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# Activity of the *Chlamydomonas* chloroplast *rbcL* gene promoter is enhanced by a remote sequence element

(β-glucuronidase reporter gene/chloroplast transformation/chloroplast gene expression/promoter structure/*in vivo* transcription)

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**ABSTRACT** **The chloroplast gene *rbcL* encodes the large subunit of ribulose bisphosphate carboxylase. In *Chlamydomonas reinhardtii*, this gene is transcribed more actively than any other protein-encoding chloroplast gene studied to date. To delineate the *rbcL* gene promoter, chimeric reporter genes containing fragments of the 5′ region of the *rbcL* gene fused to the coding sequence of the bacterial *uidA* gene, encoding β-glucuronidase, were stably introduced into the chloroplast genome of *Chlamydomonas* by microprojectile bombardment. The relative transcription rates of endogenous and introduced genes were determined in transgenic cell lines *in vivo*. The basic *rbcL* promoter is located within the region of the gene extending from positions −18 to +63, taking position +1 as the site of initiation of transcription. A chimeric reporter gene containing only the basic promoter is transcribed only 1–15% as actively as the endogenous *rbcL* gene, depending on the conditions under which cells are grown and tested. However, a chimeric gene containing *rbcL* sequences extending to position +170 or beyond is transcribed at about the same rate as the endogenous gene. Deletion of the sequence between positions +170 and +126, well within the protein-encoding region, reduces the rate of transcription to that of reporter genes with the basic promoter alone.**

Three types of chloroplast promoters have been identified to date by *in vitro* and *in vivo* functional assays. The first type, which was predicted from the presence of conserved sequences 5′ to chloroplast genes (1) and defined in plastid genes of higher plants experimentally *in vitro* (2–7) and *in vivo* in the *Chlamydomonas* chloroplast genes for rRNAs (8), resembles the typical bacterial $\sigma^{70}$ or $\sigma^{43}$ promoters, which contain the consensus motifs TATAAT and TTGACA around positions −10 and −35, respectively, relative to the start site of transcription (9, 10). The second type was defined *in vivo* first for the *atpB* gene of *Chlamydomonas* (8). It appears to be the common type of promoter of protein-coding genes in the unicellular green alga *Chlamydomonas reinhardtii*. It lacks a consensus motif around position −35 but includes the conserved sequence element TATAATAT around position −10 and a sequence element within ≈60 bp downstream of the start site of transcription. The third type—identified by deletion analyses in homologous *in vitro* transcription systems of spinach (11) and *Chlamydomonas* (12)—is thought to be an internal promoter of a subpopulation of chloroplast tRNA genes. Very few examples of each type of chloroplast promoter have been analyzed in detail, most of them in *in vitro* systems, and very little is known about the functioning and possible regulation of activity of chloroplast promoters. In none of these cases has a transcription enhancing or silencing gene element been observed.

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Fragments of the 5′ region of the *Chlamydomonas* chloroplast *rbcL* gene, cloned in front of reporter genes and stably inserted into the chloroplast genome of *Chlamydomonas*, function as promoters *in vivo* (13–15) but the rates of transcription of these constructs are only ≈1% (in cells grown under continuous illumination) to 15% (near the end of the dark period in cells grown in 12-h light/12-h dark cycles) of the rate of transcription of the endogenous *rbcL* gene (13, 15). It has been speculated (13) that poor functioning of the *rbcL* promoter in chimeric reporter gene constructs might be due to sensitivity to the sequence upstream of the gene "or within or beyond the transcribed coding regions"—i.e., to an influence of the chromosomal environment—or due to functional incompatibility of the promoter sequences with neighboring sequences of the reporter gene.

Chimeric reporter genes, consisting of various *rbcL* fragments fused to the coding sequence of the bacterial *uidA* (β-glucuronidase, GUS) gene (16) and terminated by the 3′ end of the *Chlamydomonas* chloroplast *psaB* gene, were stably inserted into the chloroplast genome of *Chlamydomonas* and rates of transcription were measured *in vivo*. We have determined that the basic *rbcL* promoter is of the *atpB* type; it is contained within the sequences from positions −18 to +63. We have also found that the activity of the *rbcL* promoter in chimeric GUS genes is not inhibited by neighboring sequences. Rather, DNA sequences located downstream of the basic promoter of the *rbcL* gene are required for full normal rates of transcription.

## MATERIALS AND METHODS

**Algae and Culture Conditions.** *C. reinhardtii* nonphotosynthetic mutant strains *ac-uc-2-21* mt⁺ (*atpB* mutant CC-373) and *rcl-u-1* mt⁺ (*rbcL* mutant CC-1815), obtained from the *Chlamydomonas* Genetics Center at Duke University (Durham, NC), and photosynthetic transformants were grown as described (13, 17).

**Chloroplast Transformation and Transformation Vectors.** The chloroplasts of mutants CC-373 and CC-1815 were transformed using standard protocols (18). The system used for generating and selecting chloroplast transformants of CC-373, into which most of the chimeric GUS reporter gene constructs were ligated, has been described (13). The *rbcL* mutant, *rcl-u-1* (19), containing a point mutation in the *rbcL* structural gene (20), was restored to the wild type by introducing the normal *rbcL* gene in a 3.2-kb *Ase* I–*Pst* I DNA fragment isolated from the *Chlamydomonas* chloroplast *Bam*HI fragment 2 and cloned into pBluescript SK+ (Strat-

Abbreviation: GUS, β-glucuronidase.

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agene) (for construction of this vector, see below). Transformants were selected as described for the *atpB* mutant (13).

**DNA and RNA Gel Blots and Hybridizations.** Isolation of total genomic DNA and total RNA from chloroplast transformants and Southern and Northern blot analyses of DNA and RNA were done as described (13, 17). The ≈1.9-kb *Bam*HI–*Sac* I restriction fragment from plasmid pBI221 (Clontech) containing the entire coding region of the bacterial *uidA* (GUS) gene and the ≈890-bp *Hin*dIII restriction fragment from plasmid pCrcrbcL (13) containing an internal portion of the *Chlamydomonas* chloroplast *rbcL* gene were used as GUS and *rbcL* probes, respectively.

**Determination of Rates of Transcription.** Relative rates of transcription of the GUS and *rbcL* genes were measured by *in vivo* labeling of newly synthesized RNA with [$^{32}$P]phosphate in the dark as described (13, 21).

**Plasmids.** Plasmids pCrc39 and pMU7 containing chimeric *rbcL* promoter–GUS genes with *rbcL* promoter fragments spanning positions −650 to +63 and positions −290 to +97 (Fig. 1), respectively, have been described (13, 15).

To construct the plasmid used as the vector for insertion of a chimeric *rbcL* promoter–GUS gene into the DNA spacer region between the *Chlamydomonas* chloroplast *atpA* and *rbcL* genes (Fig. 2), the 3.2-kb *Ase* I–*Pst* I fragment from the *Chlamydomonas* chloroplast DNA *Bam*HI fragment 2 containing the 5′ regions of the *atpA* and *rbcL* genes (Fig. 2*A*) was blunted sequentially with the Klenow fragment of *E. coli* DNA polymerase I and T4 DNA polymerase and ligated into the dephosphorylated *Hinc*II site of pBluescript SK+. This plasmid was partially digested with *Acc* I and a short polylinker region containing the restriction sites shown in Fig. 2 was blunted and inserted into the blunted *Acc* I site between the *atpA* and *rbcL* genes to create plasmid pMU2 (Fig. 2*A*). To construct plasmid pMU11, the chimeric *rbcL* promoter–GUS–*psaB* 3′-end gene was released from pMU7 as a 2.7-kb DNA restriction fragment by digestion with *Aat* II and cloned blunt-ended into the blunted and dephosphorylated *Bam*HI site of pMU2 in both orientations.

Plasmids containing *rbcL* promoter–GUS–*psaB* 3′-end genes with promoter deletions in 5′ → 3′ and 3′ → 5′ directions were constructed as follows: The 290-bp *Dde* I–*Dra* I *rbcL* promoter fragment (positions −290 to −1; Fig. 1) was blunted and subcloned into the blunted and dephosphorylated *Cla* I site of pBluescript SK+, released with *Xho* I/*Eco*RV digestion, and cloned into *Xho* I/*Sma* I-digested plasmid pCrc32 (23) to create plasmid pMU17. The 267-bp *rbcL* promoter fragment (from positions −70 to +197; Fig. 1), amplified by PCR using oligonucleotides 2 and 7 as primers (Fig. 1) and digested with *Xho* I/*Eco*RV, was subcloned into *Xho* I/*Eco*RV-cut pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to create plasmid pMU18. The 356-bp *rbcL* promoter fragment (from positions −186 to +170; Fig. 1), amplified by PCR using oligonucleotides 1 and 6 as primers and digested with *Sal* I/*Pvu* II, was subcloned into *Sal* I/*Eco*RV-cut pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to create plasmid pMU19. The 312-bp *rbcL* promoter fragment (from positions −186 to +126; Fig. 1), amplified by PCR using oligonucleotides 1 and 5 as primers and digested with *Sal* I/*Eco*RI, was subcloned into *Sal* I/*Eco*RI-cut pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to

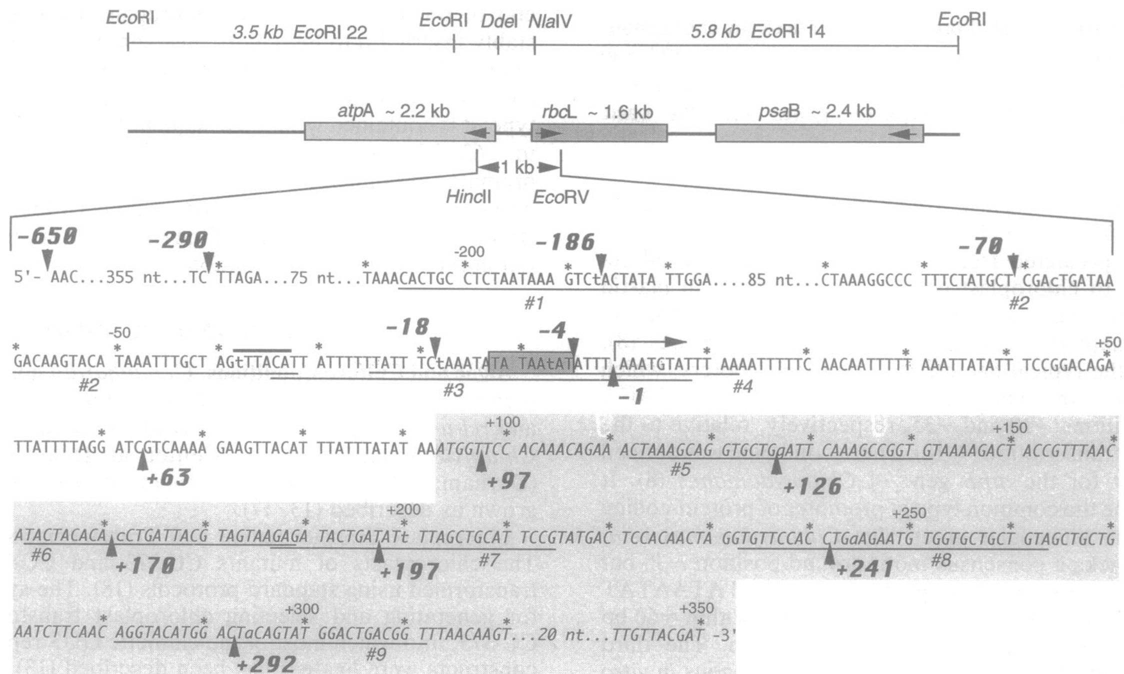


FIG. 1. Location on the chloroplast genome map and sequence of the noncoding strand of the 5′ region of the *Chlamydomonas* chloroplast *rbcL* gene (22). Important restriction sites used in this study are shown. Arrows in the boxes designating the *atpA*, *rbcL*, and *psaB* genes indicate the direction of transcription. Approximate sizes of transcripts are given in kilobase pairs (kb). Arrowheads above and below the sequence denote the end points of deletions in the 5′ → 3′ and 3′ → 5′ directions, respectively. Nucleotides are numbered relative to the start site of transcription, which is indicated by an arrow. Sequences of the coding region are shaded, starting at the ATG codon at position +93. The −10 element of the *rbcL* gene promoter is boxed and shaded. Underlined are the oligonucleotides and the sequences complementary to oligonucleotides used in this study as PCR primers to amplify fragments of the *rbcL* 5′ region. Oligonucleotides: 1, positions −206 (5′) to −177 (3′); 2, positions −78 (5′) to −32 (3′); 3, positions −34 (5′) to +8 (3′); 4, positions −24 (5′) to +12 (3′); 5, positions +141 (3′) to +112 (5′); 6, positions +189 (3′) to +162 (5′); 7, positions +214 (3′) to +187 (5′); 8, positions +263 (3′) to +233 (5′); 9, positions +311 (3′) to +281 (5′). Lowercase type in the *rbcL* sequence denotes bases that were changed in the oligonucleotides to mutate the sequence or to create recognition sites for restriction enzymes. Bases were changed as follows. Oligonucleotides: 1, T → G, creating a *Sal* I site; 2, C → G, creating a *Xho* I site, and T → C, mutating the putative −35 motif; 3, T → G, creating a *Bst*BI site; 4, T → A, creating a *Ssp* I site; 5, G → A, creating an *Eco*RI site; 6, C → G, creating a *Pvu* II site; 7, T → C, creating an *Eco*RV site; 8, A → C, creating a *Pst* I site; 9, A → C, creating a *Pst* I site.

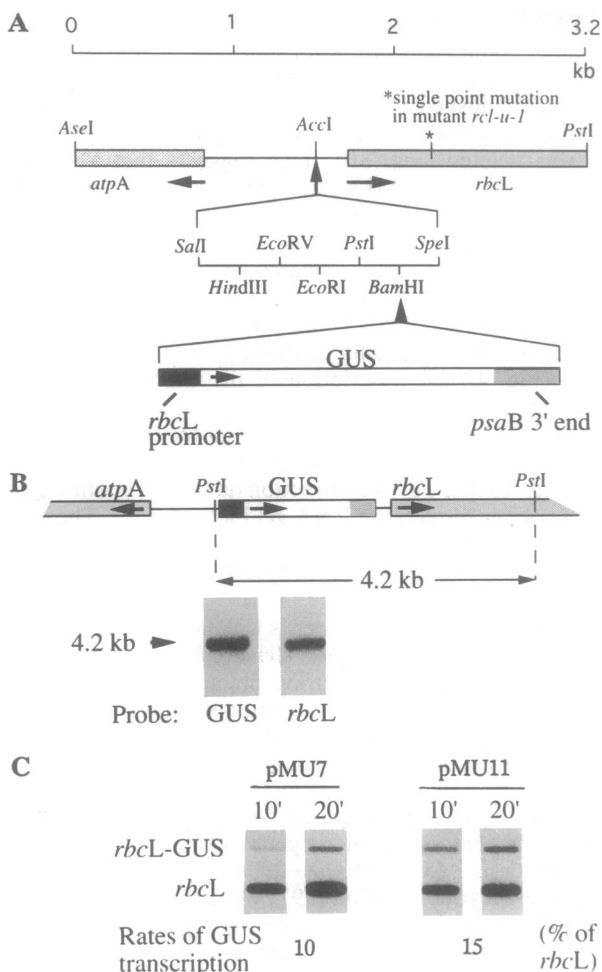


FIG. 2. (*A*) Transformation vector used for insertion of a chimeric *rbcL* promoter–GUS–*psaB* 3′-end gene into the spacer region between the *atpA* and *rbcL* genes in the chloroplast genome of *Chlamydomonas*. The chimeric *rbcL* promoter–GUS gene from plasmid pMU7 (15) was cloned into the blunted *Acc* I site located at position −188 relative to the start site of *rbcL* gene transcription on the 3.2-kb *Ase* I–*Pst* I fragment that was isolated from the *Chlamydomonas* chloroplast DNA *Bam*HI restriction fragment 2 and cloned into pBluescript SK+. Upon recombination of the ≈5.9-kb GUS-containing *Ase* I–*Pst* I sequence into the genome of nonphotosynthetic mutant *rcl-u-1* (19), a point mutation in the *rbcL* gene (20), indicated by an asterisk, is complemented and the capacity for photosynthesis is restored. In transformant MU11 whose analysis is shown in *B* and *C*, the GUS gene is transcribed in the same direction as the endogenous *rbcL* gene (directions of transcription are indicated by arrows at the genes). (*B*) DNA gel blot (Southern) analysis of transformant MU11 showing insertion of the chimeric GUS gene into the DNA spacer region between the *atpA* and *rbcL* genes on the *Chlamydomonas* chloroplast chromosome. Total DNA was isolated from the GUS-positive transformant, digested with *Pst* I, which cuts close to the 5′ end of the GUS construct and ≈1200 bp into the coding region of the *rbcL* gene (see map), and separated in a 0.8% agarose gel. After transfer of the DNA bands to a nylon membrane, the blot was hybridized to random primer-labeled DNA probes specific for the GUS or *rbcL* genes. The membrane was exposed to x-ray film with an intensifying screen at −80°C overnight. Hybridization with both the GUS- and *rbcL*-specific probes labeled the same 4.2-kb *Pst* I fragment, showing that the chimeric GUS gene is located next to the endogenous *rbcL* gene on the chloroplast chromosomes of transformants. (*C*) Relative rates of transcription of the GUS gene in transformants MU7, harboring the chimeric gene *rbcL* promoter–GUS–*psaB* 3′ end in a position adjacent to the *atpB* gene (15), and MU11 harboring the same construct in a position adjacent to the *atpA* gene on the *Chlamydomonas* chloroplast chromosome. Total RNA (10 μg), isolated from *Chlamydomonas* chloroplast transformants, that was labeled *in vivo* for 10 min (lanes 10′) and 20 min (lanes 20′) in the dark with [32P]phosphate was hybridized to DNA probes (each at 1 μg) specific for the GUS and *rbcL* genes previously fixed to nylon membranes in a slot blot apparatus. Membranes were exposed to x-ray film with an intensifying screen at −80°C overnight. Relative rates of transcription were calculated from the intensities of the hybridization signals measured with a laser scanning densitometer.

create plasmid pMU21. The 215-bp *rbcL* promoter fragment (from positions −18 to +197; Fig. 1), amplified using oligonucleotides 3 and 7 as primers and digested with *Bst*BI/*Eco*RV, was subcloned into *Acc* I/*Eco*RV-cut pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to create plasmid pMU23. The 201-bp *rbcL* promoter fragment (from positions −4 to +197; Fig. 1), amplified using oligonucleotides 4 and 7 as primers and digested with *Ssp* I/*Eco*RV, was subcloned into the dephosphorylated *Hinc*II site of pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to create plasmid pMU24. The 311-bp *rbcL* promoter fragment (from positions −70 to +241; Fig. 1), amplified using oligonucleotides 2 and 8 as primers and digested with *Xho* I/*Pst* I, was subcloned into *Xho* I/*Pst* I-cut pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to create plasmid pMU25. The 362-bp *rbcL* promoter fragment (from positions −70 to +292; Fig. 1), amplified using oligonucleotides 2 and 9 and digested with *Xho* I/*Pst* I, was subcloned into *Xho* I/*Pst* I-cut pBluescript SK+, released with *Xho* I/*Sma* I, and ligated into *Xho* I/*Sma* I-digested pCrc32 to create plasmid pMU27.

**PCRs.** PCR amplifications of *rbcL* promoter fragments were done with Vent DNA polymerase (New England Biolabs) for 30 cycles with temperatures for denaturing, annealing, and primer extension set to 94°C (1 min), 42°C (1 min), and 72°C (2 min), respectively. Concentrations of template DNA and primers were as recommended for Vent DNA polymerase. The plasmid used as template contained the 1984-bp *Dde* I restriction fragment from the *Chlamydomonas* chloroplast *Bam*HI fragment 2, including the entire *rbcL* gene, cloned blunt-ended into the blunt-ended and dephosphorylated *Cla* I site of pBluescript SK+.

## RESULTS

**Low Activity of the *rbcL* Promoter in Chimeric GUS Genes Is Not Caused by the Chromosomal Environment.** A 712-bp *Hinc*II–*Sau*3A DNA fragment from the 5′ region of the *Chlamydomonas* chloroplast *rbcL* gene, containing the putative promoter and 63 bp of 5′ untranslated region, promoted transcription of the GUS reporter gene in cells grown under continuous illumination at only 1% of the rate of the endogenous *rbcL* gene in transgenic *Chlamydomonas* (13). Similarly, a 388-bp *Dde* I–*Nla* IV DNA fragment (positions −290 to +97; Fig. 1) containing the putative promoter and the entire 5′ untranslated region of the *rbcL* gene functioned poorly in GUS transcription in chloroplast transformants (15). In both studies, the chimeric *rbcL* promoter–GUS genes were inserted into the chloroplast genome in a position close to the 3′ end of the *atpB* gene, ≈30 kb away from the endogenous *rbcL* gene. To investigate whether the chromosomal environment might affect the activity of the *rbcL* promoter, the construct containing the 388-bp *Dde* I–*Nla* IV *rbcL* promoter fragment fused to the GUS reporter gene was cloned into another *Chlamydomonas* chloroplast transformation vector designed to direct insertion of the chimeric gene into the spacer DNA sequence between the *atpA* and *rbcL* genes such that the *rbcL* promoter of the GUS construct became positioned—with respect to the *atpA* gene (Fig. 2 *A* and *B*)—in the same location as the endogenous *rbcL* promoter in wild-type *Chlamydomonas* cells. The location of the chimeric *rbcL* promoter–GUS gene in the chloroplast ge-

nome of transformants was verified by DNA gel blot (Southern blot) analysis (Fig. 2B). Chloroplast transformants having the chimeric GUS construct inserted in this position accumulated GUS transcripts but *rbcL* promoter activity, measured *in vivo* as relative rates of GUS transcription, was about as low as in transformants carrying the same construct in the alien location adjacent to the *atpB* gene (Fig. 2C). This suggests that factors other than location on the chromosome must be responsible for low activity of the *rbcL* promoter in the chimeric reporter genes *in vivo*.

**Structure of the *Chlamydomonas* Chloroplast *rbcL* Promoter.** To delineate the structure of the *rbcL* promoter, we constructed chimeric *rbcL* promoter–GUS genes with progressive 5′ and 3′ deletions into the promoter region (for end points of deletions, see Fig. 1). PCR-amplified fragments of *rbcL* sequences from positions −70 to +197, −18 to +197, and −4 to +197 were cloned upstream of a GUS–*psaB* 3′-end construct, and the resulting chimeric genes were introduced by particle bombardment (13) into the chloroplast genome of *C. reinhardtii* adjacent to the 3′ end of the *atpB* gene.

Chloroplast transformants harboring chimeric *rbcL* promoter–GUS genes with 5′ → 3′ deletions into the *rbcL* promoter region down to position −18 (Fig. 1) accumulated GUS transcripts (Fig. 3A) showing that no essential promoter element is present upstream of position −18 in the *rbcL* promoter and that the TTTACA sequence around position −35 (Fig. 1), which resembles the characteristic bacterial −35 consensus element TTGACA, has no crucial function in initiation of transcription. To substantiate this conclusion, a point mutation in the putative −35 motif of the *rbcL* promoter was introduced by oligonucleotide mutagenesis (primer 2, Fig. 1), which altered the TTTACA sequence to CTTACA. This mutation, which is expected to inhibit severely the activity of promoters of the $\sigma^{70}$ type (24), had no effect on accumulation of GUS transcripts in chloroplast transformants (Fig. 3A, lane −70), confirming the conclusion that the *Chlamydomonas* chloroplast *rbcL* promoter is not homologous to typical $\sigma^{70}$ bacterial promoters.

Deletion of sequences from positions −18 to −4 resulted in loss of *rbcL* promoter activity. No GUS transcripts accumulated in chloroplast transformants harboring this deletion construct (Fig. 3A), supporting the notion that, as in the *Chlamydomonas* chloroplast *atpB* promoter (8), the TATAATAT sequence around position −10 (Fig. 1) is an essential component of the *rbcL* promoter.

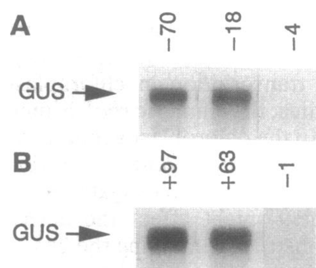


FIG. 3. RNA gel blot (Northern) analysis of total RNA isolated from *Chlamydomonas* chloroplast transformants harboring chimeric *rbcL* promoter–GUS constructs with *rbcL* promoter deletions in 5′ → 3′ direction (*A*) and 3′ → 5′ direction (*B*). Total RNA (4 μg per lane) was separated in 1.3% agarose/formaldehyde gels and transferred to nylon membranes. To detect GUS transcripts, membranes were hybridized overnight to random primer-labeled DNA probes specific for the GUS gene and exposed for 1 h (lanes −70 and −18) or overnight (lanes −4, −1, +63, and +97) to x-ray film with an intensifying screen at −80°C. Numbers above the lanes denote the end points of the 5′ or 3′ deletions relative to the start site of transcription (Fig. 1). The 3′ ends of the 5′-deleted *rbcL* promoter fragments were at position +197 (see Fig. 1); the 5′ ends of the 3′-deleted *rbcL* promoter fragments were at position −290 for the +97 and −1 constructs and at position −650 for the +63 construct (see Fig. 1).

Deletions in 3′ → 5′ direction into the *rbcL* promoter fragment abolished accumulation of GUS transcripts in chloroplast transformants when the region from positions +63 to −1 was deleted (Fig. 3B). This suggests that a sequence element in this region (i.e., downstream of the transcription start site) is required for *rbcL* promoter activity or transcript stability.

Activities of the various deleted *rbcL* promoter fragments described above were measured *in vivo* as described (13) by determining rates of GUS transcription in *Chlamydomonas* chloroplast transformants harboring *rbcL* promoter–GUS genes (Fig. 4A). Transcription of the GUS gene could be detected in all but those chloroplast transformants harboring *rbcL* promoter–GUS genes with promoter deletions beyond position −18 in the 5′ → 3′ direction (lane −4) and beyond position +63 in the 3′ → 5′ direction (lane −1), confirming the results obtained with the RNA gel blots (Fig. 3). These data show that the basic *rbcL* promoter lies between position −18 and position +63 relative to the start site of transcription of the *rbcL* gene.

**Activity of the *rbcL* Promoter Is Enhanced by a Sequence in the Coding Region of the *rbcL* Gene.** *Chlamydomonas* chloroplast transformants harboring chimeric *rbcL*–GUS constructs with *rbcL* gene sequence from positions −70 to +197 or −18 to +197 transcribed the GUS gene at 99% and 96% of the rate of the endogenous *rbcL* gene, respectively (Fig. 4A), indicating that the *rbcL* fragments in those chimeric genes included all elements required for normal *rbcL* promoter activity. In contrast, chimeric genes with the *rbcL* sequence from positions −650 to +63 or −290 to +97 (i.e., lacking *rbcL* sequences beyond position +97) were transcribed only 5% and 10%, respectively, as rapidly as the endogenous *rbcL* gene (Fig. 4A; ref. 13). This suggested that sequences in the *rbcL* structural gene beyond position +97 [the start of translation is supposed to be at position +93 (22)] might

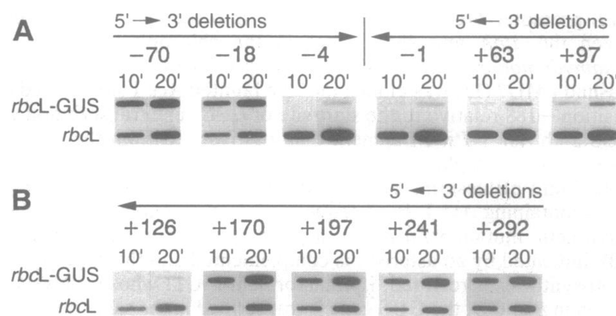


FIG. 4. Transcription of the GUS reporter gene and the endogenous *rbcL* gene in the *Chlamydomonas* chloroplast transformants analyzed in Fig. 3 (*A*) and in transformants harboring *rbcL* promoter–GUS–*psaB* constructs with *rbcL* sequences extending to various points within the coding region of the gene (*B*). The 5′ ends of the 3′-deleted *rbcL* promoter fragments were at position −70 for the constructs extending to positions +292, +241, and +197 and at position −186 for the constructs extending to positions +170 and +126 (see Fig. 1). Borders of the other *rbcL* promoter fragments were as described in Fig. 3. Slot blots and labeling conditions were as described in Fig. 2C. Numbers above the slots denote the end points at the 5′ or 3′ ends of the *rbcL* promoter fragments. Rates of GUS transcription (percent of *rbcL*) are as follows. Constructs: −70, 99%; −18, 96%; −4, 0%; −1, 0%; +63, 5%; +97, 10%; +126, 4%; +170, 101%; +197, 99%; +241, 105%; +292, 104%. These rates were calculated by laser densitometry of the hybridization signals, taking into account the differing sizes of transcripts from the chimeric *rbcL*–GUS genes and the endogenous *rbcL* gene (sizes of GUS transcripts vary from ≈2.1 kb to ≈2.4 kb; *rbcL* transcripts are ≈1.6 kb long). Values are corrected for background hybridization determined with pUC18 plasmid DNA as probe.

enhance the activity of the basic *rbcL* promoter from positions −18 to +63. To delineate the putative enhancer element, *rbcL* sequences extending from positions −70 to +292, +241, or +197 and from positions −186 to +170 or +126 were synthesized by PCR, using the primers shown in Fig. 1, and cloned in front of the GUS coding region for transformation of the *Chlamydomonas* chloroplast. Measurements of rates of GUS transcription in chloroplast transformants harboring these *rbcL*–GUS constructs revealed a significant decrease in *rbcL* promoter activity when the sequence between position +170 and position +126 was absent (Fig. 4*B*). Thus, we conclude that an element is located in this region of the *Chlamydomonas* chloroplast *rbcL* gene that enhances transcription from the basic *rbcL* promoter. Transcription is augmented 7- to 10-fold under our experimental conditions—i.e., cells grown synchronously in 12-h light/12-h dark cycles, harvested at 10 h of darkness, and assayed in the dark—converting the relatively weak basic *rbcL* promoter into one of the strongest promoters of the *Chlamydomonas* chloroplast genome.

## DISCUSSION

The basic promoter of the *C. reinhardtii* chloroplast gene *rbcL* is contained within the sequence from positions −18 to +63, taking position +1 as the site of initiation of transcription. This location of the promoter and the presence in it of a TATAATAT motif are features first described in the promoter of the *Chlamydomonas* chloroplast *atpB* gene (8). In addition to defining the limits of the basic promoter region of *rbcL*, we have found that a segment of the gene lying between position +126 and position +170—i.e., within the protein-coding region of the gene—is required to augment transcription from the rate exhibited from the basic promoter to that of the endogenous gene. The *rbcL* gene is the most actively transcribed chloroplast protein-coding gene studied in *Chlamydomonas* to date (13). To our knowledge, stimulation of expression from promoter sequences by other elements in a gene has not been reported previously in any chloroplast gene.

The best known prokaryotic transcription enhancer systems include (*i*) activation of transcription by $\sigma^{54}$-containing RNA polymerase holoenzyme of certain enteric bacterial −24/−12 genes by, e.g., nitrogen-regulating protein C or by NIFA (25), (*ii*) transcription of the late genes of bacteriophage T4 (e.g., ref. 26), and (*iii*) activation of the L-arabinose catabolic operon by the AraC protein (27). Also, in seeking to understand how the *Chlamydomonas rbcL* sequence from positions +126 to +170 influences the rate of transcription from the basic promoter, one situation to be kept in mind is the activation of transcription from human immunodeficiency virus 1 long terminal repeat gene promoters by the virus-encoded tat protein that binds to a 30-nt (positions +14 to +44) sequence of the transcript termed tar (trans-activation response element) (e.g., refs. 28 and 29). And, because part of the promoter of the *rbcL* gene is downstream of the transcription start site and the enhancing element lies within the protein coding region of the gene, the operation of the eukaryotic nuclear RNA polymerase III and its associated transcription factors (30) come to mind. It remains to be determined whether the *Chlamydomonas rbcL* promoter and transcriptional apparatus are related to any of the above prokaryotic or eukaryotic situations or are distinct.

The recent success in stably transforming tobacco chloroplasts (31–33) should permit *in vivo* analyses of chloroplast promoters of angiosperms comparable to experiments described in *Chlamydomonas* and should lead to additional information concerning their structures and functional features.

**Note Added In Proof.** Extensive mutagenesis of the sequence between positions +75 and +115 eliminates the transcription-stimulating activity of the region from positions +126 to +170 (Mahipal Singh and L.B., unpublished data). Thus, sequences between positions +75 and +115 and the nucleotides between positions +126 and +170 in the 5′ region of the *rbcL* gene appear to be required for enhancing transcription from the basic *rbcL* promoter.

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