***In vitro* characterization of the tobacco *rpoB* promoter reveals a core sequence motif conserved between phage-type plastid and plant mitochondrial promoters**

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We report here the *in vitro* characterization of *PrpoB*-345, the tobacco *rpoB* promoter recognized by NEP, the phage-type plastid RNA polymerase. Transcription extracts were prepared from mutant tobacco plants lacking PEP, the *Escherichia coli*-like plastid-encoded RNA polymerase. Systematic dissection of a ~1 kb fragment determined that the *rpoB* promoter is contained in a 15-nucleotide segment (–14 to +1) upstream of the transcription initiation site (+1). Point mutations at every nucleotide reduced transcription, except at the –5 position which was neutral. Critical for *rpoB* promoter function was a CRT-motif (CAT or CGT) at –8 to –6 (transcription <30%), defining it as the promoter core. The core CAT sequence is also present in the maize *rpoB* promoter, which is faithfully recognized by tobacco extracts. Alignment of NEP promoters identified a CATA or TATA (=YATA) sequence at the *rpoB* core position, also present in plant mitochondrial promoters. Furthermore, NEP and the phage T7 RNA polymerase exhibit similar sensitivity to inhibitors of transcription. These data indicate that the nuclear *RpoZ* gene, identified by sequence conservation with mitochondrial RNA polymerases, encodes the NEP catalytic subunit.

Keywords: *in vitro* transcription/nuclear-encoded plastid RNA polymerase/phage-type RNA polymerases/plastid promoters/*rpoB* operon

Introduction

Chloroplasts, the sites of photosynthesis, have a central role in the metabolism of plant cells. Although they contain their own genome and a gene expression system, chloroplasts are genetically semi-autonomous and require nuclear gene products for biogenesis and function (Sugiura, 1992). An important mechanism regulating the proplastid to chloroplast development is regulation of plastid gene transcription (Herrmann *et al.*, 1992; Mullet, 1993; Link, 1994, 1996). The plastid genome contains functional *rpo* genes encoding the homologs of the eubacterial RNA polymerase α , β and β' subunits (Bogorad, 1991; Igloi and Kössel, 1992; Sugiura, 1992) which form the core of the plastid-encoded RNA polymerase (PEP; Hu and Bogorad, 1990; Hu *et al.*, 1991; Pfannschmidt and Link, 1994). A set of sigma-like factors interact with PEP conferring promoter-specific binding and transcription specificity (Tanaka *et al.*, 1996, 1997; Isono *et al.*, 1997;

Kestermann *et al.*, 1998; Tozawa *et al.*, 1998). A second, plastid-localized, nuclear-encoded RNA polymerase (NEP) has been proposed by several researchers (Bünger and Feierabend, 1980; Morden *et al.*, 1991; Hess *et al.*, 1993; Lerbs-Mache, 1993). This has been established recently by showing transcription from novel promoters in plants lacking PEP due to targeted deletion of plastid *rpo* subunits (Allison *et al.*, 1996; Serino and Maliga, 1998). A gene (*RpoZ*) for the putative NEP catalytic subunit has been cloned, and its product was found to be similar to mitochondrial and phage RNA polymerases (Hedtko *et al.*, 1997).

Identification of transcription initiation sites for NEP was possible in plants lacking PEP, or in tissue culture cells, both having proplastid-like non-photosynthetic plastids (Hajdukiewicz *et al.*, 1997; Kapoor *et al.*, 1997; Hübschmann and Börner, 1998; Miyagi *et al.*, 1998; reviewed in Maliga, 1998). These studies revealed that photosynthetic genes have PEP promoters, whereas non-photosynthetic genes have promoters for both RNA polymerases. Only a few genes are known to be transcribed exclusively from a NEP promoter: *accD*, encoding a subunit of the acetyl-CoA carboxylase in dicots (Hajdukiewicz *et al.*, 1997); *clpP*, encoding the proteolytic subunit of the Clp ATP-dependent protease in monocots; and the *rpoB* operon in all higher plants (Silhavy and Maliga, 1998). The *rpoB* operon includes *rpoB*, *rpoC1* and *rpoC2*, the genes for three of the four PEP core subunits. Transcription of the *rpoB* operon is highly regulated (1000-fold), controlling the availability of the PEP (Baumgartner *et al.*, 1993). Thus, the *rpoB* NEP promoter plays a central role in chloroplast development.

Our long-term interest is to understand the mechanism by which NEP transcription rates are regulated. Given its biological importance, we decided to define the *rpoB* promoter functionally. To this end, a novel *in vitro* transcription system was developed, which is utilizing a unique source of NEP activity, plants lacking the *rpoA* gene (Serino and Maliga, 1998). Since the extracts entirely lack PEP, a comprehensive characterization of NEP transcription activity could be accomplished without the biochemical separation of the NEP and PEP activities.

Although the gene for the tentative catalytic core of the NEP has been identified (*RpoZ*; Hedtko *et al.*, 1997), no linkage has been made between the gene and NEP transcription activity. Using our novel *in vitro* transcription system, we demonstrate here that NEP, with respect to its sensitivity to drugs, is similar to phage-type RNA polymerases. Furthermore, fortuitous conservation of the *rpoB* promoter core with plant mitochondrial promoters indicates that *RpoZ* encodes the NEP catalytic core.

Results

Novel *in vitro* transcription system to characterize NEP promoter activity

Chloroplast extracts for *in vitro* PEP promoter dissection have been described (Jolly and Bogorad, 1980; Link, 1984; Gruissem and Zurawski, 1985; Orozco *et al.*, 1985). Recent identification of plastid NEP promoters created the need to develop an *in vitro* system for the phage-type plastid RNA polymerase. In order to test transcription initiating from NEP promoters *in vitro*, promoter-terminator cassettes were constructed, consisting of a NEP promoter and a pair of transcription terminators (*hisa*, *thra*). Termination at the histidine attenuator (*hisa*; Barnes and Tuley, 1983) is expected to add 145 nucleotides to the transcript; termination at the threonine attenuator (*thra*; Gardner, 1982) is expected to add an additional 72 nucleotides (Figure 1B). RNA polymerase activity was detected in run-off type experiments which involve incubation of promoter-terminator cassettes in transcriptionally active plastid extracts in the presence of labeled nucleotides, and analysis of newly synthesized RNA in sequencing gels (Gruissem *et al.*, 1986; Orozco *et al.*, 1986).

Six NEP promoters were tested for *in vitro* activity: *PrpoB*-345 (Serino and Maliga, 1998), *PaccD*-129, *PatpB*-289, *PatpI*-207, *PclpP*-173 (Hajdukiewicz *et al.*, 1997) and the *rrn* NEP promoter, mapped in tissue culture cells as *Prrn16*-64 (Vera and Sugiura, 1995) and in leaves as *Prrn16*-62 (Allison *et al.*, 1996). The promoters are identified by the gene symbol and the distance in nucleotides of the transcript 5' end from the coding region. Extracts for testing NEP transcription activity were prepared from the non-photosynthetic $\Delta rpoA$ plastids. Two transcripts were obtained from the supercoiled DNA templates, as expected if NEP transcription initiated accurately and terminated at one of the two terminators (Figure 1A, lanes 1, 3, 5 and 7). From linearized plasmids, only negligible amounts of transcripts could be detected (Figure 1A, lanes 2, 4, 6 and 8). Products matching the size of transcripts initiating from the *atpB* PEP promoter (*PatpB*-255; lane 1) as well as from the strong *psbA* PEP promoter (data not shown) have not been observed. Therefore, we conclude that $\Delta rpoA$ plants indeed possess no PEP transcription activity and that NEP transcription activity is dependent on supercoiled DNA as a template *in vitro*. Primer extension analysis confirmed that transcription from all six NEP promoters initiates accurately to the nucleotide (Figure 2). These data indicate that our transcription system contains all essential components required for accurate transcription initiation *in vitro*.

We have tested the sensitivity of NEP to inhibitors of RNA polymerase activity. Data summarized in Table I indicate that NEP transcription is sensitive to actinomycin, a non-specific RNA polymerase inhibitor, and is partially resistant to rifampicin and rifamycin SV, inhibitors of eubacterial RNA polymerases (Hartmann *et al.*, 1967). Furthermore, NEP is resistant to α -amanitin, an inhibitor of nuclear type II RNA polymerases, and tagetin, a selective inhibitor of nuclear RNA polymerase III, the *Escherichia coli* RNA polymerase and the related chloroplast RNA polymerase (PEP; Lukens *et al.*, 1987; Mathews and Durbin, 1990; Steinberg *et al.*, 1990). The T7 RNA polymerase exhibited a similar pattern of resistance to

Fig. 1. NEP transcription *in vitro* is dependent on supercoiled DNA templates. (A) *In vitro* transcription products from supercoiled and linear DNA templates. Plastid extracts of $\Delta rpoA$ plants were incubated with supercoiled (lanes 1, 3, 5 and 7) or *XhoI*-linearized (lanes 2, 4, 6 and 8) plasmids containing *atpB* (lanes 1 and 2), *atpI* (lanes 3 and 4), *accD* (lanes 5 and 6) and *rpoB* (lanes 7 and 8) promoter-terminator cassettes in the presence of [α - 32 P]UTP for 45 min at 30°C. Products were analyzed on 6% sequencing gels. The sizes of the reaction products are indicated, with ¹ and ² marking the position of products of the first and second termination event, respectively. (B) Maps of promoter-terminator cassettes. The sites of transcription initiation for NEP and PEP (■, □) and the corresponding RNA 5' ends (●, ○) are marked. The site of transcription terminators is also denoted (◆). Arrows with solid lines symbolize NEP transcripts; those with dotted lines mark potential PEP transcripts. The name of the plasmid carrying the promoter-terminator cassette is given in parentheses.

these drugs (Table I), hence biochemically corroborating the assignment of NEP as a phage-type RNA polymerase.

Deletion analysis identifies *rpoB* promoter external boundaries

To identify the sequences required for *rpoB* promoter function, a series of promoter deletion derivatives were introduced into promoter-terminator cassettes and tested *in vitro*. The first set of *rpoB* promoter deletions was carried out on a relatively large fragment (1079 bp; between -951 and +128). These experiments led to the conclusion that the *rpoB* promoter should be contained within the -14 to +3 fragment (data not shown). The second set of deletions was carried out on a much smaller 25 bp fragment, the endpoints of which correspond to positions -20 and +5 (Figure 3). Deletions at the 5' end

Fig. 2. NEP promoters are recognized accurately *in vitro*. (A) Primer extension analysis to map the 5' end of *in vitro* transcripts. For reference, the same end-labeled primer was used to generate a DNA sequence ladder. (B) Alignment of DNA sequences surrounding the NEP transcription initiation sites. The *in vitro* transcription initiation site for *rpoB* was reported by Serino and Maliga (1998), for the *rrn* operon NEP promoter by Vera and Sugiura (1995), and for all other promoters by Hajdukiewicz *et al.* (1997). Bold nucleotides denote transcription initiation sites (+1).

to -15 had no significant effect on *in vitro* transcription (85%; pKL120; Figure 3A, lane 6). Deletion of additional nucleotides to -12, -10, -8 and -6 gradually reduced NEP activity to 38, 24, 15 and 4% of the value observed with the 25 bp fragment (pKL121-pKL124; Figure 3A, lanes 7-10). On the downstream side (3' end), removal of 5 bp between +5 and -1 did not alter NEP transcription activity (pKL118; Figure 3A, lane 3). However, removing an additional 3 bp from the 3' end to -4 reduced the transcription activity drastically to ~8% (pKL173; Figure 3A, lane 4). Using the criterion of maintaining ~100% ($\pm 15\%$) of wild-type transcription activity, this deletion analysis places the *rpoB* promoter between positions -15 to -1 relative to the transcription initiation site (pKL120, pKL118; Figure 3A, lanes 5 and 3). Interpretation of data derived from a deletion analysis is complicated by the replacement of the deleted region with a random sequence, which may be biologically relevant. A further point mutational analysis confirmed this to be the case, placing the *rpoB* promoter boundaries at -14 and +1.

Point mutagenesis identifies the *rpoB* promoter core

To explore the role of individual base pairs in NEP promoter function, all possible single nucleotide changes were made in the -15 to +1 region tentatively identified

Table I. Transcriptional properties of NEP

Reaction mixture	RNA polymerase activity (%)	
	NEP	T7
Control (full system)	100.0	100.0
-template DNA	0	n.d.
-NTPs	0	n.d.
-NTPs; +dNTPs	0	n.d.
+Actinomycin D		
10 μ g/ml	0	0
100 μ g/ml	0	0
+Heparin		
0.1 μ g/ml	101.7	n.d.
1.0 μ g/ml	88.3	98.7
10 μ g/ml	14.0	39.5
100 μ g/ml	0	14.5
+ α -Amanitin		
100 μ g/ml	95.0	n.d.
250 μ g/ml	98.5	104.2
500 μ g/ml	98.3	95.0
+Rifampicin		
0.01 μ g/ml	95.0	n.d.
0.1 μ g/ml	109.2	n.d.
1.0 μ g/ml	87.5	73.2
10 μ g/ml	70.8	66.6
100 μ g/ml	65.8	45.4
+Rifampicin SV		
0.01 μ g/ml	101.7	n.d.
0.1 μ g/ml	97.5	n.d.
1.0 μ g/ml	85.8	77.1
10 μ g/ml	69.2	67.9
100 μ g/ml	64.2	58.7
+Tagetin		
1 U	96.7	n.d.
10 U	98.3	n.d.
20 U	109.2	107.9
80 U	99.2	97.4

as the *rpoB* promoter by deletions. The only exception is the -9/-10 position where two single mutations were introduced together (Figure 4). The point mutations were introduced in a slightly larger fragment (-20 to +5) to maintain the wild-type *rpoB* promoter context. Data shown in Figure 4 are an average of three or four *in vitro* transcription assays per construct. In the regions between -14 and -9 (Region I) and -4 to -2 (Region IV), at least one of the mutations at each position reduced transcription to 60-70%. The sensitivity to mutations in these regions corresponds mostly to transversions (R [A, G] to Y [T, C] and vice versa) rather than transitions (R to R and Y to Y), with the non-complementary exchange being the most effective. This suggests that the character of the base pairs (purine or pyrimidine) in these regions is more important for *PrpoB*-345 activity than the identity of the base pair itself. Interestingly, all mutations at -5 (A, Region III) are neutral, although the A at this position is conserved in the NEP core promoter motif established by sequence alignment (see below). Mutations around the transcription initiation site (-1, +1; Region V, Figure 4) did not have a significant effect, except the substitution with C, which caused in both cases a reduction of activity to 30%.

The region from -8 to -6 is particularly sensitive to mutations (Region II; Figure 4). At least two base changes at each of these positions reduced the transcription activity to <30%. Therefore, we consider the sequence CAT as

Fig. 3. Deletion analysis to determine the *PrpoB*-345 promoter external boundaries. **(A)** *In vitro* transcription products separated on 6% sequencing gels. Arrows with ¹ and ² mark the position of products of the first (*hisa*) and second (*thra*) termination event. **(B)** DNA sequence of the *rpoB* promoter region and its deletion derivatives. Numbers indicate endpoints in nucleotides relative to the transcription initiation site. Italicized lower case letters mark vector sequences replacing deleted sequences. Nucleotides in bold denote the transcription initiation sites (+1). Plasmids carrying the constructs and their transcription activities (% of largest fragment) are also listed. A graph below the sequences summarizes the effect of *PrpoB*-345 5' and 3' end deletions on the *in vitro* NEP transcription activity. The percentage values represent the average of at least three experiments and thus may be slightly different from those reflected by the gel.

the core of the *rpoB*-345 promoter. The A→G transversion at position -7 is fully acceptable, sustaining wild-type transcription activity. Therefore, the plastid NEP promoter core may be defined as CRT, indicating the acceptability of A or G (R) at the -7 position. The CRT sequence motif is comprised in the YRTA conserved sequence motif of plant mitochondrial promoters (see below).

Taken together, these results indicate that the boundaries of the *rpoB*-345 promoter domain extend from -14 to +1. Nucleotides between -8 and -6 are essential for transcrip-

tion from the *rpoB*-345 promoter *in vitro*, defining it as the core promoter element.

The maize rpoB promoter functions in the heterologous tobacco extract

The *rpoB* promoter in higher plants was mapped only in maize (Silhavy and Maliga, 1998). We were interested in finding out if the maize promoter would function in the tobacco extracts. A promoter-terminator cassette was constructed consisting of a 35 bp maize promoter fragment and the bacterial *hisa* and *thra* terminators (pKL176; Figure 5B). The maize *rpoB* promoter was then tested *in vitro* alongside the tobacco promoter in the $\Delta rpoA$ tobacco plastid extract. As shown in Figure 5A, the tobacco NEP faithfully recognized the maize *rpoB* promoter sequences. As the maize promoter contains the functionally important nucleotides (see below), its expression in the tobacco extract is in accordance with the conclusions of the mutagenesis study.

Discussion

We report here the first functional definition of a promoter for the phage-type, nuclear-encoded plastid RNA polymerase. Deletion analysis determined that the *PrpoB*-345 promoter is contained in a small (<20 bp) DNA fragment. We were surprised by the small size of the *rpoB* promoter and the lack of regulatory elements outside the promoter. Therefore, a second tobacco NEP promoter was dissected to confirm the salient features of *PrpoB*-345. We have chosen *PaccD*-129, the only promoter driving *accD* transcription. Deletion analysis of an 831 bp fragment (–735 to +96) indicates that the *accD* promoter is contained within nucleotides –17 to +2 relative to the transcription initiation site (+1; data not shown). Thus, the *PaccD*-129 promoter is similar to *PrpoB*-345: both have a small size, are positioned similarly relative to the transcription initiation site and lack regulatory elements outside the promoter. However, *PaccD*-129 has a TAT sequence instead of the CAT at the conserved core position (see below).

A combined deletion and point mutagenesis study further refined the data on the *PrpoB*-345 promoter, localizing it to a 15 bp AT-rich region (−14 to +1). While nucleotides in Regions I and IV are important for promoter strength, only Region II, a CRT sequence, is crucial for promoter function (Figure 4). The C of the CRT sequence is at the −8 position. Most of the NEP promoters identified in tobacco (Hajdukiewicz *et al.*, 1997; Kapoor *et al.*, 1997; Hübschmann and Börner, 1998) and maize (Silhavy and Maliga, 1998) have either a CAT or a TAT (YAT) at the −6 to −10 position (Figure 6). Interestingly, the CGT sequence is present in only one promoter (*Pycf2*-1577), although the mutagenesis indicates that G at the central position does not affect promoter strength in the *PrpoB*-345 context. Furthermore, the *PrpoB*-345 mutagenesis study suggested that promoters containing the TAT sequence should be weaker since the C→T mutation at the −8 position reduced transcription to ~30%. Indeed, the *PaccD*-129 which has the TAT sequence is significantly weaker than *PrpoB*-345 (Figure 1A).

YAT is included in the YRTA sequence motif, the consensus for higher plant mitochondrial promoters based

Fig. 4. Point mutagenesis of the *PrpoB*-345 promoter indicates that transcription is highly dependent on a CRT sequence. The wild-type sequence is shown on the top; the position of nucleotides relative to the transcription initiation site (+1; bold), the promoter boundaries (−14 to +1) and the promoter core are marked. The far-left column indicates the range of the mutant transcription activities. The table on top gives the transcription activity for each point mutation relative to wild-type (100%). Each letter marks a mutation; double mutations are connected by a plus sign. The graph below shows the average transcription activity (%) of all three single mutations per position on NEP transcription activity. Regions discussed in the text are indicated at the bottom.

on sequence alignments for maize (Mulligan *et al.*, 1991), wheat (Covello and Gray, 1991), *Oenothera* (Binder and Brennicke, 1993), soybean (Brown *et al.*, 1991) and *Sorghum* (Yan and Pring, 1997). The functional significance of the YRTA sequence motif has been confirmed by *in vitro* mutagenesis of the maize mitochondrial *atpA* and *cox3* promoters (Rapp and Stern, 1992; Rapp *et al.*, 1993; Caoile and Stern, 1997). The A of the YRTA motif is also present in most plastid NEP promoters, including *PrpoB*-345 (Figure 6). Interestingly, the mutation of A to any other nucleotide has no effect on *PrpoB*-345 promoter function, this being the only neutral position in the 15 bp fragment. Given its conservation in most promoters, it is possible that the A has an important role in the function of promoters other than *PrpoB*-345.

We have found that in the *rpoB* promoter mutations from T to A or G at the −1 and +1 positions (Region V; Figure 4) did not have a significant effect on transcription

activity. Although A, G and T were equally acceptable at the *rpoB* transcription initiation site, transcription from NEP promoters initiates only from A or T (Figure 6).

It was proposed that the phage-type plastid RNA polymerase gene (*RpoZ*) evolved by duplication of the nuclear gene encoding the mitochondrial enzyme (*RpoY*), and re-targeting of the gene product to plastids (Hedtke *et al.*, 1997). Conservation of the *rpoB* promoter core and the core sequence motif of plant mitochondrial promoters and NEP sensitivity to inhibitors of transcription indicate that *RpoZ* encodes the NEP catalytic subunit. We propose that, initially, transcription of plastid genes by the NEP occurred from spurious promoters, generating additional sets of RNAs for plastid genes. NEP probably became indispensable when it took over transcription of essential genes, such as the *rpoB* operon encoding the subunits for the PEP, the RNA polymerase derived from the ancestral cyanobacterium. Transcription of PEP genes by the NEP

Fig. 5. Testing the maize *PrpoB*-147 promoter in tobacco extracts. (A) *In vitro* transcription products separated on a sequencing gel. The sizes of the reaction products are indicated, with ¹ and ² marking the position of products of the first and second termination event, respectively. Schematic maps of the promoter-terminator cassettes are shown below with plasmid designation (left) and the expected size of transcripts (right). Sites of transcription initiation for NEP (■) and the corresponding RNA 5' ends (●) are marked. The site of the transcription terminators is also denoted (◆). (B) Alignment of the maize *PrpoB*-147 (Silhavy and Maliga, 1998) and tobacco *PrpoB*-345 (Serino and Maliga, 1998) promoters. The identified tobacco *rpoB* promoter is underlined. Conserved nucleotides are shaded; the CAT sequence is boxed.

was probably a critical step in the nucleus, indirectly taking control of the transcription of plastid genes, thereby fully integrating the photosynthetic endosymbiont-turned organelle into the developmental network of multicellular plants. Conservation of the YAT sequence motif in plastid NEP and mitochondrial promoters corroborates the hypothesis of a phylogenetic relationship between the nuclear-encoded phage-type transcription apparatus of plant mitochondria and chloroplasts (Hedtko *et al.*, 1997).

We have shown here that the maize (monocot) *rpoB* promoter is recognized faithfully *in vitro* by the tobacco (dicot) extract, confirming conservation of the NEP transcription machinery between monocots and dicots. Expression of a monocot promoter in dicots *in vivo* has been shown earlier by demonstrating the expression of the rice *PclpP*-111 promoter in transplastomic tobacco (Sriraman *et al.*, 1998). Both the tobacco *PrpoB*-345 and rice *PclpP*-111 NEP promoters have the conserved CAT sequence upstream of the transcription initiation site. The promoters listed in Figure 6 are all similar, and have been classified as type I NEP promoters. The tobacco *PclpP*-53 promoter does not fit into the alignment (Hajdukiewicz *et al.*, 1997) and was shown to be localized downstream of the transcription initiation site (type II NEP promoter; -5 to +25; Sriraman *et al.*, 1998).

The *in vitro* system reported here faithfully recognizes NEP promoters. Our initial attempts to detect specific NEP promoter activity in chloroplast extracts of wild-type plants failed, indicating the importance of the $\Delta rpoA$ plants as the source of plastids to obtain NEP transcription activity. Transcription by NEP from a phage T7 promoter with a purified extract from young spinach chloroplasts was reported by Lerbs-Mache (1993). Interestingly, the

Fig. 6. The YAT sequence motif is present in most type I plastid NEP promoters. The YAT sequence is boxed and the YATA sequence is shaded. The tobacco promoters were described by Hajdukiewicz *et al.* (1997), except *rpl32* (Vera *et al.*, 1996), *rrn16* (Vera and Sugiura, 1995; Allison *et al.*, 1996) and *rpoB* (Serino and Maliga, 1998); the barley promoters were described by Hübschmann and Börner (1998) and the maize promoters were described by Silhavy *et al.* (1998). The higher plant type I NEP consensus is shown below. For the mitochondrial promoter consensus, see Rapp *et al.* (1993) and Tracey and Stern (1995). Note that the conserved YATA box may be 3-7 nucleotides upstream of the transcription initiation site (nucleotides in bold). Transcription initiates from an A or T.

tobacco NEP does not recognize the T7 promoter *in vitro* (data not shown) or *in vivo* (McBride *et al.*, 1994).

The crude tobacco $\Delta rpoA$ extracts concentrated by ammonium sulfate precipitation apparently contain an active NEP and all specificity factors which are necessary to recognize the NEP promoters, or at least the six tested thus far (Figure 2). Attempts at purification in our hands led to reduction or loss of transcription activity (data not shown). This contrasts with the mitochondrial system, in which purification is necessary to eliminate non-specific transcription. However, not every promoter is recognized in purified mitochondrial extracts, probably due to the loss of promoter-specific transcription factors (Tracy and Stern, 1995). The novel *in vitro* system described here will be essential to make progress in characterizing the NEP transcription machinery.

Materials and methods

Extract preparation

Tobacco plants lacking the PEP were obtained by targeted deletion of the plastid *rpoA* gene (Serino and Maliga, 1998). $\Delta rpoA$ plants were grafted onto wild-type tobacco plants and grown in the greenhouse for 6-8 weeks prior to harvesting the leaves. Plastids were isolated from ~400 g of leaves following the protocol of Gruissem *et al.* (1986) and Nickelsen and Link (1993). After lysis, plastid proteins were precipitated with solid ammonium sulfate to 55% saturation. The precipitated proteins were resuspended in 1% of the original volume using 40 mM Tris-HCl pH 7.9, 10 mM NaCl, 5 mM dithiothreitol (DTT) and 5% glycerol, dialyzed overnight against 2 l of the same buffer at 4°C and stored at -80°C after quick-freezing in liquid nitrogen. Lack of mitochondrial contamination was indicated by the absence of fumarase activity in the extracts (Racker, 1950).

Table II. The PCR primers used^a

No.	Name	Sequence (5'–3')	5' Position in sequence ^b
449	5' <i>hisa</i>	ccgaattcACGCGTTCAATTTAAACACCACC	+37 ^c
450	3' <i>hisa</i>	ccaagcttCGCGCCAAAAAAGCCCC	+189 ^c
475	5' <i>atpB</i>	gggagctcGAGCCAATTAGATACAAATAATGAATCG	57 130
476	3' <i>atpB</i>	gggaatTCTAGTACCGGACCGATGATTTGG	56 647
477	5' <i>atpI</i>	gggagcTCGCAATTTGTGAGGGTCGTTCTAGC	16 271
500	5' <i>trnQ</i>	ggggagcTCTAAATGGGGCGTGGC	7493
501	3' <i>trnQ</i>	ggggaaTCTGGGACGGAAGGATTC	7414
502	3' <i>atpI</i>	ggggAATTCAGAAATAGTAAGCGATTCC	16 096
480	5' <i>thra</i>	cccaagctTGACGCGTACAGGAACACAG	–84 ^d
481	3' <i>thra</i>	gggctcgagCGCATGGTTACCTCGTTACC	+5 ^d
528	<i>accD</i> (–268)	cccgagCTCTTTTCTACCCATCCTG	59 378
529	<i>accD</i> (+96)	cccgaaTTCCTATCAGACTAAGC	59 752
530	<i>rpoB</i> (–194)	ggggagCTCTCTCTTCTCTACAAATC	28 040
531	<i>rpoB</i> (+128)	ggggaaTTCACAGATACAAATGGAAAG	27 718
777	<i>accD</i> (–175)	ggggagctcGATATTAGTATTAGACG	59 481
778	<i>accD</i> (–99)	ggggagctCGATGCGAATTTGACACG	59 557
779	<i>accD</i> (–29)	ggggagctcGGGGGTTCCAACATATT	59 628
780	<i>accD</i> (+45)	ggggaaTTCGGATTGTGAGTATTG	59 700
781	<i>accD</i> (+22)	ggggaaTCTGAATCTGGGGC	59 676
782	<i>rpoB</i> (–154)	cccgagctCATAACAGAAAAGGGGG	28 000
783	<i>rpoB</i> (–86)	cccgagctcGCCTTTATCTCAGCG	27 932
784	<i>rpoB</i> (–28)	cccgagctCAGGTTGGAATGTGTA	27 874
785	<i>rpoB</i> (+78)	cccgaaTCTGCTACTTAGAC	27 768
786	<i>rpoB</i> (+29)	cccgaaTCAAAACAAAATGATTCC	27 818
831	<i>accD</i> (–735)	ggggagcTCGCGAGGCTTGC	58 921
832	<i>accD</i> (–145)	ggggagctCAATTATTTTTTTATTTCTTTATAGG	59 511
833	<i>rpoB</i> (–951)	ggggagcTCGGCCATGTGC	28 797
834	<i>rpoB</i> (–101)	ggggagcTCTTTCTTATTTTTTATGC	27 947
835	<i>rpoB</i> (–65)	ggggagcTCAAATGTCGAATCC	27 911
836	<i>accD</i> (–17)	ggggagctCATATTAATATATAGTG	59 639
837	<i>accD</i> (–6)	ggggagctcATAGTGAAGTGTTC	59 650
838	<i>accD</i> (+15)	cccgaaTCTGGGGGAACACTTC	59 670
839	<i>accD</i> (+2)	cccgaaTCACTATATATTAATATGTTGG	59 657
840	<i>rpoB</i> (–14)	ggggagctcTATTATCATAATAATGG	27 860
841	<i>rpoB</i> (–4)	ggggagctcATAATGGTAGAAATGG	27 850
842	<i>rpoB</i> (+15)	ggggaATTCCATTCTTACC	27 832
843	<i>rpoB</i> (+3)	ggggaatTCCATTATTATGATAATACAC	27 844

^aUpper case letters denote chloroplast sequences, lower case indicates nucleotides added to introduce restriction sites.

^bNucleotide position in plastid genome (Shinozaki *et al.*, 1986), except:

^cBarnes and Tuley (1983) and

^dGardner (1982).

In vitro transcription assays

Standard NEP *in vitro* transcription assays were carried out for 45 min at 30°C in 20 µl of reaction mixture containing 40 mM Tris–HCl pH 7.9, 10 mM KCl, 6 mM MgCl₂, 5 mM DTT, 2 mM spermidine, 500 µM each ATP, GTP and CTP, 25 µM unlabeled UTP and 40 µCi of [α -³²P]UTP (6000 Ci/mmol), 30 U of RNAGuard (Pharmacia), 0.5 µg of template DNA and 8 µl of plastid extract (10 µg of protein). The reaction products were phenol/chloroform extracted, isopropanol precipitated, and analyzed on 6% sequencing gels. Molecular weight markers (500, 400, 300, 200 and 100 nucleotides) were prepared with the RNA Century Markers Template Set (Ambion), following the manufacturer's protocol.

For primer extension analysis, *in vitro* transcription assays were carried out in the presence of 500 µM each ATP, GTP, CTP and UTP. Reaction products were resuspended in H₂O and subjected to primer extension analysis.

In vitro transcription assays with T7 RNA polymerase and EcoRI-linearized pKL36 (pBSC-*trnQ*Δ5') were carried out for 45 min at 37°C using the MaxiScript Kit (Ambion) according to the manufacturer's protocol, except that 1 U of T7 RNA polymerase and 1 µCi of [α -³²P]UTP were used per 20 µl reaction. The reaction products were phenol/chloroform extracted, isopropanol precipitated, and analyzed on 6% sequencing gels.

In experiments designed to test the effect of RNA polymerase inhibitors, *in vitro* transcription reaction mixtures with inhibitors were incubated for 10 min on ice prior to adding the ribonucleotides. Inhibitors were stored as stock solutions (1 mg/ml; actinomycin D, heparin and α -amanitin in water, the rifamides in methanol) and freshly diluted in water before use.

Transcription activities were quantified by densitometric analysis of both termination signals using the DensoSpot program of an Alpha Imager 2000 (Alpha Innotech Co.). Values shown are the averages of at least three independent experiments with density values referring to black as 100 and percentages referring to the density values with the control set to 100%. The 3' deletions reduce the size of transcripts relative to the reference RNA derived from the largest promoter fragment. The 3' deletions shown in Figure 3 reduced transcript size at most by 5%, which is much less than the standard error ($\pm 15\%$), therefore no correction for the size reduction was made in this case.

Primer extension analysis

Primer extension reactions were carried out with RNAs from NEP *in vitro* transcription assays according to standard protocols (Sambrook *et al.*, 1989). Briefly, primer *hisa*PEII (#844; CAGCAGACATCGCTG-AAAGACTAGTCAGG), which anneals to the *hisa* region between +103 and +74 relative to the native transcription start site (Barnes and Tuley, 1983), was end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase. Primer extensions were performed using Superscript MMLV reverse transcriptase (Gibco-BRL) at 42°C and the resulting products analyzed on 6% sequencing gels. DNA sequences were generated by sequencing the transcription templates using the same primers.

Vector construction

Plasmid pKL36 used in *in vitro* transcription experiments with T7 RNA polymerase was constructed by PCR amplification of a 86 bp fragment (#500/501; 7493/7414) containing the *trnQ* coding region and subsequent cloning of the amplified *SacI*–*EcoRI* fragment into pBluescript II KS (Stratagene).

The NEP *in vitro* transcription assays were carried out using promoter-terminator cassettes as templates. The cassettes have a modular design. The promoter fragments were cloned as *SacI*-*EcoRI* fragments in plasmid pKL23, a pBluescript KS II+ plasmid derivative carrying two bacterial transcription terminators downstream of suitable (*SacI*-*EcoRI*) restriction sites. Plasmid pKL23 was constructed as follows. The first of the terminators, the histidine attenuator from *Salmonella typhimurium* (Barnes and Tuley, 1983), was PCR amplified from position '36' to '190' (#449 5'hisA/#450 3'hisA) using plasmid pRHT1 (Rogers *et al.*, 1990; kindly provided by Emil M.Orozco) and cloned as an *EcoRI*-*HindIII* fragment (5'-3') into vector pBluescript KS II (Stratagene), resulting in plasmid pKL10. The second terminator, the *E.coli* threonine attenuator (Gardner, 1982), was PCR amplified from plasmid pRHT1 (#480 5'thrrA, -84/#481 3'thrrA, +5) and cloned as a *HindIII*-*XhoI* fragment downstream of the histidine attenuator, resulting in plasmid pKL23.

To construct promoter-terminator cassettes, the promoters were PCR amplified introducing *SacI* and *EcoRI* restriction sites at the 5' and 3' ends, respectively, and cloned into pKL23 carrying the terminators. The promoter fragments are defined by their position in the tobacco plastid genome sequence (Shinozaki *et al.*, 1986; accession No. Z00044). The following is the list of pKL23 plasmid derivatives and the promoter fragments they carry: pKL56, *clpP* promoter as a 312 bp *SacI*-*EcoRI* fragment (74 404/74 709) from pPS6 (Sriraman *et al.*, 1998); pKL57, *rm16* as a 269 bp *SacI*-*EcoRI* fragment from pZS173 (139 958/139 729); pKL59, *atpB* as a 322 bp PCR fragment (#475/476; 57 130/56 647); pKL60, *atpI* as a 120 bp PCR fragment (#477/503; 16 271/16 096).

The following is the list of pKL23 plasmid derivatives with *accD* promoter fragments, the primers with which the promoters were generated (see Table II) and their position in the plastid genome: pKL62, 374 bp PCR fragment (#528/529; 59 378/59 752); pKL87_(-175/+96), 271 bp fragment (#777/529; 59 481/59 752); pKL88_(-99/+96), 195 bp fragment (#778/529; 59 557/59 752); pKL89_(-28/+96), 124 bp fragment (#779/529; 59 628/59 752); pKL90_(-268/+45), 322 bp fragment (#528/780; 59 378/59 700); pKL91_(-268/+22), 298 bp fragment (#528/781; 59 378/59 676); pKL92_(-28/+22), 48 bp fragment (#779/781; 59 628/59 676); pKL109_(-17/+96), a 113 bp fragment (#836/529; 59 639/59 753); pKL110_(-6/+96), a 102 bp fragment (#837/529; 59 650/59 753); pKL111_(-268/+15), a 292 bp fragment (#528/838; 59 378/59 670); pKL112_(-268/+2), a 278 bp fragment (#528/839; 59 378/59 657).

The following is the list of pKL23 plasmid derivatives with *rpoB* promoter fragments, the primers with which the promoters were generated (see Table II) and their position in the plastid genome: pKL63_(-194/+128), 322 bp PCR fragment (#530/531; 28 040/27 718); pKL93_(-154/+128), a 282 bp fragment (#782/531; 28 000/27 718); pKL94_(-86/+128), a 214 bp fragment (#783/531; 27 932/27 718); pKL95_(-28/+128), a 156 bp fragment (#784/531; 27 874/27 718); pKL96_(-194/+78), a 272 bp fragment (#530n/785; 28 040/27 768); pKL97_(-194/+29), a 222 bp fragment (#530/786; 28 040/27 818); pKL98_(-28/+29), a 56 bp fragment (#784/786; 27 874/27 818); pKL99_(-735/+96), a 831 bp fragment (#831/529; 58 921/59 752); pKL100_(-145/+96), a 241 bp fragment (#832/529; 59 511/59 752); pKL101_(-951/+128), a 1.1 kb fragment (#833/531; 28 797/27 718); pKL102_(-101/+128), a 229 bp fragment (#834/531; 27 947/27 718); pKL103_(-65/+128), a 193 bp fragment (#835/531; 27 911/27 718); pKL113_(-14/+128), a 142 bp fragment (#840/531; 27 860/27 718); pKL114_(-4/+128), a 132 bp fragment (#841/531; 27 850/27 718); pKL115_(-194/+15), a 208 bp fragment (#530/842; 28 040/27 832); and pKL116_(-194/+3), a 196 bp fragment (#530/843; 28 040/27 844).

The deletion and mutation series of the -20/+5 *rpoB* promoter region, and the maize and tobacco *rpoB* -25/+10 constructs were obtained by annealing complementary oligonucleotides, *SacI*-*EcoRI* digestion and subsequent cloning into pKL23, yielding plasmids pKL117-pKL173, pKL176 and pKL177. The sequence of the top strand for each construct is shown in Figures 4B, 5 and 7B.

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