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The phage-type PclpP–53 plastid promoter comprises sequences downstream of the transcription initiation site

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ABSTRACT

The existence of a phage-type plastid transcription machinery (NEP), related to the mitochondrial RNA polymerase, has been recognized only recently. Here we report the *cis* sequences required for transcription initiation by the phage-type enzyme. The promoter chosen for the study, PclpP–53, is well expressed in tobacco leaves, unlike most NEP promoters. Promoter definition was carried out *in vivo*, in transplastomic tobacco plants expressing a *uidA* reporter gene from PclpP–53 promoter derivatives. We report here that sequences from –5 to +25 (relative to the transcription initiation site) are sufficient to support specific transcription initiation. Requirement of sequences downstream of the transcription initiation site contrasts with mitochondrial promoters, which have conserved sequences predominantly upstream. The promoter defined here is conserved in liverworts and conifers, indicating that the phage-type transcription machinery appeared in plastids early on during the evolution of land plants. The PclpP–53 promoter sequences are present in rice but do not function, suggesting that PclpP–53 recognition specificity is absent in some monocots.

INTRODUCTION

Plastid genes in higher plants are transcribed by two DNA-dependent RNA polymerases (1). The plastid-encoded polymerase (PEP) core subunits (α, β, β′ and β″) are encoded in the plastid genome (2–4). The promoters for PEP are similar to *Escherichia coli* σ^{70} promoters consisting of –35 and –10 consensus elements and are recognized by nuclear-encoded σ factors (5–7). Transcription by PEP is modulated by nucleus-encoded transcription factors which interact with upstream regulatory elements (8–10).

The existence of a distinct, nuclear-encoded plastid RNA polymerase (NEP) has been established recently (11). The catalytic subunit of NEP is a single polypeptide of ~110 kDa (12). This protein resembles phage T3 and T7 and mitochondrial RNA polymerases (13). Furthermore, it has been shown that PEP and NEP do not share core subunits (11,14).

Transcription initiation sites for NEP have been identified in tobacco (11,15), barley (16) and maize (17). For most promoters, sequence alignment showed a loose 10 nt consensus predominantly upstream of the transcription initiation site (15–17). These NEP promoters are either inactive or only weakly expressed in chloroplasts (11,15,18). An exceptional NEP promoter in tobacco is one of the promoters for *clpP*, a gene encoding the proteolytic subunit of the Clp ATP-dependent protease. This promoter, PclpP–53, initiates transcription 53 nt upstream of the translation initiation codon. Transcription from this promoter is maintained at significant levels in wild-type tobacco leaves. This promoter does not fit the NEP consensus (15). Given its readily detectable expression in wild-type leaves, PclpP–53 was the promoter of choice for the functional definition of a NEP promoter *in vivo*.

To functionally define NEP promoter sequences, we tested expression of *uidA* reporter genes from an ordered set of PclpP–53 promoter deletion derivatives. We have found that a 30 bp fragment extending from –5 to +25 (with the transcription initiation site being +1) is sufficient to support specific transcription initiation. To our knowledge, it is without precedence for phage-type promoters, that sequences required for transcription initiation are predominantly downstream of the initiation site. Furthermore, we have found that the NEP transcription machinery is conserved throughout the evolution of land plants, although the ability to recognize the PclpP–53 promoter is apparently absent in some monocots.

MATERIALS AND METHODS

Plasmid construction

Plasmid pPS6 contains a *uidA* reporter gene as a *Sac*I–*Hin*dIII fragment in a pBSKS+ plasmid (Stratagene). The chimeric *uidA* gene consists of: between the *Sac*I and *Xho*I sites, the test promoter fragment (PclpP–152/+154); between the *Xho*I and the *Nco*I sites, a ribosome binding site with the sequence 5′-CTCGA-GAATTCAGTTGTAGGGAGGGATCCATGG-3′; between the *Nco*I and *Xba*I sites, the *uidA* coding region with an N-terminal c-myc tag corresponding to amino acids 410–419 (EQKLISEEDL)

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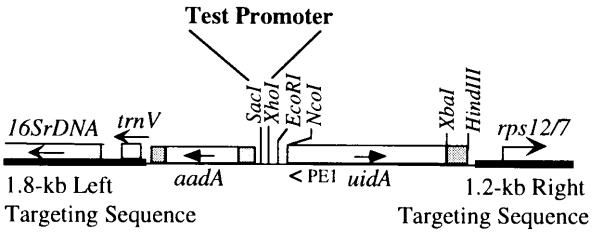

Figure 1. Plastid transformation vectors for testing *clpP* promoter fragments. The test promoters are cloned as *Sac*I–*Xho*I fragments upstream of the *uidA* reporter construct. Location of the selectable marker (*aadA*) conferring spectinomcyin resistance is marked. The *aadA* gene was expressed in a PpsbA/TpsbA cassette in vector pPRV111A derivatives and in a Prrn/Trps16 cassette in vector pPRV112A derivatives (20). Straight arrows mark gene orientation. Bent arrow marks transcription initiation site. <PE1 marks position of oligonucleotide for mapping transcript 5′-ends shown in Figures 2 and 3.

within the C-terminal domain of the human c-myc protein (19); between the *Xba*I and *Hin*dIII sites, the 3′-untranslated region of the *rps16* ribosomal protein gene (Trps16). Plasmid pPS16 was obtained by cloning the *uidA* gene as a *Sac*I–*Hin*dIII fragment into *Sac*I and *Hin*dIII-digested pPRV111A plastid transformation vector (20). The *Sac*I–*Xho*I promoter fragment of pPS16 was replaced with PclpP–152/+10 to give pPS17, with PclpP–22/+25 to give pPS18, with PclpP–152/+41 to give pPS37, with PclpP–39/+154 to give pPS38, with PclpP–22/+21 to give pPS41, with PclpP–22/+16 to give pPS42, with PclpP–10/+25 to give pPS43, with PclpP–5/+25 to give pPS44, with PclpP+1/+25 to give pPS45 and with PclpP+6/+25 to give pPS46. The chimeric *uidA* genes in pPS41, pPS42, pPS44, pPS45 and pPS46 are in vector pPRV112A (20).

The *Sac*I–*Xho*I fragments for pPS16–38 were obtained by PCR amplification. PCR amplification of promoter fragments was carried out using Amplitaq polymerase (Perkin Elmer) and the polymerase buffer supplied by the manufacturer. The fragments were amplified for 30 cycles (1 min at 92°C, 1 min at 55°C, 1 min at 72°C). PCR primers are listed according to the position of the terminal nucleotide relative to the transcription initiation site (it is the complement of nt 74557 in the tobacco plastid genome; 21; accession no. Z00044): clpP–152, ccgagctcGAATGAGTCCATACTTAT; clpP–39, ccgagctcAAAACCAATATGAATATTATA; clpP–22, ccgagctcTATAAAGACAATAAAAAAAAT; clpP+10, ccctcgaGAAACGTAACAATTTTTTTT; clpP+25, ccctcgagTTTCACTTTGAGGTGGA; clpP+41, ccctcgagAGAACTAAATACTATATTTC; clpP+154, ccctcgagATATGACCCAATATATCTG. Anchor sequences derived from the plastid genome are in upper case; added nucleotides to create restriction sites (underlined) are in lower case. *Sac*I–*Xho*I promoter fragments for pPS41–pPS46 were obtained by annealing two complimentary oligonucleotides.

Plasmid pDS44 is a pLAA24 derivative (20) which carries a *uidA* reporter gene expressed from a P*rrn* promoter. Plasmid pDS44 was obtained by excising the P*rrn* promoter as a *Sac*I–*Eco*RI fragment and replacing it with the rice *clpP* promoter region engineered as a *Sac*I–*Eco*RI fragment. The 251 nt *Sac*I–*Eco*RI DNA fragment containing the rice *clpP* promoter region (including 19 bp of the coding region) was obtained by PCR amplification. The sequences of the PCR primers and the positions of their first nucleotide (or of its complement) in the rice plastid genome (22; GenBank accession no. X15901) are: P1, 68520(C), gggagcTCGAATCACCATTCTTT; P2, 68270, gggaattcTTGGAACACCAATGGGCAT. Nucleotides derived from the plastid genome are in upper case; those included to create a restriction site are in lower case. *Sac*I or *Eco*RI restriction sites are underlined.

Tobacco plastid transformation

For plastid transformation, tungsten particles were coated with DNA and introduced into the leaves of *Nicotiana tabacum* plants using the Dupont PDS1000He Biolistic gun at 1100 p.s.i.

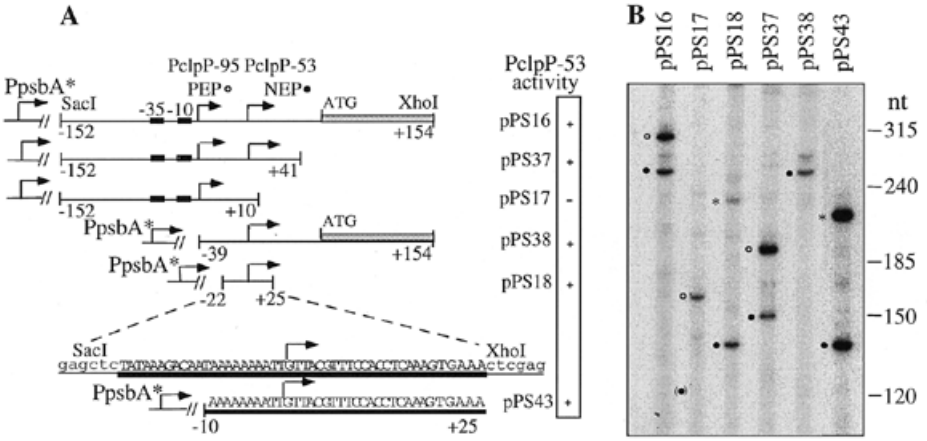

Figure 2. *In vivo* dissection of the tobacco *clpP* promoter region. (**A**) The *clpP* fragments tested for promoter activity. The largest segment contains the PclpP–53 and PclpP–95 transcription initiation sites derived from NEP and PEP promoters, respectively. Relative position of divergent PpsbA* transcript is also marked. Numbering is according to distance (nt) from the PclpP–53 transcription initiation site (+1). Sequence of the smallest fragments is shown in capital letters. Bases added to create restriction sites are in lower case. Transcription from PclpP–53 is indicated by +. (**B**) Primer extension analysis to test *clpP* promoter activity. Primer position is marked in Figure 1. 5′-Ends derived from the PclpP–53 NEP promoter (●), the PclpP–95 PEP promoter (○) and the divergent *psbA* promoter (PpsbA*) (*) are marked. Where the transcript is absent (pPS17), the symbol is bracketed. Molecular weight marker in nt is provided on the side.

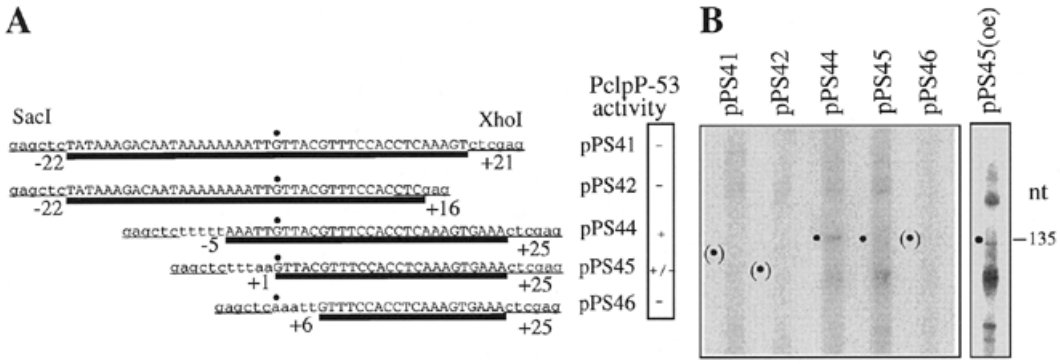

Figure 3. Definition of the minimal PclpP–53 promoter. (**A**) DNA sequence of tested promoter fragments. The numbers indicate distance (nt) from the PclpP–53 transcription initiation site (+1). Plastid DNA sequences are in upper case and are underlined in bold. Added sequences are in lower case. The restriction sites used for cloning are underlined. Transcription from test promoter is indicated by +. (**B**) Primer extension analysis to test PclpP–53 promoter activity. Expected position of PclpP–53 transcript 5′-end is marked (●). Where the transcript is absent, the filled circle is in brackets. An overexposed (oe) lane of the pPS45 primer extension is shown on the far right.

Transgenic shoots were selected aseptically on RMOP medium containing 500 µg/ml spectinomycin dihydrochloride (23). A uniform population of transformed plastid genomes was verified by Southern analysis. Transgenic shoots were rooted on RM medium consisting of agar solidified MS salts and 3% sucrose (24). The plants were transferred to the greenhouse where they flowered and produced seed.

Primer extension analysis

Total leaf RNA was isolated from the leaves of transgenic plants maintained on RM medium (25). Primer extension reactions were carried out (9) using 15 µg of total RNA and primer PE1 (5′-GGCCGTCGAGTTTTTTGATTTCACGGGT-TGGGG-3′), complimentary to the 5′ end of the *uidA* coding sequence. Sequence ladders were generated with the same primer and template DNA using the Sequenase II kit (US Biochemical) and were used as molecular size markers.

RNA gel blot analysis

Total leaf RNA was isolated from leaves of plants maintained on RM medium (25). The RNA was electrophoresed on 1% agarose–formaldehyde gels, then transferred to Hybond N (Amersham) using the Posiblot Transfer apparatus (Statagene). The blots were hybridized to a double-stranded DNA probe prepared by random primed ^{32}P-labeling of the 1.8 kb *Nco*I–*Xba*I fragment containing the *uidA* coding region from plasmid pPS16. Hybridization was carried out overnight at 65°C in Rapid Hybridization Buffer (Amersham).

RESULTS

Transcription from PclpP–53 is separable from the adjacent PEP promoter

In tobacco *clpP* is transcribed from multiple promoters, including PclpP–53 and the PclpP–95, which initiate transcription 42 nt apart (15). The objective of the first set of deletions was to see if the two promoters are separable and also to define the approximate boundaries of the NEP promoter. The largest studied fragment included 152 nt upstream (–152) and 154 nucleotides downstream

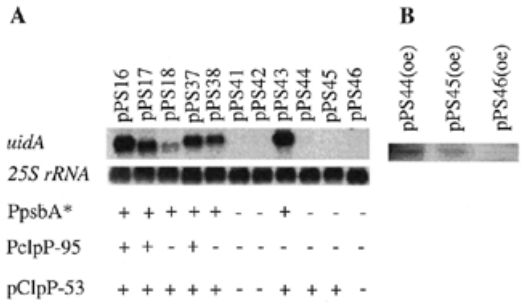

Figure 4. Steady-state levels of *uidA* mRNA in the transplastomic lines. RNA gel blots were hybridized with the *uidA* probe and a probe for the cytoplasmic 25S rRNA to control for loading. (**A**) Standard exposure to detect abundant *uidA* mRNA. Contributing promoters (+) are listed below the lanes. (**B**) Overexposure of some of the lanes.

(+154) of the NEP transcription initiation site, +1 being the first nucleotide transcribed by NEP. The promoters were PCR amplified and cloned upstream of a *uidA* coding region as *Sac*I–*Xho*I fragments to create chimeric genes (Fig. 1). To stabilize the mRNAs, the *uidA* genes were supplied with the *rps16* plastid gene 3′-untranslated region (Trps16). The salient features of the test promoter fragments are shown in Figure 2A. The chimeric *uidA* genes were cloned into plastid transformation vector pPRV111A and introduced into the tobacco plastid genome.

Primer extension analysis shown in Figure 2B detects up to three transcript 5′-ends derived from the PclpP–53 NEP promoter, the PclpP–95 PEP promoter and a divergent promoter (PpsbA*) contained in the *psbA* promoter fragment driving *aadA* (1; Fig. 2A). Data on plants transformed with plasmid pPS38 indicate that the NEP and PEP promoters are separable. The PclpP–95 PEP transcript is absent while transcription from the PclpP–53 NEP promoter initiates at the correct nucleotide. Indeed, the upstream border of PclpP–53 is closer: in transgenic plant Nt-pPS43 the promoter has only 10 nt upstream (–10) of the transcription initiation site, still allowing faithful initiation. The downstream boundary of the PclpP–53 promoter is mapped between nucleotides

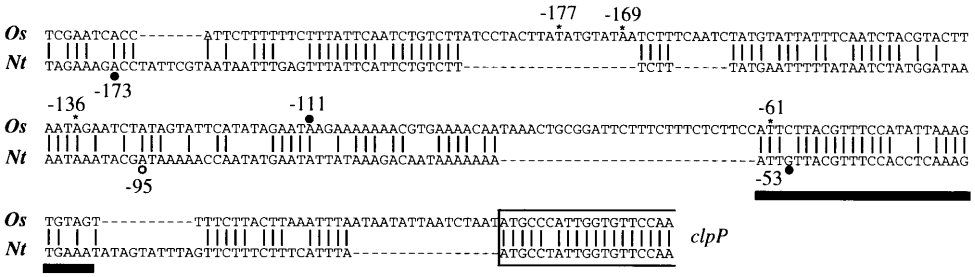

Figure 5. Sequence alignment of the tobacco (*Nt*) and rice (*Os*) plastid *clpP* promoter regions. The 251 bp rice sequence shown here was included as the *clpP* promoter fragment in plasmid pDS44. The position of the native tobacco PclpP–53 (●, NEP) and PclpP–95 (❍, PEP; 15) and the rice PclpP–111 (●;17) is marked. 5′-Ends mapped in tobacco from the rice promoter fragment are shown (*) (–61, –111, –136, –169 and –177). The *clpP* coding region is boxed. The 28 bp promoter core sequence is underlined.

+10 and +25, since the PclpP–53 NEP promoter is active in the line transformed with plasmid pPS18, but is not functional in plants transformed with plasmid pPS17.

Definition of the PclpP–53 core promoter boundaries

To further define the boundaries of the NEP promoter within the 35 bp fragment, additional PclpP–53 promoter deletions were made and tested *in vivo* (Fig. 3A). The new set of *uidA* constructs was introduced into plastids using transformation vector pPRV112A. In this vector, *aadA* (spectinomycin resistance gene) is expressed from the plastid rRNA operon (P*rrn*) promoter. P*rrn* does not have any divergent promoter activity, nor do the tested fragments contain the PclpP–95 PEP promoter. Therefore, primer extension analysis from this promoter set will detect only the PclpP–53 NEP promoter activity. Primer extension data in Figure 3B show that sequences extending from –5 to +25 (pPS44) are still sufficient for accurate initiation by the NEP. In the +1/+25 construct (pPS45) lane the signal is very weak and can be seen only upon overexposure of the gel (Fig. 3B). No transcription is observed from the promoter fragment extending from +6 to +16 (pPS46). Since transcript accumulation from the pPS45 promoter is negligible (Fig. 4B), we place the 5′ PclpP–53 boundary at position –5. If sequences between +21 and +25 are removed (pPS41), transcription is completely lost, hence the 3′ boundary of the NEP promoter is between +21 and +25.

Northern blot analysis was carried out to test steady-state levels of *uidA* mRNA in the transplastomic lines (Fig. 4). Promoters contributing to mRNA accumulation are listed below the lanes. Analysis of data in Figures 2–4 indicate a significant drop in PclpP–53 promoter strength upon deletion of sequences between the –10 and –5 positions, suggesting that these sequences may be important for relatively high rates of transcription. This conclusion is based on reduced *uidA* mRNA accumulation in pPS44 plants relative to pPS43 plants. The *uidA* message in the pPS44 plants was detectable only upon overexposure (Fig. 4B). Alternatively, reduced transcript accumulation in the pPS44 plants may be due to the different genomic context in the vector. In any event, transcription in the pPS44 plants initiated faithfully, indicating that –10 to –5 sequences are not essential for PclpP–53 promoter recognition.

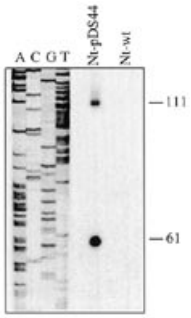

Figure 6. Primer extension to map RNA 5′-ends in the rice *clpP* promoter fragment in tobacco. RNA was isolated from the leaves of wild-type (Nt-wt) and transplastomic (Nt-pDS44) tobacco plants. The numbers –61 and –111 refer to the position of the RNA 5′-ends relative to the translation initiation codon (nucleotide upstream of ATG is at position –1).

Transcription of *uidA* from the rice *clpP* promoter in tobacco plastids

The *clpP* gene in rice and maize is transcribed by NEP initiating transcription at position –111 with respect to the translation initiation codon (17). Alignment of the rice and tobacco *clpP* promoter regions indicates that sequences required for PclpP–53 promoter function in tobacco are present in rice (Fig. 5). However, this homologous stretch of sequence is transcriptionally silent in rice. To check if the rice sequence could serve as a NEP promoter, the 251 bp rice *clpP* DNA fragment (Fig. 5) was tested for promoter function in tobacco plastids. For testing, the rice *clpP* fragment was cloned upstream of a *uidA* coding region and linked to a selectable spectinomycin resistance (*aadA*) gene in plastid vector pPRV111A. The schematic outline of the transformation vector is shown in Figure 1. The resulting plasmid pDS44 was introduced into tobacco plastids and transplastomic plants were selected on spectinomycin.

Primer extension analysis was carried out to map *uidA* 5′-ends initiating from the test promoter region (Fig. 6). A transcript 5′-end mapped to nucleotide position –111, the same position as in rice. This indicates that the rice PclpP–111 promoter is properly recognized in tobacco. Interestingly, a second transcript was

Figure 7. DNA sequence conservation around the Type II *clpP* transcription start site. Sequence alignment is shown for spinach (26), tobacco (15), *Arabidopsis thaliana* (GenBank accession no. AF090188), rice (22), *Marchantia polymorpha* (27), *Pinus contorta* (28) and *Chlamydomonas reinhardtii* (29). Transcript 5′-ends are marked (●). Nucleotides conserved in at least four species are boxed. The 28 nt promoter core is underlined.

found at the rice –61 position initiating from the region with homology to the tobacco PclpP–53 promoter (28 bp; underlined in Fig. 5).

Evolutionary conservation of *clpP* promoters

Unexpectedly, we found transcription activity in the rice *clpP* promoter fragment from the region corresponding to the tobacco PclpP–53. In this region, rice and tobacco share a 28 nt homologous region with 22 conserved nucleotides. To test the conservation of this same region during evolution, sequences around the *clpP* transcription initiation sites were aligned (Fig. 7), including those of the liverwort *Marchantia polymorpha* (27), the conifer *Pinus contorta* (28) and the dicots spinach (26), tobacco (15) and *Arabidopsis thaliana* (GenBank accession no. AF090188). The sequence alignment indicates that the 28 nt segment around the *clpP* transcription initiation sites is conserved, suggesting that *clpP* is transcribed by NEP in all of these species. Included in the alignment are *clpP* sequences for the monocot rice (22) which are not functional in rice plastids (17) but function in tobacco, as we have shown in the paper. *Chlamydomonas clpP* sequences (29) with partial homology to the 28 nt segment are also included in Figure 7. However, no promoter activity could be detected in this region (data not shown).

DISCUSSION

We report here the first definition of core promoter requirements of NEP, the phage-type nucleus-encoded plastid RNA polymerase. The study was carried out on the PclpP–53 promoter which is well expressed in the chloroplasts of tobacco (15), spinach (26) and *Arabidopsis* (unpublished data). The availability of plastid transformation made tobacco the system of choice for the *in vivo* PclpP–53 study (23,30). The chloroplast is a plastid type in which most NEP promoters are weak or inactive. These weak or inactive promoters could only be characterized in mutant plants (15–17) or in the BY2 tobacco tissue culture line (18,31).

In vivo promoter dissection shows that a 30 bp fragment around the transcription initiation site is sufficient to support specific transcription initiation by the NEP. The essential sequences for the PclpP–53 promoter are predominantly downstream, extending from –5 to +25 around the transcription initiation site. Since the sequence conservation between tobacco and rice is from –3 to +27, we expect that sequences from –3 to +25 are sufficient for minimal PclpP–53 promoter function. Conservation of sequences downstream of the transcription initiation site contrasts other plastid NEP promoters (15–18) and the promoters in mitochondria (32–35) and phages (36). The position of conserved sequences relative to the transcription initiation site and the lack of sequence conservation prompted us to classify PclpP–53 as a Type II NEP promoter and all the other NEP promoters as Type I.

In higher plants, there are numerous Type I NEP promoters, while the Type II group thus far is represented only by PclpP–53 characterized here. The 30 bp region shown here to be important for PclpP–53 promoter function contains a 28 bp stretch which is conserved and functions in liverwort (27) and conifers (28), indicating that the phage-type plastid polymerase appeared early on during evolution in land plants (37). This region is poorly conserved in the unicellular alga *Chlamydomonas reinhardtii*, in which it does not function as a promoter (Fig. 7), supporting the lack of a phage-type plastid transcription system (38). Interestingly, the *atpB* gene in *Chlamydomonas* chloroplasts is expressed from a significantly larger promoter extending well into the transcribed region (–20 to +60; 39).

While the tobacco PclpP–53 region is well conserved in rice, it does not function as a promoter. The six point mutations in the rice sequence relative to tobacco do not abolish promoter function in tobacco, but shift the site of transcription initiation by 3 nt (Fig. 5). Lack of PclpP–53 function is probably due to the lack of a Type II NEP specificity factor in rice, maize and barley. Since the plastid NEP is related to phage-type yeast and mammalian mitochondrial enzymes, it probably associates with at least two other factors, one of which confers promoter specificity to the polymerase (40–42). Given the distinct Type I and Type II recognition specificities, it is likely that the plastid NEP utilizes a similar mechanism.

ACKNOWLEDGEMENTS

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