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Two types of chloroplast gene promoters in *Chlamydomonas reinhardtii*

(chloroplast transformation/*in vivo* deletion analysis/β-glucuronidase reporter gene/*atpB* and 16S rRNA-encoding gene promoters/chloroplast transcription)

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ABSTRACT **Structures of the promoters of *Chlamydomonas reinhardtii* plastid *atpB* and 16S rRNA-encoding genes were analyzed *in vivo*. Chimeric constructs, containing the *Chlamydomonas* chloroplast *atpB* or 16S rRNA-encoding gene promoter coupled to the *Escherichia coli uidA* (β-glucuronidase, GUS) reporter gene and bordered by *C. reinhardtii* chloroplast sequences, were stably introduced into the chloroplast of *Chlamydomonas* by microprojectile bombardment. Activity of the promoters in the chloroplast of GUS gene-positive transformants was assayed by measuring the abundance of GUS transcripts and determining the relative rates of GUS transcription *in vivo*. Deletion analyses of the 16S rRNA gene and *atpB* promoter fragments showed that the two promoters differ structurally. The 16S rRNA gene promoter resembles the bacterial σ^{70} type with typical −10 and −35 elements. The *atpB* promoter, on the other hand, lacks a conserved motif in the −35 region but contains, in the −10 region, a characteristic octameric palindrome (TATAATAT) that is conserved in the promoter sequences of some other *C. reinhardtii* chloroplast genes. For maximum activity, the *atpB* promoter requires sequences of ≈22 base pairs upstream and ≈60 base pairs downstream of the transcription start site.**

Previous studies of the sequences of a few chloroplast promoters of a small number of higher plants (1, 2) indicated that they are similar to typical bacterial promoters of the *Escherichia coli* σ^{70} or *Bacillus subtilis* σ^{43} types (3). Putative higher plant chloroplast promoters generally contain hexanucleotide sequences upstream of the transcription start site resembling bacterial −10 (TATAAT) and −35 (TTGACA) consensus motifs. These sequences have been shown to be necessary for transcription in homologous *in vitro* transcriptional assays (4–9). To date, however, only a few chloroplast promoters have been analyzed in detail *in vitro*. Some promoters deviate from the general bacteria-like structure: (*i*) deletion of the −35 element in the mustard *psbA* chloroplast promoter significantly decreased the transcription rate *in vitro* but, unlike in other chloroplast promoters, did not abolish transcription completely (7); (*ii*) all sequences 5′ to two spinach chloroplast tRNA genes (*trnS1* and *trnR1*) could be deleted without any effect on *in vitro* transcription, indicating the absence of any promoter sequence in front of these genes (10).

In our studies on the regulation of chloroplast transcription in *Chlamydomonas* we are using a chloroplast transformation vector containing the widely used *E. coli uidA* gene, encoding β-glucuronidase (GUS) protein (11), as a transcriptional reporter gene coupled to different *Chlamydomonas* chloroplast promoters. The chloroplast of *Chlamydomonas* can be stably transformed with these constructs by particle bombardment (12). Stable introduction of promoter–GUS gene constructs into the *Chlamydomonas* chloroplast genome enables us to measure the activity of chloroplast promoters *in vivo* and to analyze the effects of promoter deletions and modifications on the ability to direct and initiate transcription correctly. By using this system we found, unexpectedly, that the −35 region in the *Chlamydomonas* chloroplast *atpB* promoter can be deleted without any decrease in promoter activity (12). To characterize the structure of the *Chlamydomonas* chloroplast *atpB* promoter in more detail we have measured the effect of 5′ → 3′ and 3′ → 5′ deletions on promoter activity *in vivo*. For comparison with the *atpB* promoter, we have also analyzed the promoter of the *Chlamydomonas* chloroplast 16S rRNA gene by 5′ → 3′ deletions.

MATERIALS AND METHODS

Algae and Culture Conditions. *C. reinhardtii*, nonphotosynthetic mutant strain CC-373 (ac-uc-2-21), was obtained from the *Chlamydomonas* Genetics Center, Durham, NC. Cultures (50–1000 ml) of the mutant strain and of transformants were grown on high salt medium (13) (transformants) or high salt medium plus 2.5 g of potassium acetate/liter (mutant) in a temperature-controlled shaker or water bath at 32°C. Water bath cultures were mixed by bubbling with air/2% CO_2.

***Chlamydomonas* Chloroplast Transformation.** Photosynthetic transformants of mutant CC-373 were obtained by bombarding agar-plated cells with DNA-coated tungsten particles (14). Individual steps of the procedure were done essentially as described (15). GUS gene-positive transformants were grown for several months and repeatedly checked for homoplasmicity by Southern analysis.

Determination of *in Vivo* Transcription Rates. Transcription rates of chloroplast genes were determined by measuring the rate of incorporation of [^{32}P]orthophosphate into newly synthesized RNA as described (12, 16).

DNA and RNA Isolation and Hybridization Analyses. Total DNA and RNA were isolated essentially as described (12). Hybridizations—to screen for the GUS gene in transformants—and RNA gel-blot analyses were done as reported (12).

Primer-Extension Analysis. The 5′ ends of transcripts were located by primer-extension analysis as described (12). The following oligonucleotides, synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer, were used as primers: 21-mer GUS primer, 5′-CGCGCTTTCCCACCAACGCTG-3′, complementary to positions 412–392 (11); 25-mer *psaA1* primer, 5′-CGCGCTCTGGAGTACTAATTGTCAT-3′, complementary to positions 262–238 (17); 21-mer *atpA* primer, 5′-GACGCCGATTGCTTTGTTAGG-3′, complementary to positions 624–604 (18); 21-mer *petA* primer, 5′-CAGCTAATGTTGCTGCGCGTA-3′, complementary to positions 1040–1020 (19).

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Abbreviation: GUS, β-glucuronidase.

Plasmids and Promoter Deletions. *atpB promoter:* plasmid pCrc44 was the starting plasmid for fine deletions into the *atpB* promoter region. Construction of this plasmid has been described (12), and its structure is outlined in Fig. 1. To create 5′ → 3′ deletions into the *atpB* promoter region, plasmid pCrc44 was linearized with *Eco*RI, which cuts in the polylinker 17 bp upstream of the *atpB* promoter fragment, and treated with exonuclease Bal-31 (21). After nuclease treatment, the plasmid was digested with *Xho* I, which cuts 33 bp upstream of the above-mentioned *Eco*RI site, blunted by Klenow fragment fill-in DNA synthesis, and ligated with T4 DNA ligase. Unidirectional 3′ → 5′ deletions were made with exonuclease III (21) after subcloning the *atpB* promoter as an *Eco*RI–*Sma* I fragment from pCrc44 into pUC19 and digestion with *Sma* I/*Sph* I. Suitably deleted promoter fragments were removed with *Eco*RI/*Hin*dIII and subcloned into pBluescript II SK+ (Stratagene). The *atpB* promoter fragments were taken out of the above plasmid with *Eco*RI/*Hin*cII and finally cloned in front of the GUS gene into *Eco*RI/*Sma* I-digested pCrc44.

16S rRNA gene promoter: The ≈2.1-kilobase (kb) *Bam*HI–*Eco*RI restriction fragment isolated from the *Chlamydomonas* chloroplast *Bam*HI 11/12 fragment was cloned into pUC19 and digested with *Kpn* I and *Nco* I to release a 457-bp fragment of the 16S rRNA gene 5′-flanking region. The *Kpn* I–*Nco* I restriction fragment was blunted with T4 DNA polymerase and cloned into the T4-modified *Pst* I site of pBluescript II SK+ vector to create plasmid pCrc79. The fragment was removed from the above plasmid by digestion with *Sma* I/*Eco*RI and cloned into the *Sma* I/*Eco*RI-digested pCrc44 transformation plasmid ahead of the GUS reporter gene to create plasmid pCrc89, containing the chimeric 16S rRNA gene promoter–GUS–*rbcL* 3′-end gene. Deletions in the 5′ → 3′ direction into the 16S rRNA promoter fragment were made in either pCrc79 or pCrc89 with exonuclease III.

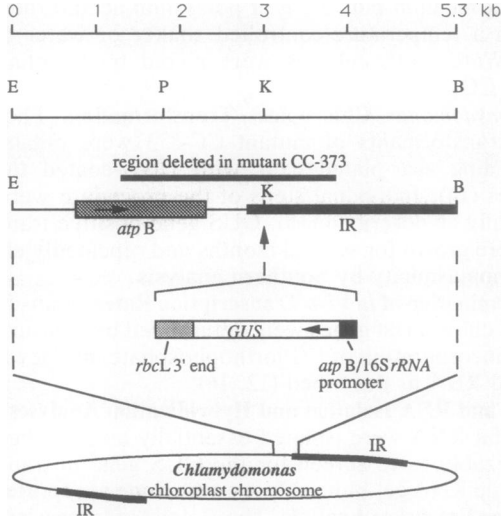

FIG. 1. Vector used to transform *Chlamydomonas* chloroplast with chimeric promoter–GUS genes. The GUS reporter gene-coding sequence terminated by 315 base pairs (bp) from the 3′ end of the *Chlamydomonas* chloroplast *rbcL* gene (12) and coupled downstream of either *atpB* or 16S rRNA gene promoter sequences was inserted in the orientation shown into the *Kpn* I site beside the 3′ end of the *atpB* gene in the 5.3-kb *Eco*RI–*Bam*HI *Chlamydomonas* chloroplast DNA fragment (12, 20). Upon recombination of the ≈7.8-kb final construct into the chloroplast genome of nonphotosynthetic mutant CC-373, a 2.5-kb deletion in that mutant is complemented, and photosynthetic competency is restored (14). The chimeric GUS gene becomes positioned at the end of the inverted repeat (IR) and is transcribed toward the 3′ end of the endogenous *atpB* gene. E, *Eco*RI; B, *Bam*HI; K, *Kpn* I; P, *Pst* I.

RESULTS

Abundance of GUS Transcripts in *atpB*- and 16S rRNA Promoter–GUS Transformants. Total RNA isolated from GUS gene-positive transformants was analyzed by RNA gel blots for GUS transcript abundance (Figs. 2*b* and 3 *b* and *c*). Abundant transcripts of the GUS reporter gene were found in transformants carrying the largest *atpB* or 16S rRNA promoter–GUS constructs.

Deletions in the 5′ → 3′ direction into the 16S rRNA promoter, leaving 70 or 39 bp ahead of the transcription start site (Fig. 2*a*), did not affect the abundance of GUS transcripts (Fig. 2*b*). However, no GUS transcripts could be detected, even on overexposed autoradiograms, when the −35 motif (TTGACA) in the 16S rRNA promoter was deleted in constructs extending to −27 or −16.

In contrast, deleting the *atpB* promoter 5′ → 3′ down to 22 bp upstream of the transcription start site (Fig. 3*a*) had no effect on GUS transcript abundance (Fig. 3*b*), confirming reported results (12). A series of 5′ → 3′ deletions into the *atpB* promoter were created to define its structure in more detail. Deletions beyond the −22 nucleotide caused a gradual decrease in GUS transcript abundance (Fig. 3*b*, see also Fig. 5*b*). GUS transcripts could still be detected at ≈3% of the maximum level with a promoter extending only to −10. No

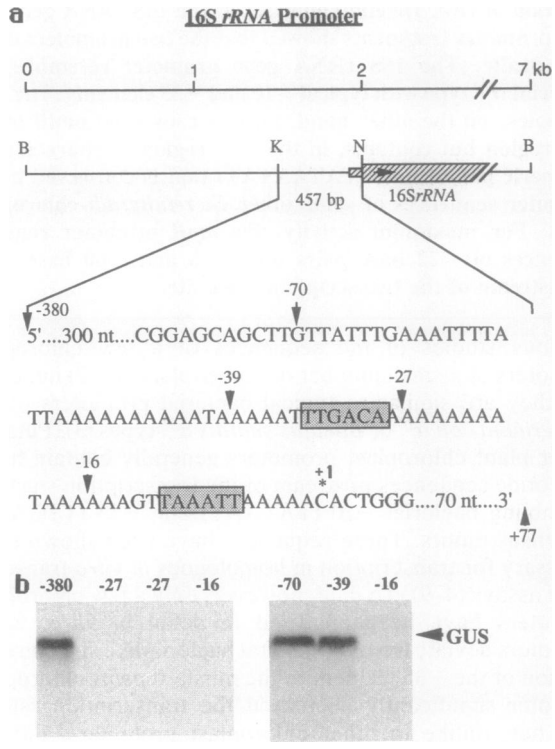

FIG. 2. Locations of 16S rRNA gene promoter deletions (*a*) and RNA gel-blot analysis (*b*) to determine the amount of GUS transcripts in 16S rRNA promoter–GUS transformants with 5′ → 3′ deletions of the promoter fragment. (*a*) Numbers above sequence indicate deletion end points relative to the site of transcription initiation (22), which is marked by an asterisk and numbered +1. Characteristic −10 and −35 promoter elements are in shaded boxes. Wide and narrow hatched boxes denote the processed and primary transcripts of the 16S rRNA gene (23), respectively. B, *Bam*HI; K, *Kpn* I; N, *Nco* I; B, *Bam*HI; nt, nucleotides. (*b*) Four micrograms of total RNA isolated from different GUS gene-positive transformants harboring promoter–GUS constructs with deletions as indicated was separated in a 1.3% agarose/formaldehyde gel and transferred to a nylon membrane. Membranes were hybridized with random primer-labeled GUS DNA, washed, and exposed to x-ray film for 1–3 hr. Numbers above lanes denote numbers of base pairs remaining ahead of the transcription start site (compare Fig. 2*a*).

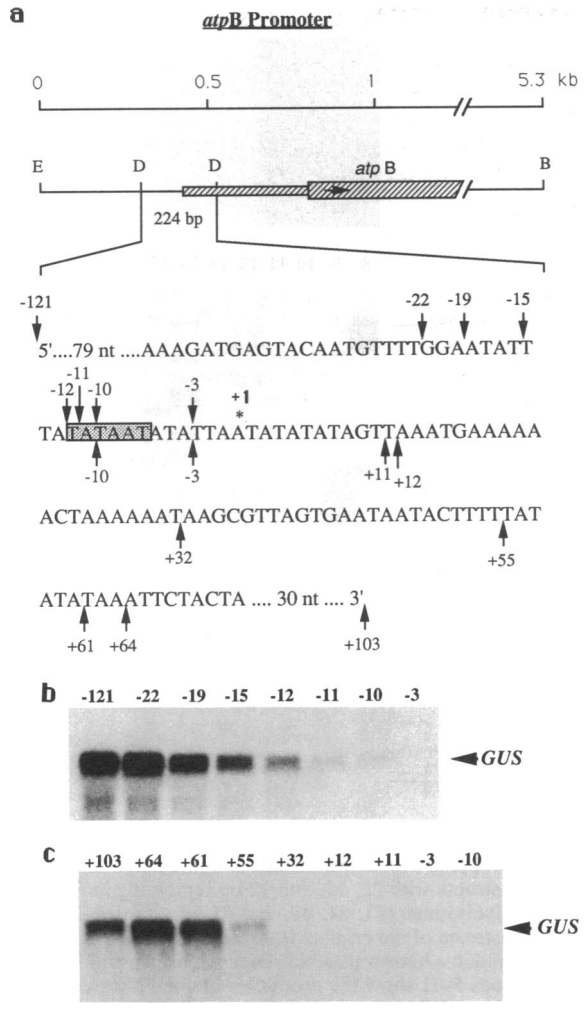

FIG. 3. Locations of *atpB* promoter deletions (*a*) and RNA gel-blot analyses to determine abundance of GUS transcripts in *Chlamydomonas* cells transformed with *atpB* promoter–GUS constructs with 5′ → 3′ (*b*) and 3′ → 5′ (*c*) deletions into the promoter fragment. (*a*) The *atpB* promoter region was isolated as a 224-bp *Dra* I restriction fragment from the 5.3-kb *Eco*RI–*Bam*HI *Chlamydomonas* chloroplast DNA restriction fragment (20). Transcribed regions of the *atpB* gene are shown as hatched boxes (narrow box, untranslated region; wide box, translated region). Numbers above and below sequences indicate the end points of 5′ → 3′ and 3′ → 5′ deletions, respectively, relative to the transcription start site, which is marked by an asterisk and numbered +1. The −10 element is shown in a shaded box. E, *Eco*RI; D, *Dra* I; B, *Bam*HI; nt, nucleotides. (*b* and *c*) Electrophoresis, blotting, and hybridization were as described for Fig. 2. Exposure to x-ray film was for 24–48 hr. Numbers above lanes denote the deletions as shown in sequence of Fig. 3*a*.

transcripts were found with a 5′ → 3′ deletion that retained just 3 nucleotides ahead of the transcription start site.

To assess the importance of sequences downstream of the transcription start site for transcriptional activity from the *atpB* promoter, deletions were made at the 3′ end (starting at position +103) in the 3′ → 5′ direction (Fig. 3*a*). No effect on transcript abundance was seen when sequences up to the +61 position were removed (Fig. 3*c*; see also Fig. 5*b*). Beyond this point the level of GUS transcripts dropped precipitously. Transformants carrying an *atpB* promoter fragment with 32 or fewer base pairs of original sequence remaining downstream of the transcription start site did not accumulate GUS transcripts. We conclude from these data that ≈60 bp 3′ to the transcription start site are needed either for maximum transcription from the *atpB* promoter or for transcript stability.

To further confirm the lack of a specific required sequence in the −35 region of the *Chlamydomonas* chloroplast *atpB* promoter and to determine whether a functional −35 sequence might have been produced inadvertently in making the 5′ → 3′ deletion constructs (Fig. 3*a*), promoter fragments in which the original −35 region was replaced during exonuclease treatment with different upstream polylinker sequences were analyzed for transcriptional activity (Fig. 4). GUS transcripts were detected in all transformants carrying these constructs, despite different −35 regions in front of a conserved sequence of 22 nucleotides upstream of the transcription start site (Fig. 4); this finding reinforced our conclusion that the *Chlamydomonas* chloroplast *atpB* promoter does not extend to include a specific required sequence in the −35 region.

***In Vivo* Transcription Rates.** We used a described (12) *in vivo* labeling method to determine the transcription rate of the GUS reporter gene in cells transformed with chimeric promoter–GUS constructs (Fig. 5 *a* and *b*). Rates of transcription correlate with abundance of GUS transcripts: in transformants carrying the 16S rRNA gene promoter ahead of the GUS gene, transcription could be seen only with promoter fragments that included the −35 region (Fig. 5*b*). In the *atpB* promoter, deletion of the −35 region did not result in a lower rate of GUS transcription *in vivo* (Fig. 5 *a* and *c*). A significant decrease in the rate of GUS transcription occurred only with promoter fragments that had <22 nucleotides in front of the transcription start site (Fig. 5*c*). With 3′ → 5′ deletions into the *atpB* promoter fragment, rates of transcription and transcript abundance decreased in parallel, indicating that in this promoter the region downstream of the transcription start site affects mainly transcription and not transcript stability.

Transcription Start Sites. It had to be demonstrated (*i*) that the normal start site is used for transcription from active but extensively deleted *atpB* promoters and (*ii*) that the decreases in transcript abundance and rates of transcription are not from use of less effective and abnormal start sites. Using the primer-extension method, we located the 5′ termini of GUS transcripts from seven different transformants carrying deleted *atpB* promoter fragments (Fig. 6). The 5′ → 3′ deletions do not affect size of the transcript: the primer-extension products in lanes 5–7 are of the same size and

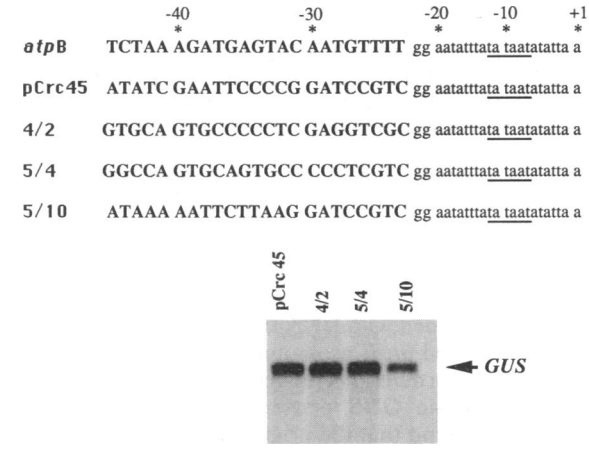

FIG. 4. Abundance of GUS transcripts in *Chlamydomonas* chloroplast transformants carrying *atpB* promoter–GUS constructs with different sequences in the −35 region. (*Upper*) Sequences of endogenous *atpB* promoter region and of constructs in which the region upstream of −22 nucleotide were replaced with different sequences. (*Lower*) RNA gel-blot analysis of total RNA isolated from cells transformed with the different constructs. Construct pCrc45 is −22 in Fig. 3*b*. A characteristic −10 motif is underlined. Agarose gel electrophoresis, blotting, and hybridization were as described for Fig. 2*b*.

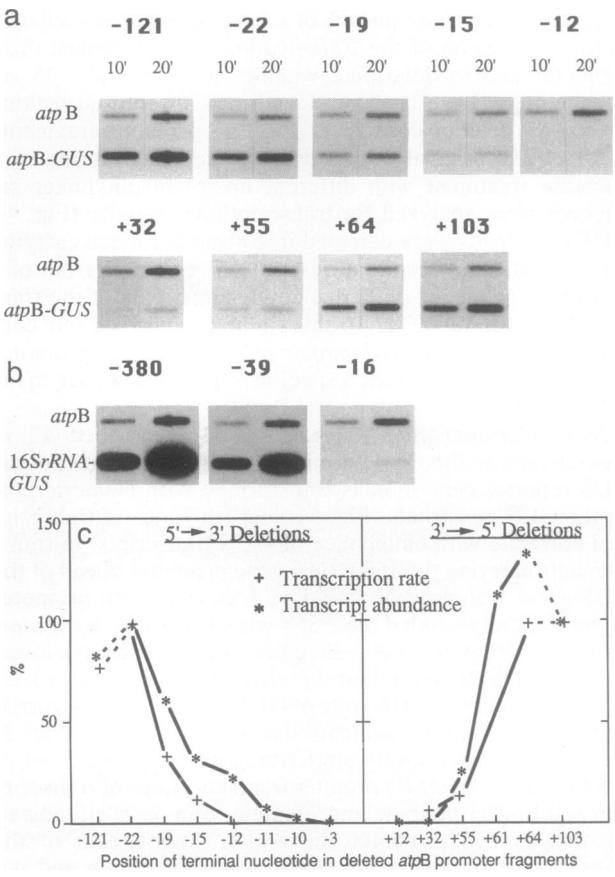

FIG. 5. (*a* and *b*) Slot-blot hybridization of total ^{32}P-labeled RNA to determine relative rates of GUS transcription *in vivo* in transformants containing *atpB* (*a*) and 16S rRNA (*b*) promoter–GUS constructs with deletions into the promoter region. End points of promoter deletions relative to the transcription start site are indicated above each pair of slots. Total RNA was extracted from transformants exposed to [^{32}P]orthophosphate for 10 min (10′) and 20 min (20′) and hybridized (20 μg) on a slot blot to 1 μg of a probe specific for either endogenous *atpB* (used as internal standard) or GUS reporter gene. (*c*) Relative transcript abundance and relative rates of transcription of GUS reporter gene in transformants harboring chimeric *atpB* promoter GUS constructs with 5′ → 3′ and 3′ → 5′ deletions into the *atpB* promoter fragment. Values were calculated from laser scanner densitometric measurements of autoradiograms of RNA gel blots (Fig. 3 *b* and *c*) and slot blots (Fig. 5 *a* and *b*). Rates of GUS transcription were calculated relative to the rate of *atpB* transcription and normalized to the promoter–GUS constructs, which served as starting plasmid for the deletions (−22 for 5′ → 3′ deletions, +103 for 3′ → 5′ deletions, compare Fig. 3). Numbers along abscissa indicate number of base pairs remaining upstream (−) and downstream (+) of transcription start site. Note that points on the abscissa are not placed proportionally to length of *atpB* promoter fragments.

migrate to exactly the same position in the high-resolution gel. In contrast, different sizes of GUS primer-extension products are obtained with RNA from transformants harboring 3′ → 5′ deleted *atpB* promoter fragments (Fig. 6, lanes 8–11) because such deletions reduce the distance between the binding site of the GUS primer and the 5′ end of the transcript. We found that the original transcription start site is retained in all *atpB* promoter–GUS genes, demonstrating that neither the 5′ → 3′ nor the 3′ → 5′ deletions abolish correct initiation of transcription.

DISCUSSION

Using *in vivo* assays with the GUS reporter gene to determine the activity of deleted promoters, we defined the structures of *Chlamydomonas* chloroplast 16S rRNA gene and *atpB* promoters. It is evident that the two promoters analyzed differ structurally. The promoter of the 16S rRNA gene resembles the *E. coli* σ^{70} type of promoter (3) and appears similar to the pea chloroplast 16S rRNA gene promoter that has been analyzed structurally in a homologous *in vitro* chloroplast transcription system (9). The *Chlamydomonas* chloroplast *atpB* promoter, on the other hand, has an unconventional structure, lacking a −35 element and extending well into the transcribed region. This promoter differs from the chloroplast *atpB* promoters of maize and spinach, which have been reported to contain typical −35-like elements essential for transcriptional activity in homologous *in vitro* systems (4, 8, 24).

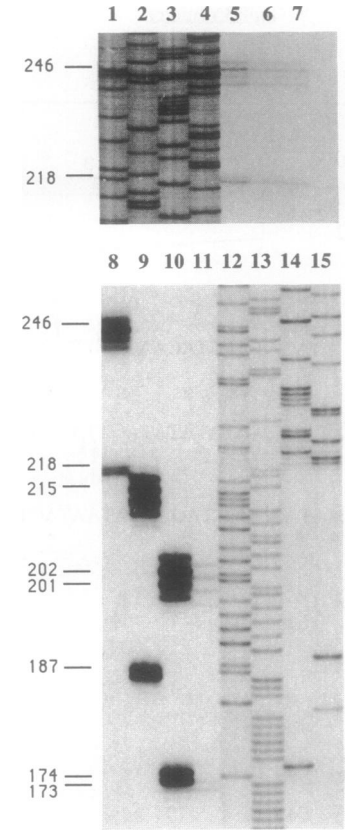

FIG. 6. Mapping transcription start site in deleted *atpB* promoter–GUS constructs with 22, 15, and 12 bp remaining in front (lanes 5–7, respectively) and 103, 64, 61, and 55 bp (lanes 8–11, respectively) downstream of the original transcription start site. Lanes 1–4 and 12–15 contain a known plasmid sequence used as molecular size markers. Lanes 5–11 show the products obtained after extension of a 21-nucleotide GUS primer that was hybridized to 10 μg of total RNA isolated from cells transformed with promoter deletions, as indicated above. Sizes of the primer-extension products (in nucleotides) are marked at left of blots. In all cases two sets of products were obtained; sizes differed by 28 nucleotides. We do not know whether the smaller products are artifacts or belong to real RNA 5′ termini. Low levels of primer-extension products were obtained with RNA from transformants shown to accumulate only small amounts of GUS transcripts (lane 7, lane −12 in Fig. 3*b*; lane 11, lane +55 in Fig. 3*c*). Distances on the gel between primer-extension products in lanes 8–11 do not represent actual differences in base pairs among 3′ → 5′-deleted *atpB* promoter fragments because in some constructs sequence variations were introduced during subcloning between the promoter fragment and the GUS reporter gene.

A comparison of putative promoter region sequences of eight *Chlamydomonas* chloroplast genes (Fig. 7) shows that six of these sequences lack a typical conserved −35 element. Seven promoters contain a palindromic TATAATAT motif in the −10 region, consisting of two overlapping −10-like elements, TATAAT and TAATAT. In most cases, additional −10-like sequences can be identified around the −10 region. Whether

FIG. 7. Sequences upstream of the site of transcription initiation in chloroplast genes of *C. reinhardtii*. Only sequences identified by mapping the locations of the 5′ ends of their transcripts are included. Where two or more 5′ ends were found by primer-extension analysis or S1 nuclease mapping, the position yielding the largest transcript is shown. Boxed is a conserved octameric palindrome in all protein genes: putative −10 and −35 motifs in the 16S rRNA sequence, and in *rbcL* nucleotides at positions −33 to −38 with homology to a −35 motif but at a distance of 20 bp from the −10 element. Numbers at the 5′ end, below each sequence, denote the position of this nucleotide in the original sequence report. Refs., references. †, Unpublished (oligonucleotides used as primers in our unpublished work are listed in *Materials and Methods*).

addition of an AT dinucleotide to the typical −10 element and/or the clustering of −10-like elements in this region is necessary for transcription from this type of promoter remains to be determined.

Lack of a conserved −35 element in the *atpB* promoter is reminiscent of positively controlled bacterial (27) and bacteriophage promoters (28–30) that also lack typical −35 consensus sequences. Some of these promoters require RNA polymerase plus additional transcription factors for full activity. σ-like transcription factors have also been discovered and partially purified from spinach (31) and mustard chloroplasts (32).

Considering the effects of 3′ → 5′ and 5′ → 3′ deletions on *atpB* promoter activity in the *Chlamydomonas* chloroplast (Fig. 5*b*), sequences upstream and downstream of the transcription start site could enhance transcription by stabilizing contacts between the promoter and RNA polymerase or be sites for transcription factor binding. As an alternative, chloroplasts may contain more than one type of RNA polymerase, recognizing different promoter sequences—e.g., promoters with and without a −35 element. In *Euglena* chloroplasts, for example, two different types of RNA polymerases have been suggested to occur on the basis of differences in catalytic properties of different protein fractions (33). In extracts from maize chloroplasts a protein fraction has been identified that can bind and transcribe relaxed DNA templates, whereas the bulk of chloroplast RNA polymerases prefer supercoiled templates (34).

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