

Transcriptional Analysis of Endogenous and Foreign Genes in Chloroplast Transformants of *Chlamydomonas*

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Transcription from modified chloroplast genes has been studied *in vitro*, but only with the recently developed ability to stably introduce foreign DNA into *Chlamydomonas reinhardtii* chloroplast chromosomes *in situ* has it become possible to do so *in vivo*. Cloned chloroplast DNA sequences, into which had been inserted chimeric genes composed of the GUS coding sequence reporter under transcriptional control of chloroplast promoters for the *C. reinhardtii* *atpA*, *atpB*, and *rbcL* genes, were introduced into the cells on microprojectiles. These constructs become integrated into chloroplast chromosomes by homologous recombination. RNA gel blot analyses demonstrated that a single major β -glucuronidase (GUS)-hybridizing transcript accumulates in each chloroplast transformant. We have found that: (1) Transcription of the chimeric gene begins at the same site as in the corresponding endogenous chloroplast gene; (2) the rates of transcription *in vivo* of the *atpA*:GUS and *atpB*:GUS genes relative to one another and to other genes are the same as those for the endogenous *atpA* and *atpB* genes, respectively, indicating that these promoters are fully functional despite being fused to a foreign gene and being at an alien location on the chloroplast chromosome; (3) in contrast to the *atpA* and *atpB* promoters, the *rbcL* promoter directs transcription of the *rbcL*:GUS gene at only 1% of the expected rate, suggesting that other features are required for optimal activity of this promoter; and (4) 22 base pairs upstream of the 5' end of the *atpB*:GUS transcript in the *atpB* promoter element is sufficient to confer wild-type levels of promoter activity.

INTRODUCTION

Elucidation of molecular mechanisms controlling gene expression in the chloroplast is of great importance for understanding the complex developmental processes of plant growth and of organelle/nuclear-cytoplasmic relations in eukaryotes in general. Levels of transcripts of individual chloroplast genes are affected by light (Rodermel and Bogorad, 1985; Sheen and Bogorad, 1985; Klein and Mullet, 1987; Deng and Gruissem, 1987), developmental stage (Berry et al., 1985; Nikolau and Klessig, 1987), and cell type (Link et al., 1978; Jolly et al., 1981; Sheen and Bogorad, 1988). Modifications in chloroplast gene expression through transcriptional and post-transcriptional mechanisms are thought to account for changes in relative sizes of transcript pools (Berry et al., 1985; Deng and Gruissem, 1987; Klein and Mullet, 1987, 1990; Mullet and Klein, 1987).

Studies of transcription *in vitro* using chloroplast RNA polymerase preparations from maize (Crossland et al., 1984; Bradley and Gatenby, 1985), mustard (Link, 1984),

and spinach (Gruissem and Zurawski, 1985a, 1985b) have led to the conclusion that sequences composed of -35 and -10 elements [resembling promoters for *Escherichia coli* genes transcribed by $\sigma 70$ polymerase (Reznikoff et al., 1985)] direct transcription *in vitro* and, by extension, function as chloroplast promoters *in vivo*. However, it has been observed that removal of the first two, generally very highly conserved, nucleotides of the spinach *trnM2* -35 sequence reduced transcription *in vitro* by a partially purified spinach chloroplast RNA polymerase by only 60% (Gruissem and Zurawski, 1985a), and a mustard chloroplast *psbA* gene construct without a -35 sequence (Link, 1984) was transcribed to some extent *in vitro*. Furthermore, *trnR1* and *trnS1* of spinach (Gruissem et al., 1986) are transcribed *in vitro* although they have no recognizable -10 or -35 sequences. Not only may plastid extracts lack essential transcription components, but extrapolating results of *in vitro* transcription studies to events *in vivo* is further complicated by the high sensitivity of plastid RNA polymerases to template topology (e.g., Jolly and Bogorad, 1980; Stirdivant et al., 1985; Sun et al., 1986; Russell and Bogorad, 1987). The latter point is reinforced by the identification of a *Chlamydomonas reinhardtii* chloroplast promoter that is regulated *in vivo* by torsional stress (Thompson and Mosig, 1987).

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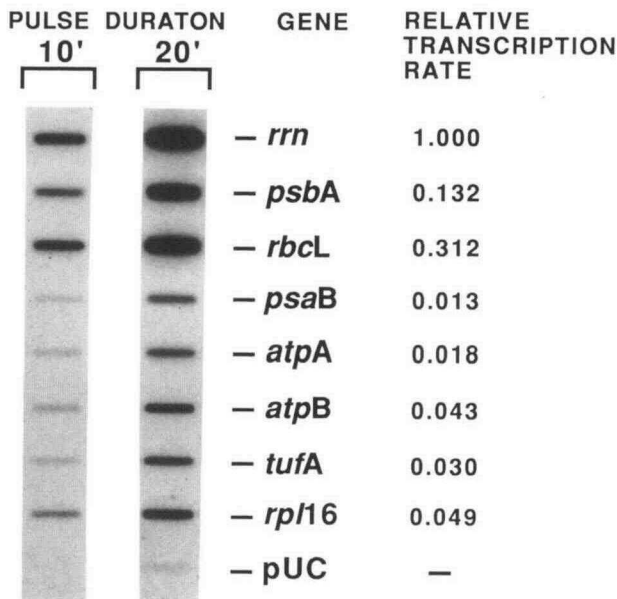


Figure 1. Relative Rates of Synthesis of Chloroplast mRNAs in Vegetatively Growing Wild-Type *C. reinhardtii* Cells.

³²P-RNA, labeled in vivo with 10-min or 20-min pulses of ³²P-orthophosphate, was hybridized to nylon filters containing immobilized DNA of the following plasmids (see Methods): pCrcrrn, pCrcpsbA, pCrcrbcL, pCrcpsaB, pCrcatpA, pCrcatpB, pCrcrctufA, pCrcrpl16, and pUC7. Hybridization signals were quantified by scanning laser densitometry, normalized with respect to hybridization to ribosomal DNA, and standardized according to transcript length and gene copy number, as described in the text.

The relative transcription rate for each gene was calculated by using the following formula:

$$\frac{\left(\frac{\Delta \text{ dpm gene [20 min - 10 min]}}{\Delta \text{ dpm } rrn \text{ [20 min - 10 min]}} \right) \left(\frac{\text{transcript length [in kb]}}{1.5 \text{ kb}} \right)}{\text{number of copies of the genes per chromosome}}$$

To examine more closely the contribution of transcription to levels of chloroplast gene mRNAs in *C. reinhardtii* and to begin to analyze DNA sequences that affect transcription in vivo, we have measured the relative rates of transcription in vivo of eight chloroplast genes. Furthermore, we have compared the effectiveness of presumed promoter regions of the *Chlamydomonas* chloroplast *rbcL*, *atpA*, and *atpB* genes for transcription in situ and at another location on the chromosome when fused to the structural sequence of the *uidA* gene (which encodes the β -glucuronidase or GUS protein) from *E. coli* (Jefferson et al., 1986). Plasmids containing chimeric genes composed of *C. reinhardtii* plastid gene promoter regions fused to GUS coding sequences flanked by *C. reinhardtii* chloroplast DNA sequences, including sequences to complement

photosynthetic deficiency because of a deletion in *atpB*, were introduced into an *atpB* deletion mutant of *C. reinhardtii* by bombardment with high-velocity tungsten microprojectiles (Boynton et al., 1988; Blowers et al., 1989). Stable, photosynthetically competent chloroplast transformants containing these novel chimeric genes were selected, and transcription of introduced genes was studied.

RESULTS

Relative Transcription Rates of Endogenous Chloroplast Genes

No information has been available regarding the relative rates of transcription of chloroplast genes in vivo. We obtained such data for eight chloroplast genes in log phase *C. reinhardtii* cells. In addition to the gene that encodes the chloroplast 16S ribosomal RNA, genes that encode polypeptides for components of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*), photosystem I (*psaB*), photosystem II (*psbA*), the alpha and beta subunits of the ATP synthase CF₁ complex (*atpA* and *atpB*), and the translational apparatus (*tufA* and *rpl16*) were studied.

Total cellular radiolabeled RNA, prepared from *Chlamydomonas* cells that had been pulse labeled with ³²P-orthophosphate (see Methods), was hybridized to nylon filters containing immobilized plasmid DNA of the gene-specific restriction fragments, as shown in Figure 1. Hybridizations were under conditions of DNA excess with respect to the protein-coding mRNA transcripts (data not shown). Under these conditions, hybridization to the gene-specific fragments at completion (assuming all transcripts are removed from solution at equal efficiency) is proportional to the relative amounts of mRNA, including ³²P-RNA, present for each gene (assuming that all transcripts are removed from solution with equal efficiency). Hybridizations were in RNA excess with respect to the 16S ribosomal DNA sequences (data not shown), and under these conditions, hybridization to the rDNA is representative of the specific radioactivity of the rRNA.

The amount of hybridization to each plasmid DNA probe was quantified by scanning laser densitometry of autoradiograms, and the relative transcription rates per gene in the chromosome for the chloroplast genes are presented in Figure 1. The amount of ³²P-RNA hybridizing to a protein-coding gene has been normalized to the amount of ³²P-16S rRNA that hybridized to the rDNA and to a unit length of RNA of 1.5 kb, the approximate size of 16S rRNA. Furthermore, we conclude from titration experiments that only approximately 25% of the total 16S rRNA transcript in solution hybridizes to the immobilized rDNA under the conditions used, thus underestimating the amount of *rrn* transcription by a factor of 4. All of these corrections, including accounting for the number of copies

of the gene per chromosome (each *C. reinhardtii* chloroplast chromosome contains two *rrn* and two *psbA* genes but only one of each of the other genes studied), have been made in arriving at the relative transcription rates. In standardizing all measurements to the rRNA specific radioactivity, it is assumed that rRNA synthesis remains constant during the short labeling period and that the ribonucleotide precursors are drawn from the same pool. Two observations support the validity of these assumptions: (1) in long-term continuous labeling, rRNA synthesis remained constant for at least 4 hr (data not shown); and (2) during short-term pulse labeling, the proportional increase in the specific radioactivity of rRNA from 10 min to 20 min was identical to that observed for the protein-coding mRNAs (see Figure 1).

Ribosomal RNA is synthesized about threefold more rapidly than mRNA from the most actively transcribed protein-coding gene, *rbcl*. And *rbcl* is transcribed about 25-fold more actively than the least rapidly transcribed gene we studied, *psaB*. Interestingly, the three genes with the highest transcription rates (16S rDNA, *rbcl*, and *psbA*) are also represented by the largest mRNA pools in the *Chlamydomonas* chloroplast (A.D. Blowers and L. Bogorad, unpublished observations).

Chlamydomonas Chloroplast Promoters Can Direct Transcription of Foreign Genes in Vivo

We have taken advantage of the ability to stably introduce foreign DNA into *Chlamydomonas* chloroplast chromosomes (Blowers et al., 1989) to examine aspects of the regulation of chloroplast gene transcription in vivo. Fusions in which the β -glucuronidase structural gene (GUS) was placed downstream of *Chlamydomonas* chloroplast promoters from the *atpB*, *atpA*, and *rbcl* genes were introduced into *Chlamydomonas* cells on tungsten microprojectiles. Transcription from these promoters in chimeric genes integrated into the chloroplast chromosome at an alien location were compared with transcription of the corresponding endogenous gene in situ.

As shown in Figure 2, a 224-bp *Dra*I fragment containing the putative promoter, transcription initiation site, and a portion of the 5'-noncoding region of the transcript of the *atpB* gene (Woessner et al., 1986) was fused in the proper orientation to the GUS structural gene to create plasmid pCrc34. In pCrc34, as well as in all of the other GUS constructs employed here, 315 bp of the 3' terminus of the chloroplast *rbcl* gene was added to the 3' end of the GUS gene, as described briefly in Methods and in detail elsewhere (A.D. Blowers, G.S. Ellmore, and L. Bogorad, manuscript in preparation). To study transcription directed by the 5' regions of *rbcl* or *atpA*, a 709-bp fragment containing the putative promoter regions, transcription initiation sites, and portions of the 5'-noncoding regions of the transcripts of the divergently transcribed *atpA* [first

described as gene X by Dron et al. (1982a) and later identified as *atpA* by Hallick (1984)] and *rbcl* genes (Dron et al., 1982a) was fused in both orientations to the GUS reporter gene. In one orientation, the reporter sequence was under transcriptional control of the *rbcl* promoter (pCrc39) and in the reverse orientation, the *atpA* promoter (pCrc40) was upstream of the reporter.

Chimeric genes were inserted into a polylinker region from a Bluescript plasmid vector that had been introduced into the first *Kpn*I site downstream of the endogenous *atpB* gene located on the 5.3-kb *Bam*HI-*Eco*RI restriction fragment of the *C. reinhardtii* chloroplast Bam10 fragment (Blowers et al., 1989). Thus, the chimeric gene was bordered by *C. reinhardtii* chloroplast DNA sequences including an entire *atpB* gene. The chimeric genes were con-

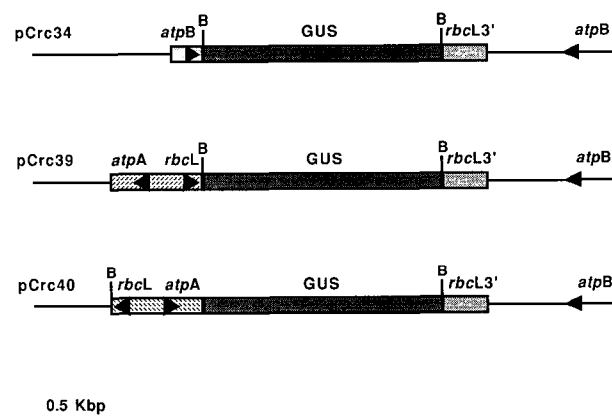


Figure 2. Structure and Organization of the Chimeric GUS Reporter Genes on the Recombinant Chloroplast Chromosomes.

Transcriptional fusions between the presumptive chloroplast promoters for the *atpB* (in pCrc34), *rbcl* (in pCrc39), and *atpA* (in pCrc40) genes and a promoterless GUS structural gene containing 12 bp of (bacterial) 5'-untranslated sequences (including 3'-flanking sequences from the *rbcl* gene) were constructed as described in Methods. In pCrc34, 103 bp of the 362 bp of the *atpB* 5'-transcribed but untranslated leader sequence (Woessner et al., 1986) are present in the *atpB*-GUS gene. In pCrc39, 62 bp of the 92 bp of 5'-untranslated leader sequence of *rbcl* (Dron et al., 1982a) are included in the *rbcl*-GUS gene. Finally, about 265 bp of the approximately 455 bp in the 5'-untranslated leader sequence of the *atpA* gene (Dron et al., 1982a) are present in the *atpA*-GUS gene in pCrc40 transformants. In each of the above, the sequences missing from the 5'-untranslated regions are those adjacent to the ATG codons of the endogenous chloroplast gene transcripts. In all constructs, the chimeric genes were inserted downstream of the 3' end of the endogenous *atpB* gene (as shown by the arrowhead on the thin black line) such that transcription proceeded toward the *atpB* gene. Endogenous chloroplast DNA sequences (represented by the thin black line) flank both ends of the chimeric genes. B denotes *Bam*HI restriction sites.

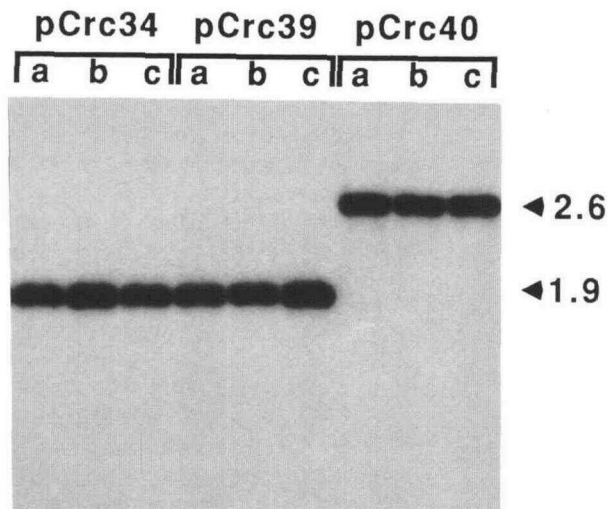


Figure 3. DNA Gel Blot Analysis of pCrc34, pCrc39, and pCrc40 *Chlamydomonas* Chloroplast Transformants.

Total cell DNA (0.1 μ g) from three independent isolates (a to c) from pCrc34, pCrc39, and pCrc40 transformants was digested with BamHI and probed with radiolabeled GUS DNA. Molecular sizes (in kilobases) of the GUS-hybridizing bands are indicated on the right.

structed so as to be transcribed convergently to the endogenous *atpB* gene (Figure 2). Plasmids pCrc34, pCrc39, and pCrc40 were each precipitated onto tungsten micro-particles and bombarded into the recipient nonphotosynthetic mutant *C. reinhardtii* *ac-u-c-2-21* cells, as described previously (Blowers et al., 1989). These mutant recipient cells are incapable of photosynthesis because of a deletion in *atpB* (Shepherd et al., 1979; Woessner et al., 1984). Transformants were selected for restoration of *atpB* function, i.e., photosynthesis, and then screened for the presence of GUS DNA-hybridizing sequences by DNA dot-blot analysis (data not shown). As reported previously (Blowers et al., 1989), 50% to 70% of such transformants selected for restoration of photosynthesis contain the complete construct. As shown in Figure 3, the expected 1.9-kb BamHI restriction fragment is detected in DNA from pCrc34 and pCrc39 transformants and the expected 2.6-kb BamHI restriction fragment is observed in pCrc40 transformants.

The RNA gel blot analyses presented in Figure 4 show that a single major GUS transcript accumulates in each chloroplast transformant. These transcripts in pCrc34 (*atpB* promoter-directed) and pCrc39 (*rbcL* promoter-directed) transformants are 2.1 kb in length and in pCrc40 (*atpA* promoter-directed) transformants they are 2.3 kb in length, as expected if transcription initiates at or near the previously reported transcription initiation sites for the

endogenous *atpB* (Woessner et al., 1986) and *rbcL* and *atpA* (Dron et al., 1982a) genes.

To confirm that each chloroplast promoter was directing transcription initiation accurately, primer extension analyses were performed using a GUS sequence-specific primer (see Methods) to locate the 5' ends of the GUS-containing transcripts in each transformant. As can be seen in Figure 5, a number of common primer extension products are present in the control wild-type RNA sample as well as in the RNA samples from the transformants. However, a unique primer extension product can be observed in each of the RNA samples prepared from the individual transformants. A unique cluster of bands in the region around the 238 and 242 nucleotide markers can be observed in RNA from transformants of the pCrc34 series (*atpB* promoter). (The 5' ends of the primer extension products are shown in Figure 8.) Transformants pCrc39 (*rbcL* promoter) and pCrc40 (*atpA* promoter) have single, unique primer extension products of 201 nucleotides and 395 ± 5 nucleotides, respectively. The 5' ends of the transcripts are 103 nucleotides, 62 nucleotides, and approximately 265 nucleotides from the 3' ends of the *atpB*, *rbcL*, and *atpA* promoter fragments, respectively. The 5' end of the *rbcL*-GUS chimeric transcript in pCrc39 transformants is identical to that previously reported for transcripts of the endogenous *rbcL* gene (Dron et al., 1982a). The 5' end of the GUS transcript from the *atpB* promoter is just upstream of the 20-bp region within which transcription was previously judged to start (Woessner et al., 1986). Finally, the 5' end of the *atpA*-GUS transcript is approximately 25 nucleotides upstream of the previously esti-

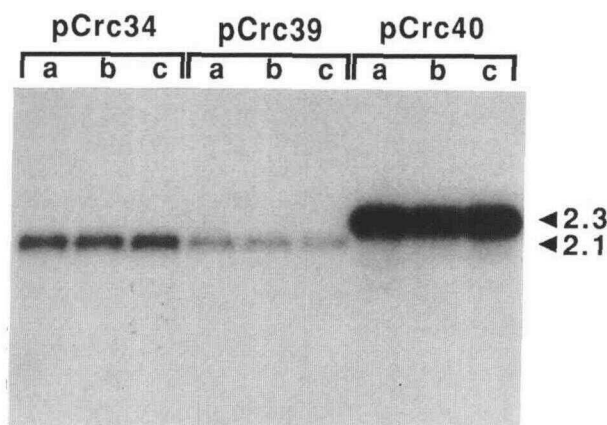


Figure 4. RNA Gel Blot Analysis of GUS Transcripts in pCrc34, pCrc39, and pCrc40 Chloroplast Transformants.

Total RNA (3 μ g) from three independent isolates (a to c) of pCrc34, pCrc39, and pCrc40 transformants (shown in Figure 3) was probed with radiolabeled GUS DNA. Molecular sizes (in kilobases) of the GUS-hybridizing bands are indicated on the right.

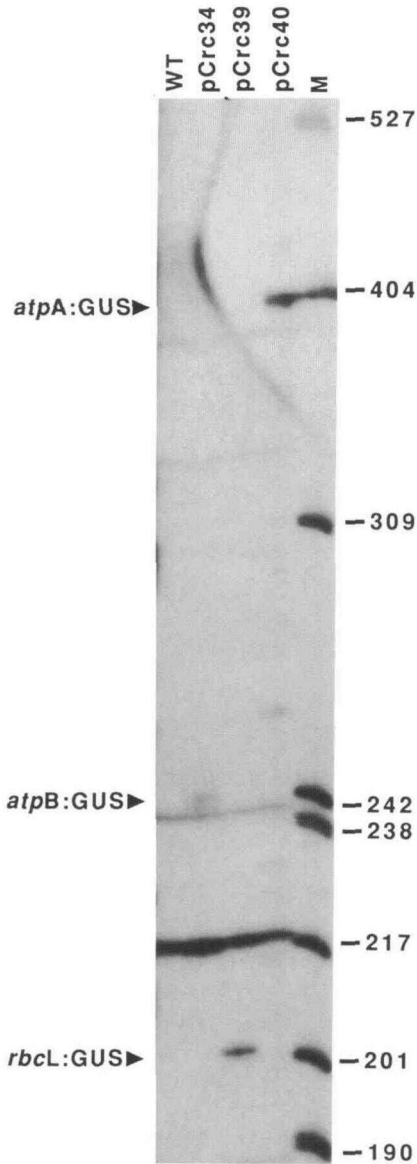


Figure 5. Determination of the 5' Ends of the GUS-Containing Transcripts in pCrc34, pCrc39, and pCrc40 Transformants.

A 5'-radiolabeled 21-nucleotide primer homologous to GUS sequences was hybridized with 10 μ g of total RNA isolated from *C. reinhardtii* wild-type cells (WT) or chloroplast transformants (pCrc34, pCrc39, pCrc40) and then elongated with AMV reverse transcriptase and unlabeled deoxynucleotides. The reaction products were denatured, electrophoresed through a denaturing polyacrylamide-urea gel, and visualized by autoradiography. The molecular size markers (M) are 5' end-labeled pBR322/MspI restriction fragments (in base pairs) and are indicated on the right. The primer extension product unique to each chloroplast transformant is indicated on the left.

mated transcription initiation site for the endogenous gene (Dron et al., 1982a). These results show that the chloroplast *atpB* and *rbcL* promoters can accurately initiate transcription, even when joined to a foreign coding sequence and relocated outside of its normal position on the chloroplast chromosome; also, it seems highly likely that transcription from the *atpA* promoter is correct considering the similarities in the rates of transcription of *atpA* and *atpA*:GUS, as shown in Figure 6.

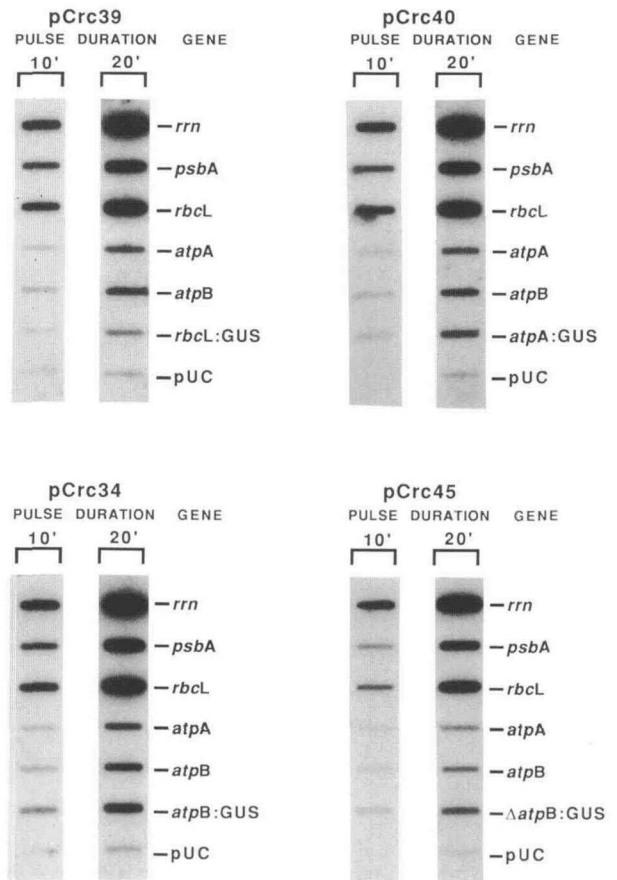


Figure 6. Relative Rates of Synthesis of Chloroplast mRNAs in pCrc34, pCrc39, pCrc40, and pCrc45 Chloroplast Transformants.

³²P-RNA, labeled in vivo with 10-min or 20-min pulses of ³²P-orthophosphate, was hybridized to nylon filters containing immobilized DNA of selected plasmids listed in Figure 1 and, in addition, pBI221. pBI221 detects the *rbcL*:GUS, *atpA*:GUS, *atpB*:GUS, and Δ *atpB*:GUS. Hybridization signals were quantified and normalized as described in Figure 1 and the text. The relative transcription rates for the endogenous genes in the four transformants are: *rrn*, 1.000; *psbA*, 0.130 to 0.134; *rbcL*, 0.310 to 0.313; *atpA*, 0.017 to 0.018; and *atpB*, 0.043 to 0.044. The transcription rates for the GUS gene constructs in the transformants relative to *rrn* are: pCrc39 (*rbcL*:GUS), 0.003; pCrc40 (*atpA*:GUS), 0.018; pCrc34 (*atpB*:GUS), 0.042; pCrc45 (Δ *atpB*:GUS), 0.043.

The *rbcl* Promoter in pCrc39 Transformants Is Only Partially Functional

The relative transcription rates observed for the endogenous *atpB*, *atpA*, and *rbcl* genes (see Figure 1) suggest that if all the GUS transcripts were equally stable, pCrc39 (*rbcl* promoter-GUS) transformants should contain the highest steady-state levels of GUS-containing transcripts followed by pCrc34 (*atpB* promoter-GUS) and pCrc40 (*atpA* promoter-GUS) transformants, respectively. On the contrary, Figure 4 shows clearly that the relative steady-state GUS mRNA levels in the transformants are pCrc40 (*atpA*-GUS) >> pCrc34 (*atpB*-GUS) > pCrc39 (*rbcl*-GUS), the inverse of the prediction.

To determine whether the transformants transcribed these endogenous genes at the same relative rates as do wild-type cells, transformants pCrc34, pCrc39, and pCrc40 were individually pulse labeled with ³²P-orthophosphate, and total RNA was isolated and hybridized to immobilized plasmid DNA of selected endogenous chloroplast genes and the GUS structural gene. As can be seen in Figure 6, the relative transcription rates for the endogenous chloroplast genes (16S rRNA, *psbA*, *rbcl*, *atpA*, and *atpB*) are about the same in the various transformants and in the wild-type strain (see Figure 1).

The ratio of transcription of the GUS gene from the *atpB* promoter (*atpB*:GUS) in pCrc34 transformants (lower left panel) to transcription of GUS from the *atpA* promoter (*atpA*:GUS) in pCrc40 transformants (upper right panel) is 2.3. This is close to the ratio of 2.4 (Figure 1) observed for the transcription of the endogenous *atpB* and *atpA* genes in these cells. Thus, the *atpB* and *atpA* promoters are transcribing the GUS structural gene at the same relative rates as they are their endogenous genes. However, the transcription rate of *rbcl*:GUS in pCrc39 transformants (upper left panel) is only approximately 1% of that of the endogenous *rbcl* gene. Thus, although the *rbcl* promoter initiates transcription correctly from the *rbcl*:GUS chimera (Figure 5), it does so remarkably infrequently.

Finally, the relative transcription rates of the GUS gene in pCrc34 (*atpB*-GUS) and pCrc40 (*atpA*-GUS) transformants lead to the expectation that the steady-state GUS mRNA levels would be approximately 2.3-fold higher in pCrc34 than in pCrc40 transformants. However, the GUS transcript accumulates to much higher levels in pCrc40 transformants than in pCrc34 transformants (Figure 4). Similarly, the difference in the rate of GUS gene transcription between pCrc34 (*atpB*-GUS) and pCrc39 (*rbcl*-GUS) transformants (approximately 14-fold higher in pCrc34) is not reflected in the relative steady-state levels of the two hybrid transcripts. Inasmuch as these chimeric genes encode transcripts that differ only in short sequences in their 5'-noncoding regions derived from the original *Chlamydomonas* genes, this region or specific sequences within it, perhaps in combination with the introduced GUS se-

quences, may dramatically affect the stability of the GUS-containing transcript.

Deletion Mutagenesis of the Chloroplast *atpB* Promoter

Because the primer extension analyses and in vivo pulse-labeling studies demonstrated that the *atpB* promoter initiates transcription of the GUS gene correctly and at the expected relative rate in pCrc34 transformants, we investigated what upstream sequence elements were required for efficient transcription. This investigation was also prompted by the observation that the DNA sequence upstream of the *atpB* transcription initiation site, shown in Figure 7A, contains a few sequences that resemble "−10" prokaryotic and plastid promoter elements but none that is similar to the "−35" elements of σ 70 *E. coli* promoters. The results obtained could be compared with those of Bradley and Gatenby (1985), which showed that deletion of the maize *atpB* gene to −24 rendered it inactive as a template for transcription by a maize chloroplast preparation in vitro.

Progressive deletions were made with Bal31 nuclease into the 5' end of the upstream region of the 224-bp *atpB* promoter fragment to remove part or all of the promoter element from its 5' end. The deletion fragments tested are shown in Figure 7A. Identical deletion endpoints were observed for plasmids pCrc42 and pCrc43 at −39 and for plasmids pCrc44 and pCrc45 at −22. These deletion fragments were fused to a promoterless GUS reporter gene, and the resulting plasmids, pCrc41 to pCrc47, were cloned into the same KpnI site of the 5.3-kb BamHI-EcoRI *C. reinhardtii* chloroplast DNA sequence. DNA gel blot analyses of total cellular DNA isolated from these transformants show that all contain the expected 1.9-kb GUS-hybridizing BamHI restriction fragment (Figure 7B).

Total RNA was isolated from the transformants shown in Figure 7B, and the RNA gel blot analysis of those transformants is shown in Figure 7C. The pCrc42/pCrc43 transformants, which contain 39 bp upstream of the *atpB* transcription initiation site, display GUS transcripts of the same length and abundance as the pCrc41 transformant that contains the full-length *atpB* upstream sequence (structurally identical to pCrc34). Surprisingly, pCrc44/pCrc45 transformants, which—almost like one of the maize *atpB* deletions that did not support transcription in vitro (Bradley and Gatenby, 1985)—contain only 22 bp upstream of the *atpB* transcription initiation site, accumulate a GUS transcript of the same length and abundance as the pCrc41 transformant. Finally, transformants pCrc46 and pCrc47, with deletions that extend into the 5'-noncoding region of the *atpB*-GUS gene, have no detectable GUS-hybridizing transcripts. To show that all lanes contained RNA, the same nylon filter was reprobated with radiolabeled

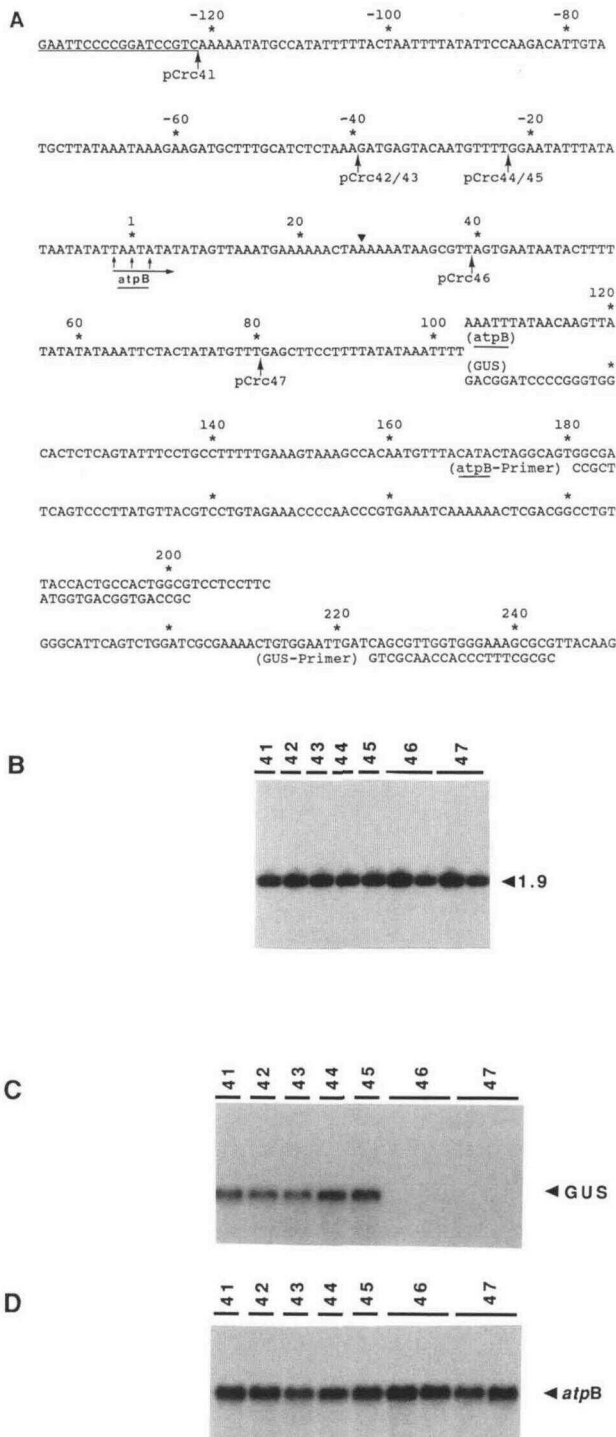


Figure 7. Transcriptional Analysis of Chloroplast *atpB* Gene Promoter Deletion Mutants.

(A) The nucleotide sequences of the 5' region of the *Chlamydomonas atpB* gene and of the *atpB*:GUS constructs in pCrc41 to

atpB DNA. Figure 7D shows that a strong 1.9-kb hybridization band corresponding to the *atpB* transcript is present in all RNA samples, including those samples that contained no detectable GUS-hybridizing mRNA.

Figure 6 (lower right panel) shows that the GUS gene is transcribed at the same relative rate in pCrc45 ($\Delta atpB$:GUS) as in the pCrc34 transformant (*atpB*:GUS), which contains the full-length *atpB* promoter fragment (Figure 6, lower left panel). In addition, the primer extension products of the GUS transcript synthesized in pCrc45 have the same 5' termini as transcripts of the endogenous *atpB* gene, as shown in Figure 8. Thus, neither the site of initiation nor the rate of transcription initiation of the *atpB* gene is affected by a deletion that removes all but 22 bp upstream of the 5' end of the transcript. Inspection of vector sequences did not reveal -35 -like elements at an appropriate location to function as part of a promoter (Figure 7A).

DISCUSSION

Relative Transcription Rates of *Chlamydomonas* Chloroplast Genes

To facilitate the identification of critical *cis*-acting and *trans*-acting elements required for *Chlamydomonas* chloroplast gene transcription *in vivo*, we have utilized a system in which a foreign reporter gene is stably introduced into the *Chlamydomonas* chloroplast chromosome and is faithfully transcribed from *Chlamydomonas* chloroplast promoter

pCrc47 are shown. The deletion endpoints of *atpB* in pCrc41 to pCrc47 are indicated by the vertical arrowheads. The start of *atpB* transcription is indicated by the vertical lines [the middle line being the major start site as determined by primer extension analysis (see Figure 8)] connected to the horizontal black arrow showing the direction of transcription. Vector sequences at the 5' border of the *atpB* region are underlined. The sequences of the GUS primer and the *atpB* primer are shown at the sequences to which they are complementary. The significance of the filled, inverted triangle (\blacktriangledown) is discussed in the legend to Figure 8.

(B) DNA gel blot analysis of pCrc41 to pCrc47 transformants was digested with BamHI and probed with radiolabeled GUS DNA. The molecular size (in kilobases) of the GUS-hybridizing bands is indicated on the right.

(C) RNA gel blot analysis of pCrc41 to pCrc47 transformants. Total RNA (3 μ g) from the pCrc41 to pCrc47 transformants in **(B)** was probed with radiolabeled GUS DNA, and GUS-hybridizing transcripts of 2.1 kb are indicated on the right.

(D) The filter from **(C)** was rehybridized with radiolabeled *atpB* DNA, and *atpB*-hybridizing transcripts of 1.9 kb are indicated on the right.

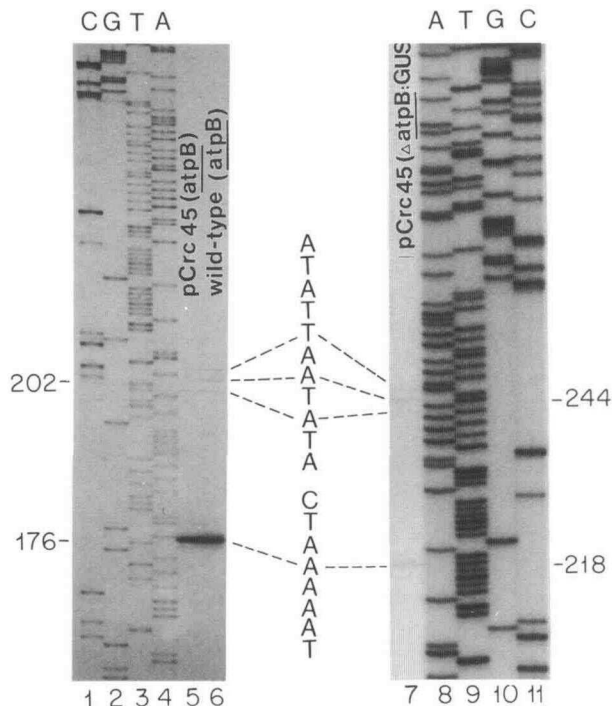


Figure 8. Autoradiograms of Primer Extension Analyses to Locate the 5' Ends of Transcripts of *atpB* in Wild-Type and pCrc45 Cells and of Δ *atpB*:GUS in pCrc45 Cells.

Lanes 1 to 4 and 8 to 11 show part of the nucleotide sequence of plasmid pCrc45 (Δ *atpB*:GUS). Lanes 8 to 11 are used to locate the 5' ends of the GUS primer extension products of Δ *atpB*:GUS RNA in pCrc45 (shown in lane 7). Part of the same sequence is shown in lanes 1 to 4, where it is used only to provide a set of DNA fragment size markers for the primer extension products of *atpB* transcripts in wild-type (lane 6) and pCrc45 (lane 5) cells using the *atpB* primer. The sequences of the *atpB* and *atpB*:GUS primers are shown in Figure 7A.

The 5' terminal nucleotides identified by primer extension are indicated on the sequences shown between lanes 6 and 7. The numbers at the outside edges of the figure show the sizes of the fragments and relate to the sequence shown in Figure 7A. The primer extension products terminating in positions 176 and 218 may be artifacts of the analyses; the position of the 5' end of these extension products is marked by a filled, inverted triangle (\blacktriangledown) in Figure 7A.

elements. This system will permit a rigorous examination of the DNA sequence elements necessary for chloroplast gene transcription *in vivo*. It will also permit testing of conclusions drawn from *in vitro* experiments in which DNA templates that may differ in conformation from their forms *in vivo* are transcribed by plastid extracts or partially purified fractions of chloroplast RNA polymerase that may lack essential components.

We have determined the relative transcription rates of eight *Chlamydomonas* chloroplast genes in log phase veg-

etative cells to establish baselines for studies of transcription of introduced chimeric genes. As shown in Figure 1, transcription rates for these genes vary about 80-fold.

Relative Transcription Rates of *Chlamydomonas* Chloroplast Chimeric GUS Genes

To determine whether the relative transcription rate of a chloroplast gene *in vivo* is determined exclusively by its 5' promoter sequence, the 5'-flanking regions of the *atpA*, *atpB*, and *rbcL* genes were fused to the same GUS structural gene to provide a readily detectable common novel transcript in the *Chlamydomonas* chloroplast, and the chimeric genes were introduced at a common site on the chromosome. When fused to the promoter region of the *atpA* or *atpB* genes, transcription of the chimeric gene began at the correct site and proceeded at the same relative rates as the endogenous *atpA* and *atpB* genes. Thus, the functions of these two promoters were not altered by being moved to a foreign site on the chromosome and dissociated from their normal structural gene. However, although the relocated promoter for the *rbcL* gene retains the ability to initiate transcription at the expected site when part of a chimeric GUS gene, it does so at only about 1% of the normal rate. These data suggest that the *rbcL* promoter may be sensitive to its sequence context more than 600 bp upstream of the gene, or within or beyond the transcribed coding regions. One possibility is that the local negative superhelicity of the promoter region is influenced by the chromosomal environment, resulting in its being somewhat silenced or unactivated. It is also possible that this promoter is incompatible with the GUS coding sequence. Further experiments should enable us to determine the basis for this behavior.

The apparent DNA context/position effect that we have observed with the *C. reinhardtii* *rbcL* promoter may also help to explain why we have not detected transcripts originating from maize *rbcL* (Blowers et al., 1989) and *psbA* (A.D. Blowers and L. Bogorad, unpublished data) gene promoters that we have introduced into the *Chlamydomonas* chloroplast at the same site as in the experiments described in this report. Alternatively, the promoter structure of the maize genes may not be recognized by the RNA polymerase or other DNA-binding transcription factors of *Chlamydomonas* chloroplasts, although the "−10" sequences of the maize and *Chlamydomonas* genes tested are very similar.

Transcription Analysis of GUS with Truncated *atpB* Promoter Elements

A startling result of these *in vivo* transcription experiments is that the functions of the *atpB* promoter are unaltered even when all but 22 bp of chloroplast DNA upstream of the transcription initiation site are removed. [GUS is also transcribed from genes with more drastic deletions of

sequences 5' to the -10 sequence (U. Klein, J. DeCamp, and L. Bogorad, unpublished results).] Based on comparisons of chloroplast gene sequences as well as in vitro transcription experiments with chloroplast genes of angiosperms (Link, 1984; Gruissem and Zurawski, 1985a, 1985b), it has been thought that chloroplast promoters resemble the *E. coli*-like -35 TTGACA and -10 TATAAT sequence motifs. Our results stand in especially sharp contrast to the observation of Bradley and Gatenby (1985) that their -24 deletion construct of maize *atpB* did not support transcription in vitro and indicate that a more diverse view of chloroplast promoters may be appropriate: Two or more classes of *C. reinhardtii* plastid promoters may exist. Alternatively, *Chlamydomonas* and angiosperm chloroplast promoters may be different. A clearer picture of chloroplast promoter structure will emerge as similar deletion analyses are performed on additional *Chlamydomonas* chloroplast promoters.

In conclusion, we have determined that transcription of endogenous chloroplast genes in vivo can vary by almost 2 orders of magnitude, and we have developed a system for studying transcription of endogenous and chimeric *C. reinhardtii* chloroplast genes in vivo. Two unexpected features of chloroplast gene transcription have been revealed: First, *atpB* sequences 22 nucleotides or less upstream of the transcription start site are sufficient for accurate initiation at a normal rate, and, second, unlike *Chlamydomonas atpA* and *atpB* promoters, the *rbcL* promoter region functions comparatively poorly when removed from its normal context and attached to GUS coding sequences and when the construct is inserted next to the endogenous *atpB* gene. One particularly attractive feature of this system is that the controls are built into it. Transcription of the endogenous gene from the endogenous promoter can be measured in the same cell as transcription of the introduced constructs. This system should facilitate the identification of promoter elements of *Chlamydomonas* chloroplast genes, and it may also permit elucidation of higher order regulatory mechanisms operating within the chloroplast. A greater understanding of the interactions between the chloroplast RNA polymerase and other *trans*-acting factors with their target sites on the chloroplast chromosome should also be attainable.

METHODS

Algae and Culture Conditions

Chlamydomonas reinhardtii nonphotosynthetic mutant strain CC-373 (*ac-u-c-2-21*) and wild-type strain CC-125 were obtained from the *Chlamydomonas* Genetics Center, Durham, NC. The mutant strain and photosynthetic transformants of this strain were maintained on HSHA (acetate) and HS (minimal) culture media, respectively (Sueoka, 1960). For in vivo pulse labeling of *Chlamydomonas*, cells were grown on medium I (Sager and Granick, 1953) and a low-phosphate variant of this medium (Baker et al., 1984).

Chlamydomonas Chloroplast Transformation Procedure

Bombardment of *Chlamydomonas* cells was carried out essentially as described by Blowers et al. (1989), except that cells were no longer transported for bombardment. Bombardment was carried out on the same day or the following day (after overnight incubation at room temperature in the dark) after plating the cells onto 9 × 50 mm Petri dishes of HSHA medium. No obvious differences in transformation frequency were observed.

In Vivo Labeling

C. reinhardtii cells, either wild type or photosynthetic transformants, were grown in medium I (Sager and Granick, 1953) as precultures. These cells were used to inoculate larger (200 mL to 500 mL) cultures of a low-phosphate variant of medium I (Baker et al., 1984). Wild-type cells were grown to about 1×10^6 cells/mL and photosynthetic transformants were grown to about 4×10^5 cells/mL such that all cells were grown for four to five generations in low-phosphate medium. For pulse labeling, cells were concentrated to 2×10^7 cells/mL in fresh medium containing no phosphate. Cells were labeled with ^{32}P -orthophosphate (Du Pont-New England Nuclear) (in dilute HCl) at a concentration of 40 $\mu\text{Ci}/\text{mL}$ cells. For pulse-labeling studies, 4×10^8 cells were labeled with 800 μCi while being agitated rapidly under illumination for up to 20 min. The pulse was terminated by removing the cells to a centrifuge tube containing 2 volumes of ice-cold medium I. Cells were immediately collected by centrifugation, resuspended/lysed in 50 mM Tris-HCl (pH 8.0)/0.3M NaCl/5 mM EDTA/5 mM EGTA/2% SDS at a concentration of 1×10^8 cells/mL, and frozen in liquid nitrogen. All steps were carried out within 5 min from the end of the pulse.

DNA and RNA Isolation

For DNA (Southern, 1975) and RNA (Thomas, 1980) gel blot analyses, total cellular DNA and RNA were prepared from cells grown in HS medium shaken vigorously in bright light (about 200 $\mu\text{E}/\text{m}^2 \text{ sec}^{-1}$). DNA was prepared essentially as described by Dellaporta et al. (1983), and total RNA was isolated essentially as described by Merchant and Bogorad (1986). Total RNA was isolated from pulse-labeled cells by extracting twice with buffer-saturated phenol/chloroform/isoamyl alcohol (50:49:1) and once with chloroform/isoamyl alcohol (24:1). After precipitation of nucleic acid with 0.3 M sodium acetate and 1 volume of 2-propanol and resuspension, RNA was purified by LiCl precipitation.

DNA and RNA Hybridization Analyses

All DNA and RNA gel blot hybridizations were carried out as described previously (Blowers et al., 1989). For hybridization of uniformly labeled *Chlamydomonas* RNA, plasmid DNA (1 $\mu\text{g}/\text{slot}$) was denatured at 65°C in the presence of 0.3 N NaOH for 1 hr. After cooling to room temperature, an equal volume of 20 × SSC (Maniatis et al., 1982) was added and the sample was applied to GeneScreen nylon membrane (pretreated with 10 × SSC) in a slot-blot apparatus (Schleicher & Schuell). After application, the plasmid DNA was covalently linked to the membrane by exposure to UV light. Hybridization and prehybridization buffers contained

0.5 M sodium phosphate (pH 7.0)/7% SDS/1% BSA (Church and Gilbert, 1984). Prehybridization was carried out for 4 hr to 16 hr and hybridization was carried out for 72 hr to 96 hr at 65°C. After hybridization, filters were washed extensively in 40 mM sodium phosphate (pH 7.0)/1% SDS at 65°C. Autoradiography for all hybridizations was carried out as described previously (Blowers et al., 1989).

Primer Extension and Mapping the 5' Termini of Transcripts

A 21-nucleotide oligomer, 5'-CGCGCTTTCCACCAACGCTG-3', which is complementary to the sense strand sequence of the GUS gene (Figure 7A, GUS primer) was the gift of Jun Ma, Harvard University. To locate the 5' ends of transcripts containing GUS sequences, this oligonucleotide was radiolabeled at its 5' terminus with T4 polynucleotide kinase and γ -³²P-ATP (Maniatis et al., 1982). The 5'-radiolabeled primer (0.3 pmol) was allowed to hybridize with 10 μ g of total RNA isolated from wild-type or transformed cells by incubation at 50°C in 0.3 M KCl for 4 hr. After this time, the annealed primer was elongated at 41°C by the addition of avian myeloblastosis virus (AMV) reverse transcriptase and unlabeled deoxynucleotide triphosphates. The reactions were terminated by the addition of EDTA and the nucleic acid precipitated with ethanol. The reaction products were resuspended in sequencing gel loading buffer, denatured at 95°C, electrophoresed through a 5% polyacrylamide/7 M urea sequencing gel, and visualized by autoradiography. A 22-nucleotide oligomer (Figure 7A, *atpB* primer) was used in a similar manner to locate the 5' ends of transcripts of endogenous *atpB* genes in wild-type and pCrc45 cells (Figure 8).

Plasmids

For assaying the *in vivo* pulse-labeling of RNA in *Chlamydomonas* cells, chloroplast gene-specific restriction fragments were isolated and cloned as follows. The approximately 930-bp EcoRI restriction fragment, which contains the 3' portion of the chloroplast 16S rRNA gene (Dron et al., 1982b), was cloned into Bluescript vector (Stratagene) to create plasmid pCrc16SrRNA. The approximately 1100-bp HindIII-KpnI restriction fragment, which contains exon 5 of the *psbA* gene (Erickson et al., 1984), was cloned into pUC19 (Vieira and Messing, 1982) to create plasmid pCrcpsbA. The approximately 890-bp HindIII restriction fragment, which contains an internal portion of the *rbcl* coding region (Dron et al., 1982a), was cloned into pUC19 to create plasmid pCrcrbcl. The approximately 910-bp HindIII-HincII restriction fragment containing the 3' end of the *psaB* gene (Dron et al., 1982a) was cloned into plasmid pUC7 to create plasmid pCrcpsaB. The approximately 910 EcoRI-AccI restriction fragment containing the 5' portion of the *atpA* gene (Dron et al., 1982a) was cloned into plasmid pUC19 to create plasmid pCrcatpA. The approximately 700-bp EcoRV-HpaI restriction fragment containing *atpB* protein-coding sequences (Woessner et al., 1986) was cloned into plasmid pUC7 to create plasmid pCrcatpB. The approximately 500-bp EcoRI-PstI restriction fragment, which contains an internal portion of the *tufA* gene (Silk and Wu, 1988) was cloned into Bluescript vector to create plasmid pCrcatpA. The approximately 520-bp DraI restriction fragment, which contains the *rp16* gene (Low et al., 1987) was cloned into pUC7 vector DNA to create plasmid pCrcrp16. Lastly, plasmid pBI221, which contains the approxi-

mately 1.9-kb β -glucuronidase (GUS) structural gene in pUC19 (Jefferson, 1987) was used to detect GUS mRNA transcripts.

For introducing the chimeric GUS genes into the chloroplast, pUC8 vector DNA containing the 5.3-kb BamHI-EcoRI restriction fragment from the *atpB*-encoding chloroplast Bam10 fragment (Woessner et al., 1986) was partially digested with KpnI, and the protruding ends were removed by treatment with T4 DNA polymerase. The polylinker region of the Bluescript vector was liberated from vector sequences by digestion with ApaI and SacII, and the ends were blunted by treatment with T4 DNA polymerase and inserted into the aforementioned KpnI/T4 DNA polymerase-treated *atpB*-containing plasmid. The polylinker fragment was inserted into KpnI site 1 (Blowers et al., 1989) nearest the 3' end of the *atpB* gene such that the NotI restriction site in the polylinker region was closest to the *atpB* gene. This plasmid, designated pCrc20, was the starting vector for all GUS-containing constructs that were destined for bombardment into the chloroplast.

To create plasmid pCrc34, a 224-bp DraI restriction fragment containing the *atpB* promoter region and a portion of the 5'-noncoding sequences (Woessner et al., 1986) was cloned into the HincII site of pUC7 DNA and then removed by digestion with BamHI. This restriction fragment was inserted in the correct orientation into the BamHI site 23 bp upstream of the ATG codon of the GUS gene in plasmid pBI221. The *atpB*-GUS region was removed by digestion with XbaI and SacI, and the protruding ends were treated with T4 DNA polymerase and cloned into the SmaI site of pCrc20 DNA such that the direction of GUS transcription is toward the endogenous *atpB* gene, thereby creating plasmid pCrc27.

The 315-bp TaqI fragment spanning the 3' end of the *rbcl* gene and its transcript was converted to blunt ends by Klenow fill-in DNA synthesis and cloned into the modified NotI site (also blunt ended by a Klenow fill-in reaction) located immediately downstream of the *atpB*-GUS gene in plasmid pCrc27. The orientation of the TaqI fragment was determined so that the coding strand of the 3' end of the *rbcl* transcript was transcribed. This plasmid was designated pCrc34.

To construct plasmids in which the GUS gene was under transcriptional control of either the *atpA* or *rbcl* gene promoter, the 723-bp Sau3AI fragment containing the promoter regions, portions of the 5'-noncoding regions, and intergenic region of the divergently transcribed *atpA* and *rbcl* genes (Dron et al., 1982a) was cloned into the BamHI site of pUC19 vector DNA. This plasmid was redigested with HincII, which cuts near the Sau3AI site in the *atpA* 5'-noncoding region and within the polylinker region of the vector adjacent to the *rbcl* 5'-noncoding sequence. The 709-bp HincII fragment was inserted in both orientations into pCrc34 DNA that had been treated as follows. Plasmid pCrc34 was digested with SmaI and XhoI to remove the *atpB* promoter region in front of the GUS gene. The XhoI site was blunt ended by Klenow fill-in DNA synthesis and the HincII fragment containing the *atpA* and *rbcl* genes' promoters and 5' ends was cloned into the SmaI-XhoI/Klenow-treated pCrc34 vector. In one orientation, the GUS gene is under transcriptional control of the *rbcl* gene promoter (pCrc39), and in the reverse orientation under transcriptional control of the *atpA* gene promoter (pCrc40).

To create a series of deletions into the 5'-upstream sequences of the *atpB* promoter, pCrc34 DNA was digested with XhoI, which linearizes the plasmid near the 5' end of the *atpB* insert. This linear molecule was digested with Bal31 exonuclease and aliquots of the reaction were removed at different timepoints. The ends of

the deletion molecules were blunt ended by Klenow fill-in DNA synthesis and then digested with HincII, which cuts near the 5' end of the GUS gene. Linear fragments containing the *atpB* sequences and attached GUS sequences were cloned into pUC7 vector DNA for DNA sequence analysis to determine the endpoints of deletion. The *atpB*-containing fragments were liberated from the pUC7 vector and GUS sequences by digestion with SmaI (which cuts between *atpB* and GUS) and EcoRI (which cuts in the polylinker region adjacent to the deletion endpoint), gel purified, and cloned into SmaI-digested and EcoRI-digested pCrc34' DNA (the EcoRI site in the pUC8 polylinker region had been previously destroyed by Klenow fill-in DNA synthesis) to create seven plasmids: pCrc41 to pCrc47. Plasmids pCrc42 and pCrc43, along with plasmids pCrc44 and pCrc45, were independently isolated clones that were found to contain the identical deletion fragment (see Figure 7A) and can be considered to be structurally identical.

Enzymes and Chemicals

Radiochemicals ^{32}P -orthophosphate, γ - ^{32}P -ATP (about 3000 Ci/mmol), and α - ^{32}P -dCTP (about 800 Ci/mmol) were from Du Pont-New England Nuclear. Random primers for oligolabeling (Feinberg and Vogelstein, 1983) and deoxynucleotide triphosphates were from Pharmacia LKB Biotechnology Inc. GeneScreen nylon membrane was from Du Pont-New England Nuclear. Restriction enzymes and DNA-modifying enzymes were from Pharmacia and New England Biolabs, and AMV reverse transcriptase was from Promega Biotec.

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