bases) starting at positions between -320 (A) and -326 (A), apparently terminate at the end of the Bst EII - Pvu II fragment. This indicates that the $tRNA^{Val}$ is co-transcribed with the rRNA. No sequences similar to those which occur at *E. coli* transcription termination sites, {dyad symmetry followed by an AT-rich stretch or the sequence CAATCA (20)},

Fig. 6 Determination by a S_1 nuclease mapping procedure of the start sites of the *in vitro* synthesized RNA product. The coding strand of the 392 bp Hinf I fragment, labeled at its 5' end, was obtained by strand separation. The unlabeled *in vitro* transcript was prepared as described in Fig. 5, and (to remove the template DNA) was treated with 6 µg of DNase I in 200 µl of 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 3 mM $MgCl_2$. Hybridization between the unlabeled *in vitro* transcript and the [5'^{32}P] labeled coding strand was performed at 50°C for 3 hr in 10 µl of a solution containing 80% formamide, 0.3 M NaCl, 40 mM Tris-HCl (pH 8), 1 mM EDTA. The hybridization mixture was then diluted with 100 µl of 0.15 M NaCl, 30 mM Na-acetate buffer (pH 4.6), 1 mM $ZnSO_4$ and digested with 15 units of S_1 nuclease at 37°C for 1 hr. After addition of 1.5 µg *E. coli* carrier tRNA and ethanol precipitation, hybrids were dissolved in 3 µl of 0.1 M NaOH, 1 mM EDTA, and an equal vol. of 10 M urea containing, 0.05% xylene cyanol and 0.05% bromophenol blue was added. The protected fragments were electrophoresed in a 12% polyacrylamide gel containing 7 M urea (b). The top band represent the full length DNA hybridized with the end-to-end transcription product. The size markers (bp) were denatured [5'^{32}P] labeled Hinf I + Hpa II digests of the 766 bp Bst EII - Pvu II fragment (a).

were found in the spacer between the tRNA and 16S rRNA genes.

As already shown in Fig. 6, only one major RNA band was detected after the S_1 nuclease mapping, showing that the smaller *in vitro* transcript (about 240 bases) from the Bst EII - Pvu II fragment was not protected from S_1 nuclease digestion. Consequently this small RNA may be transcribed from the DNA region outside the 392 bp Hinf I fragment or from the non-coding strand of the template.

Further analyses of transcription *in vivo* as well as *in*

vitro using chloroplast RNA polymerase will be necessary to establish exact positions where transcription starts.

ACKNOWLEDGEMENTS

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