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Studies on *Chlamydomonas* Chloroplast Transformation: Foreign DNA Can Be Stably Maintained in the Chromosome

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As shown originally by Boynton and co-workers (Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B., and Sanford, J.C. [1988]. Science 240, 1534–1538), a nonphotosynthetic, acetate-requiring mutant strain of *Chlamydomonas reinhardtii* with a 2.5-kilobase pair deletion in the chloroplast Bam10 restriction fragment region that removes the 3′ half of the *atp*B gene and a portion of one inverted repeat can be transformed to photosynthetic competency following bombardment with microprojectiles coated with wild-type Bam10 DNA. We have found that assorted other circular plasmids, single-strand DNA circles, or linear, duplex DNA molecules containing the wild-type *atp*B gene can also complement the same mutant. DNA gel blot hybridization analysis of all such transformants indicates that the complementing DNA has integrated into the chromosome at the *atp*B locus and suggests that a copy-correction mechanism operating between the inverted repeats maintains sequence identity in this region. Sequences from the intact inverted repeat may be recruited to restore the incomplete copy when exogenous DNA with only a portion of the deleted sequence is introduced. Furthermore, a foreign, unselected-for, chimeric gene flanked by chloroplast DNA sequences can be integrated and maintained stably in the chloroplast chromosome. The bacterial neomycin phosphotransferase structural gene fused to the maize chloroplast promoter for the large subunit gene of ribulose-1,5-biphosphate carboxylase (*rbc*L) has been integrated into the inverted repeat region of the Bam10 restriction fragment. RNA transcripts that hybridize to the introduced foreign gene have been identified.

INTRODUCTION

The relative ease with which exogenous DNA molecules can be introduced into plant cells via *Agrobacterium*-mediated DNA transfer (for review, see Fraley et al., 1986), electroporation (Fromm et al., 1986), calcium phosphate coprecipitation (Hain et al., 1985; Lörz et al., 1985), or most recently, high-velocity microprojectiles (Klein et al., 1987), has greatly facilitated studies on nuclear gene expression during plant growth and development. However, these methods have not been extended to enable reproducible transfer of foreign DNA into the chloroplasts of higher plants.

Despite the availability of a large amount of DNA sequence data, including the complete sequence of the chloroplast genomes of two plant species (Ohyama et al., 1986; Shinozaki et al., 1986), the mechanisms that govern the expression of chloroplast genes are largely unknown. Numerous studies have shown that specific chloroplast transcript levels are regulated by light (Rodermel and Bogorad, 1985; Sheen and Bogorad, 1985), developmental stage (Berry et al., 1985; Nikolau and Klessig, 1987), and cell type (Jolly et al., 1981; Sheen and Bogorad, 1988). From these and other studies, evidence has accumulated that altered transcription rates (Mullet and Klein, 1987; Deng and Gruissem, 1987) and posttranscriptional mechanisms (Berry et al., 1985, 1986; Klein and Mullet, 1987), including RNA stability (Mullet and Klein, 1987; Deng and Gruissem, 1987) and translational efficiency (Berry et al., 1988), can be important regulatory elements to varying degrees for different plastid genes in an array of plants. In addition, an in vitro transcription system using partially purified maize chloroplast RNA polymerase and supercoiled DNA templates of cloned maize chloroplast genes has shown that some chloroplast genes could be regulated at the level of transcription initiation (Crossland et al., 1984; Stirdivant et al., 1985). Chloroplast in vitro transcription systems from spinach (Gruissem and Zurawski, 1985) and mustard (Link, 1984) have been used to identify prokaryotic-like promoter sequences (Hawley and McClure, 1983) that direct transcription and probably function in vivo as chloroplast promoters. Although these numerous approaches have uncovered important determinants of chloroplast expression, basic studies on chloroplast gene reg-

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ulation would be facilitated greatly by the development of a transformation-proficient in vivo expression system to permit rigorous examination of the molecular events that modulate gene expression in chloroplasts.

Recently, Sanford and co-workers (Klein et al., 1987) have developed a DNA transfer technique that relies upon bombardment of recipient cells with high-velocity tungsten microprojectiles coated with DNA. Using this delivery system, Klein et al. (1988) have measured transient expression of the chloramphenicol acetyltransferase gene in intact maize cells. In addition, Boynton et al. (1988) have reported the stable transformation to photoautotrophism of nonphotosynthetic, acetate-requiring mutants of *Chlamydomonas reinhardtii* with genetic lesions in the chloroplast-encoded *atp*B, *psb*A, and *rbc*L genes by wild-type DNA sequences delivered on microprojectiles. DNA gel blot analysis of photosynthetically active transformants obtained from an *atp*B deletion mutant, designated *ac-u-c-2-21* (Shepherd et al., 1979; Woessner et al., 1984), revealed that the input wild-type Bam10 DNA had integrated into the chromosome at its normal location, presumably by homologous recombination.

The chloroplast of *C. reinhardtii* contains 50 to 80 copies of the approximately 190-kb circular DNA chromosome (Behn and Hermann, 1977; Rochaix, 1978). As in most higher plants, the chromosome includes an inverted repeat region (22 kb) that separates the large and small single-copy regions (for review, see Rochaix, 1987). Nucleic acid sequence analysis of several chloroplast genes has revealed a high degree of conservation between the algal and higher plant genes. As examples, the coding regions of the *ps1*A1 and *ps1*A2 genes (Kuck et al., 1987) show 83 and 84% homology, respectively, to the corresponding *ps1*A1 and *ps1*A2 genes from maize (Fish et al., 1985). Furthermore, the 5′- and 3′-flanking regions of protein-coding genes (Dron et al., 1982; Erickson et al., 1984a) have sequences that closely resemble those thought to play regulatory roles in higher plants.

Taken together, the availability of chloroplast DNA mutants, the ability to correct stably deletions in the *C. reinhardtii* chloroplast genome with introduced homologous DNA, and the high degree of DNA sequence homology to the chloroplast genomes of higher plants suggest that this organism may be well suited as an in vivo model system for detailed genetic and molecular studies of chloroplast gene expression using modified genes. We have undertaken steps to define further the *C. reinhardtii* chloroplast transformation system and to develop it for studying the roles of gene sequences in their expression in vivo. The objectives were: (1) to determine whether fragments shorter than Bam10 (7.6 kb), and thus easier to manipulate, would complement *ac-u-c-2-21*; (2) to achieve higher transformation frequencies by assaying the effectiveness of linear and single-strand DNA molecules in correcting the lesion; (3) to determine whether foreign DNA would be stably integrated; and (4) to determine whether transcripts containing sequences corresponding to those in introduced foreign DNA could be detected.

RESULTS

Subclones of the Bam10 Fragment Can Complement *ac-u-c-2-21*

The nonphotosynthetic, acetate-requiring mutant of *C. reinhardtii*, designated *ac-u-c-2-21* (Shepherd et al., 1979; Woessner et al., 1984), contains a 2.5-kb deletion in the Bam10 fragment that extends from within the 3′ half of the *atp*B gene (encoding the β subunit of the coupling factor 1 complex of the chloroplast ATP synthase) to beyond this gene into the inverted repeat region of the chloroplast chromosome. Previously, Boynton et al. (1988) utilized this mutant as the recipient strain for high-velocity microprojectile transformation of the *C. reinhardtii* chloroplast because of its strong selectable phenotype (restoration of photosynthetic activity) and an undetectable reversion frequency ($<1 \times 10^{-9}$).

Figure 1A presents a diagram of the restriction map of the Bam10 region and flanking DNA (Woessner et al., 1986), including the location of the 2.5-kb deletion in *ac-u-c-2-21* in relation to the *atp*B coding region. Initially, we confirmed the observation of Boynton et al. (1988) that the 7.6-kb Bam10 fragment complements the genetic lesion in *ac-u-c-2-21*. To determine the effectiveness of smaller DNA sequences, the 5.3-kb BamHI-EcoRI subclone of Bam10 (from 2.0 to 7.3 kb in Figure 1A) was tested and was found to complement the *atp*B deletion and restore photosynthetic activity at a frequency about twofold lower than Bam10 DNA (data not shown).

Next, the 2.9-kb EcoRI-KpnI restriction fragment (from 4.4 to 7.3 kb in Figure 1A) in pUC18 (pCrc5) was coated onto tungsten microprojectiles for bombardment into recipient *ac-u-c-2-21* cells. Photosynthetic colonies were obtained at approximately sixfold and 12-fold lower frequencies than observed for the BamHI-EcoRI subclone and Bam10, respectively. As shown in Figure 2, DNA gel blot analysis (Southern, 1975) of the DNA of 10 transformants (lanes c to l) restricted with BamHI and PstI shows the wild-type hybridization pattern of 4.1- and 3.5-kb bands derived from Bam fragment 10 (lane b). Moreover, no 5.1-kb hybridization signal indicative of the mutant Bam10 fragment (ΔBam 10, lane a) is observed in pCrc5 transformants. The restriction fragment length polymorphism observed for the 3.5-kb BamHI-PstI fragment is not unexpected since this fragment contains the 0.77-kb KpnI restriction fragment (between KpnI sites 2 and 3 in Figure 1A) near the end of the inverted repeat that has been shown to be highly variable in length in other *C. reinhardtii* chloroplast transformants (Boynton et al., 1988). Indeed, DNA gel blot analysis of these DNA samples digested with

KpnI confirms the size heterogeneity to be associated exclusively with this restriction fragment (data not shown). The higher molecular weight hybridization signal in each lane represents the Bam9 BamHI-PstI fragment that shares homology with the Bam10 in the inverted repeat region. The size of the Bam9 fragment varies directly with the size of the Bam10 fragment due to the heterogeneity of the 0.77-kb KpnI fragment in Bam9 (data not shown), as has been observed previously (Boynton et al., 1988).

In previous experiments using DNA of Bam10 and the 5.3-kb BamHI-EcoRI subclone for transformation, sequences homologous to those bordering the 2.5-kb chromosomal deletion were available for a double cross-over recombination event to restore the wild-type sequences (Figure 1A). However, only one end of the 2.9-kb EcoRI-KpnI subclone of Bam10 used in this experiment is homologous to DNA in the mutant (Figures 1A and 2). Despite this, DNA gel blot analysis of pCrc5 transformants shows a wild-type hybridization pattern (Figure 2). Inasmuch as

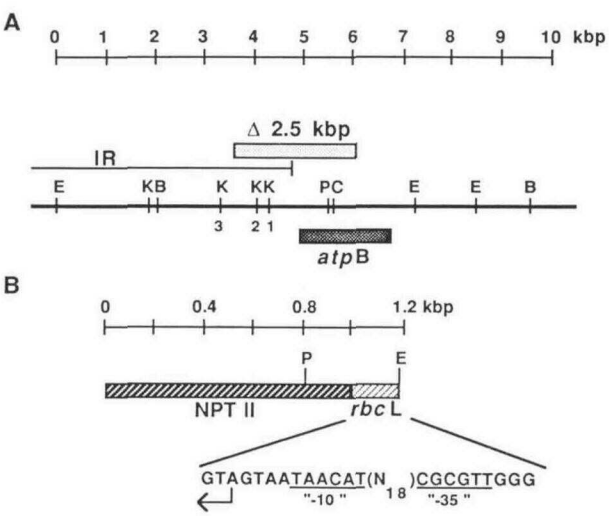

Figure 1. Restriction Map of the *Chlamydomonas* Chloroplast Bam10 Region and the Maize *rbc*L Promoter-NPT II Chimeric Gene.

(A) The 2.5-kb deletion (gray stippled box) in *ac-u-c-2-21* and the *atp*B coding region (black cross-hatched box) are shown above and below the restriction map of the Bam10 region, respectively. The *atp*B gene is transcribed from right to left. The inverted repeat region (IR) is marked by the single, thin line above the restriction map.

(B) The 170-bp maize *rbc*L promoter fragment, including the DNA sequence of its −35 and −10 regions and its transcription initiation site marked by the arrow, is fused to the NPT II structural gene. This construct is inserted into one of three KpnI sites designated 1, 2, and 3 in **(A)** to produce clones pCrc1, pCrc2, and pCrc3, respectively. The direction of NPT II transcription in pCrc2 and pCrc3 is the same as *atp*B, whereas pCrc1 is oriented in the opposite direction. Restriction sites are: B, BamHI; C, ClaI; E, EcoRI; K, KpnI; P, PstI.

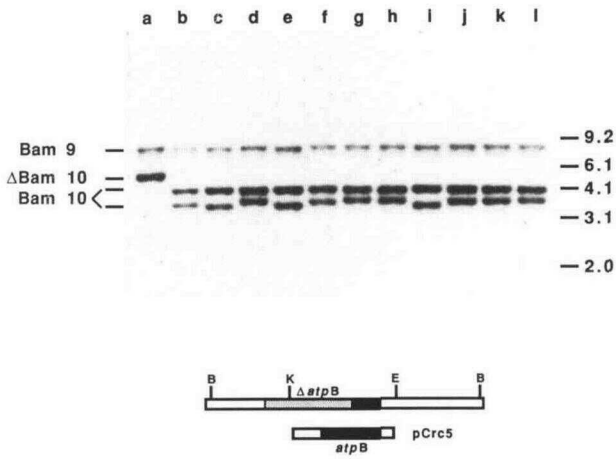

Figure 2. The pCrc5 Transformants Contain a Normal Inverted Repeat Region.

Total cell DNA (0.1 μg) from mutant (lane a), wild-type (lane b), and pCrc5 transformants (lanes c to l) was digested with BamHI and PstI and probed with radiolabeled Bam10 DNA. Molecular sizes (kb) are indicated on the right and the hybridization bands are identified on the left. Bam9 indicates the location of the major DNA fragment remaining after digestion with PstI as well as BamHI. Bam10 identifies the two BamHI-PstI fragments that originate from Bam fragment 10. The 5.1-kb Bam10 fragment of *ac-u-c-2-21* is designated ΔBam10; it lacks the PstI restriction site in the wild-type Bam10 fragment. The diagram at the bottom shows the 2.9-kb KpnI-EcoRI fragment contained in plasmid pCrc5 used for complementation. The *atp*B coding region is shown by the black box and the 2.5-kb deletion is indicated by the gray box on the mutant Bam10 fragment. Note that the *atp*B gene on the mutant Bam10 fragment is truncated and that the left end of the complementing DNA terminates within the deletion region of Bam10. Restriction sites are: B, BamHI; E, EcoRI; K, KpnI.

sequences complementary to those adjacent to the KpnI end in the fragment are found in the opposite inverted repeat, it seems likely that the missing DNA sequences in the Bam10 deletion are restored from the Bam9 region of the opposite inverted repeat, presumably by a recombination-mediated copy-correction mechanism between the inverted repeat sequences of Bam9 and Bam10 (Palmer et al., 1985).

Linear DNA Molecules Can Transform the Chloroplast

Although the exact mechanism whereby the chloroplast sequences of pCrc5 DNA integrate into the chromosome is unknown, the question arises as to whether linear DNA molecules containing free ends might be more recombinogenic and facilitate integration into the chromosome. To this end, pCrc5 plasmid DNA was linearized by digestion

with either EcoRI, KpnI, or ClaI restriction enzymes. Digestion with EcoRI generates a linear DNA molecule with one end composed of chloroplast sequences present in the mutant chromosome and pUC18 vector sequences at the other end. When pCrc5 is digested with KpnI, the chloroplast sequences at the terminus are present in the mutant chromosome only in the inverted repeat as part of the Bam9 fragment. Finally, digestion with ClaI cleaves within the 3′ end of the *atp*B gene and generates a linear DNA molecule whose ends terminate within the *atp*B deletion and have no sequence homology in the chloroplast chromosome.

Photoautotrophic colonies were obtained after cell bombardment with all types of the linearized pCrc5 DNA molecules as well as undigested pCrc5 plasmid DNA. The transformation frequencies with linear DNA molecules digested with KpnI or EcoRI were fourfold and 10-fold greater, respectively, than that observed for uncut pCrc5 DNA. Conversely, ClaI-digested DNA molecules yielded threefold fewer transformants relative to undigested pCrc5 plasmid. Figure 3 shows a DNA gel blot of DNA isolated from these transformants. All transformants (lanes c to n) displayed the wild-type hybridization pattern (lane b), regardless of which chloroplast sequences were at the terminus of the DNA used for transformation. As shown previously (Figure 2), the expected restriction fragment length polymorphisms for the 3.5-kb BamHI-PstI fragment of Bam10 and Bam9 BamHI-PstI fragment are evident. Additional DNA gel blot analysis of these DNA samples restricted with KpnI localized this heterogeneity to the 0.77-kb restriction fragment noted previously (data not shown). In view of the higher transformation frequency observed for EcoRI- and KpnI-digested linear DNA molecules relative to uncut plasmid, it seems unlikely that integration of the transforming DNA was preceded by extensive recircularization in vivo. If recircularization were involved, we would expect to observe more similar transformation frequencies for the different types of transforming DNA. Contrary to such an expectation, we observed a 30-fold difference between EcoRI-digested and ClaI-digested DNA. Our results show clearly that, not only are linear DNA molecules capable of transforming the chloroplast chromosome via integration, but that the free ends of the homologous chloroplast DNA supplied in these experiments stimulate recombination between the input DNA and the chromosomal DNA.

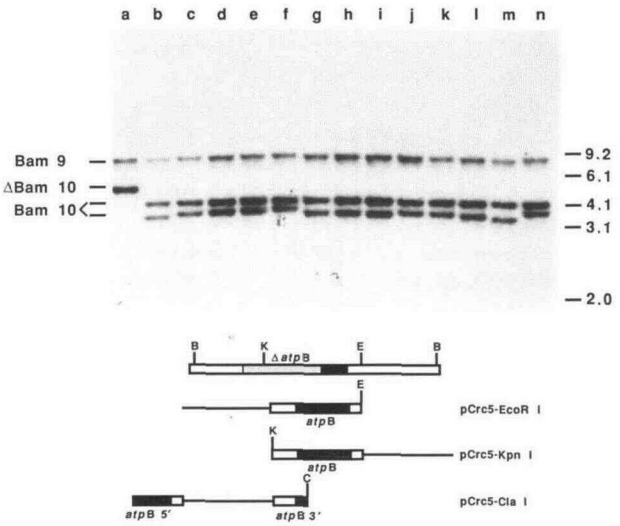

Figure 3. Linear pCrc5 DNA Molecules Can Transform the Chloroplast.

Total cell DNA (0.1 μg) from mutant (lane a), wild-type (lane b), EcoRI-linearized pCrc5 transformants (lanes c and d), ClaI-linearized pCrc5 transformants (lanes e to h), and KpnI-linearized pCrc5 transformants (lanes i to n) was digested with BamHI and PstI and probed with radiolabeled Bam10 DNA. Molecular sizes (kb) are indicated on the right and the hybridization bands are identified on the left as described in the legend of Figure 2. The diagram at the bottom shows the linear pCrc5 DNA molecules used to transform strain *ac-u-c-2-21*. Vector sequences (pUC18) are indicated by the thin black line on the linear DNA molecules. The mutant Bam10 fragment and *atp*B coding region are as shown in Figure 2. Restriction sites are: B, BamHI; C, ClaI; E, EcoRI; K, KpnI.

Single-Strand DNA Molecules Can Transform the Chloroplast

Current evidence indicates that *Agrobacterium*-mediated DNA transfer of T-DNA from the bacterium to the plant cell occurs via a single-strand DNA intermediate (Stachel et al., 1986). With this in mind, the 5.3-kb BamHI-EcoRI subfragment of Bam10 was subcloned into plasmids pTZ18R and pTZ19R to generate single-strand circular DNA molecules designated pCrcss18 and pCrcss19, respectively, containing either strand of the complementing DNA. The transformation frequency for the single-strand DNA molecules was approximately two- to fourfold lower than with the double-strand form of the plasmid and four- to eightfold lower than that observed for the Bam10 fragment in vector pUC19. We do not know whether the reduced transformation frequency results from lower stability of the single-strand DNA during handling or from its fate in vivo.

As shown by DNA gel blot analysis in Figure 4, all 12 transformants examined (lanes c to n) contain *atp*B-hybridizing BamHI-PstI bands of 4.1 and 3.5 kb, similar to those observed for the wild-type DNA (lane b), with the exception, as before, for the polymorphic 3.5-kb fragment and variations in the size of the Bam9 BamHI-PstI fragment. In all DNA samples from single-strand DNA transformants, no hybridization signal corresponding to the mutant 5.1-kb Bam10 fragment is observed (lane a), indicating that all chromosomes were transformed.

Integration of Foreign DNA into the Chloroplast Chromosome

We sought to determine whether foreign DNA could be integrated into the chromosome by embedding a foreign gene within *C. reinhardtii* chloroplast sequences for homologous recombination. A chimeric gene consisting of the promoter for maize chloroplast *rbc*L (large subunit gene of ribulose 1,5-biphosphate carboxylase) fused to the bacterial neomycin phosphotransferase II (NPT II) structural gene was constructed (Figure 1B) and inserted into one of three KpnI sites located on the 5.3-kb BamHI-EcoRI *atp*B fragment (Figure 1A) in pUC8. In pCrc2 and pCrc3, the *rbc*L promoter-NPT II construct was inserted, in the orientation shown, into KpnI sites 2 and 3 (Figure 1A), respectively.

C. reinhardtii ac-u-c-2-21 cells were bombarded with microprojectiles coated with pCrc2 and pCrc3 DNA and photosynthetic transformants were selected. Preliminary slot-blot DNA hybridization analysis of DNA isolated from 24 transformants indicated that 12 of the 24 contained a large number of copies of NPT II DNA. As shown in Figure 5A, DNA gel blot analysis of BamHI-restricted DNA isolated from seven pCrc2 and five pCrc3 high NPT II copy number

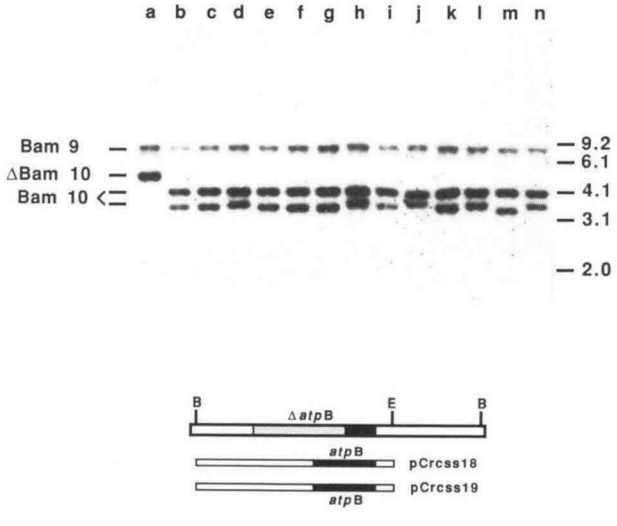

Figure 4. Single-Strand DNA Molecules Can Transform the Chloroplast.

Total cell DNA (0.1 μg) from mutant (lane a), wild-type (lane b), pCrcss18-transformed (lanes c to h), and pCrcss19-transformed (lanes i to n) cells was digested with BamHI and PstI and probed with radiolabeled Bam10 DNA. Molecular sizes (kb) are indicated on the right and the hybridization bands are identified on the left as in Figures 2 and 3. The diagram at the bottom shows the complementing 5.3-kb single-strand DNA. The mutant Bam10 fragment and *atp*B coding region are as shown in Figure 2. Restriction sites are: B, BamHI; E, EcoRI.

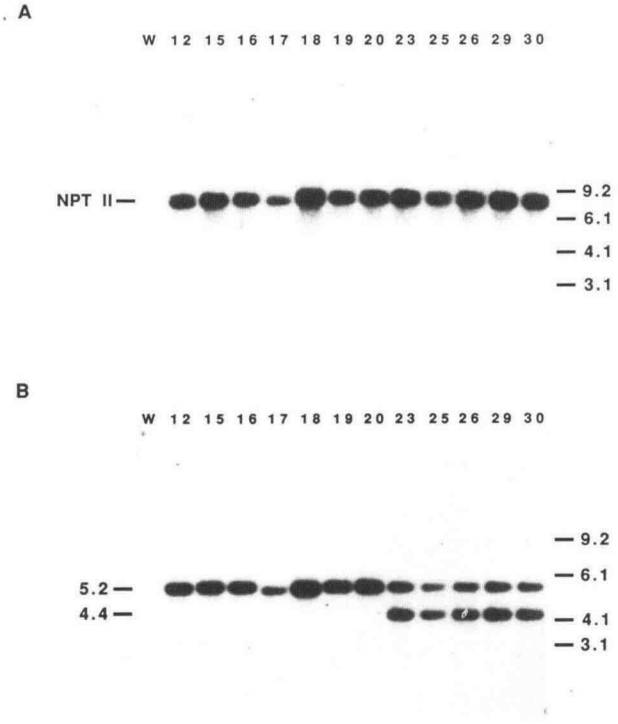

Figure 5. The *rbc*L-NPT II Chimeric Gene Integrates into the Chloroplast Chromosome.

Total cell DNA (0.1 μg) from wild-type cells (lane W), pCrc2 transformants (lanes 12, 15 to 20), and pCrc3 transformants (lanes 23, 25, 26, 29, 30) was digested with either BamHI **(A)** or EcoRI **(B)** and probed with radiolabeled NPT II DNA. The NPT II probe is a 0.9-kb PstI fragment, which extends from the PstI site in the NPT II fragment (Figure 1B) to a second PstI site approximately 130 bp beyond the end of the NPT II insert in Figure 1. Molecular sizes (kb) are indicated on the right and the hybridization bands are identified on the left.

transformants probed with radiolabeled NPT II DNA showed a strong hybridization band of approximately 8.8 kb, the expected size of the Bam10 fragment containing the 1.2-kb *rbc*L-NPT II insert. No NPT II-hybridizing band is evident in the lane containing DNA prepared from the wild-type strain (lane W).

When DNA from the 12 NPT II-containing transformants shown in Figure 5A was restricted with EcoRI (Figure 5B), the 5.2-kb hybridization band (in Figure 1, from the EcoRI site at the end of *rbc*L, through NPT II and Bam10, to the EcoRI site flanking Bam10) expected for pCrc2 transformants was observed. Conversely, all pCrc3 transformants showed two hybridization bands of 5.2 and 4.4 kb, instead of the expected single 4.4-kb band. Each of the pCrc3 hybridization bands is approximately 50% of the intensity of the pCrc2 transformant bands and the two bands in each transformant are about equally strong. Further DNA

gel blot hybridization analysis of these transformants indicates that the *rbc*L-NPT II insert itself has not undergone any DNA rearrangements (data not shown). Isolation and cloning of this region are in progress currently to determine the exact nature of this alteration. All pCrc2 transformants show the expected DNA gel blot hybridization patterns following detailed restriction analysis, indicating that this phenomenon is limited exclusively to pCrc3 transformants (data not shown).

Of the remaining 12 transformants that showed smaller copy numbers of NPT II DNA or lacked it, four showed approximately 10- to 50-fold lower amounts of NPT II DNA relative to the transformants shown in Figure 5 and the remaining eight showed no NPT II hybridization signal (data not shown). The input DNA was integrated properly in all four transformants with reduced NPT II levels (data not shown). Despite the variable number of copies of NPT II DNA in these 12 transformants, all chloroplast chromosomes contained the wild-type *atp*B gene (data not shown).

Chloroplast Transformants Contain NPT II mRNA Transcripts

To determine whether the NPT II DNA was expressed, RNA gel blot hybridization analysis (Thomas, 1980) of total RNA isolated from wild-type and transformed cells was performed and the results are shown in Figure 6. No NPT II-hybridizing RNA is observed in the wild-type RNA sample, but a rather similar complex pattern of NPT II-specific transcripts is observed in all transformants examined. The predominant transcripts are approximately 2.7 to 3.0 kb, much larger than the anticipated 1 kb transcript length, indicating that adjacent, endogenous chloroplast DNA sequences are contained within this transcript. Although the cloning sites for the *rbc*L-NPT II gene are about 0.8 kb apart in pCrc2 and pCrc3, the transcript patterns are virtually identical. Four pCrc1 transformants, in which the *rbc*L-NPT II gene is oriented in the opposite direction of pCrc2 and pCrc3 (Figure 1A), also display the same transcript pattern (data not shown). Taken together, these results indicate that neither location nor orientation of the chimeric gene affects the transcript pattern, suggesting that the NPT II RNA is transcribed as an added part of an endogenous chloroplast transcript from this region. Indeed, DNA sequence analysis has revealed the presence of several potential open reading frames and RNA gel blot hybridization analysis indicates transcripts homologous to this region (J. Erickson, personal communication). It is not obvious immediately why we fail to detect any major NPT II transcripts that initiate from within the maize *rbc*L promoter fragment, especially since the maize promoter's −35 and −10 sequences (Figure 1B) resemble other chloroplast promoters closely. Finally, RNA gel blot hybridization analysis indicates that *atp*B transcripts accumulate to wild-type levels in pCrc2 and pCrc3 transformants (data not shown).

DISCUSSION

Fate of *rbc*L-NPT II DNA on the Chloroplast Chromosome

Conservation in the size, structure, information content, and general organization of chloroplast chromosomes suggests the existence of mechanisms for minimizing duplications as well as the promiscuous incorporation and maintenance of stray DNA sequences. By contrast, substantial segments of chloroplast DNA sequences have been found embedded in maize (Lonsdale et al., 1983) and rice (Moon et al., 1987) mitochondrial chromosomes. However, the present work demonstrates that foreign DNA can be introduced into the chloroplast chromosome of *C. reinhardtii* and be maintained there in high copy number even without selective pressure for retention of the foreign sequences.

The chimeric *rbc*L-NPT II gene, embedded within *C. reinhardtii* chloroplast Bam10 sequences, was introduced on an easily manageable 5.3-kb DNA fragment that was integrated and maintained stably in the chloroplast chromosome even with selection only for *atp*B function. The pCrc1, pCrc2, and pCrc3 transformants have not been found to have increased tolerance to kanamycin-containing media. Despite lack of selection pressure, 67% (16 of 24) of the pCrc2 and pCrc3 transformants examined contained the *rbc*L-NPT II gene integrated into the Bam10 region of the chromosome. This high percentage is even more remarkable considering that, as we have shown, the 2.9-kb EcoRI-KpnI fragment can complement the *atp*B mutation in *ac-u-c-2-21* (Figures 2 and 3), and the transforming pCrc2 and pCrc3 DNAs contained the *rbc*L-NPT II sequence inserted into KpnI sites beyond the region required for complementation.

The chloroplast chromosomes of pCrc2 and pCrc3 transformants differ in their physical structure (Figure 5B). The *rbc*L-NPT II-containing regions together with their flanking chloroplast DNA sequences must be cloned and analyzed to understand why. We do not know whether the differences arise because of local features around the two KpnI sites into which the *rbc*L-NPT II sequences have been cloned or because of the locations of these sites in the inverted repeat regions. Interestingly, the KpnI cloning site for pCrc3 transformants (KpnI site 3 in Figure 1A) lies immediately adjacent to a 12-bp repeat sequence reiterated 16 times in tandem (J. Erickson, personal communication) within the 0.77-kb KpnI restriction fragment (Figure 1A). Boynton et al. (1988) have suggested that these repeats could be the sites of unequal pairing and crossing over between inverted repeat copies, thus giving rise to

the size heterogeneity observed in the 0.77-kb KpnI fragment of Bam9 and Bam10 sequences. Inserts into this region could be affected by or affect the nearby recombinational events.

It was impossible to conclude a priori whether the inverted repeat region would be a hospitable or hostile site for introduced foreign DNA. Would it be more likely to be retained by recopying or eliminated by intramolecular recombination and/or copy-correction events? We have not observed any duplication of the *rbc*L-NPT II gene inserted in Bam10 into the homologous Bam9 sequences in the opposite inverted repeat. Operationally, the ends of the inverted repeats—i.e. the span within which intramolecular recombination presumably occurs regularly—differ among pCrc2 and pCrc3 transformants, various deletion mutants of *C. reinhardtii* (Myers et al., 1982), and different plant species (Palmer, 1985). Introduction of even the short 2.9-kb EcoRI-KpnI fragment into the mutant chromosome restores the inverted repeats to their full length (Figures 2 and 3), but insertion of a construct comprising *rbc*L-NPT II followed by *C. reinhardtii* chloroplast DNA beyond the KpnI site of the EcoRI-KpnI fragment fails to do so. This may be a promising situation for studying sequences that facilitate or block interchanges between repeats.

Intramolecular Recombination between Copies of the Inverted Repeat

From the time of their initial discovery by restriction mapping (Bedbrook and Bogorad, 1976) and electron microscopy (Kolodner and Tewari, 1979), the large inverted repeat segments of chloroplast chromosomes have attracted attention as regions for intramolecular recombination and copy correction. Intramolecular recombination between copies of the inverted repeat has been hypothesized to maintain sequence identity between repeats and to account for the occurrence of symmetrical point (Erickson et al., 1984b) and deletion (Myers et al., 1982) mutations within the inverted repeats of *C. reinhardtii*. It has been suggested (Palmer et al., 1985) that small (100 to 300 bp) repeats scattered throughout the *C. reinhardtii* chloroplast genome (Gelvin and Howell, 1979), including the inverted repeat region (Rochaix and Malnoe, 1978; Palmer et al., 1985), serve as target sites for recombination. A repeat of this class has been identified (Boynton et al., 1988) near KpnI site 1 (Figure 1A) and may provide the region of homology for recombination to occur between this site on input pCrc5 DNA and another site in the inverted repeat of the mutant chromosome. The extent of interactions between the inverted repeats in this study is dramatized by the observation that, although the 2.9-kb KpnI-EcoRI fragment does not contain the entire deletion in *ac-u-c-2-21*, it does restore the chloroplast chromosome to the wild type, presumably by the recruitment and replication of sequences from within Bam9 in the unaltered inverted repeat. Similarly, Boynton et al. (1988) observed that the 7.6-kb Bam10 fragment was able to integrate properly in an *atp*B mutant with a 13-kb deletion that extends from within the *atp*B gene, downstream through Bam10 and the rRNA operon in the inverted repeat. Although it seemed likely, it remained unknown whether the remaining uncomplemented sequences in the inverted repeat had been restored.

In conclusion, the present experiments represent the first steps toward the establishment of a useful in vivo system for studying the roles of chloroplast DNA sequences in gene expression and for identifying any *trans*-acting factors that act on these sequences. Foreign and modified *C. reinhardtii* sequences can be introduced on short, manageable DNA fragments that are integrated and maintained stably. The frequency of obtaining transformants, even with smaller and thus less likely to complement fragments, can be raised by introducing linear DNAs rather than circular plasmids. Selection for transformants can be independent of the introduced gene whose transcription is to be studied because unselected-for sequences are found in 60 to 70% of the transformants. Transcripts of foreign genes are not destroyed; thus, it should be possible to measure transcripts from introduced sequences that accumulate in vivo.

METHODS

Algae and Culture Conditions

Chlamydomonas reinhardtii wild-type strain CC-124(−) and nonphotosynthetic mutant strain CC-373 (*ac-u-c-2-21*) were obtained from the *Chlamydomonas* Genetics Center, Durham, NC and maintained on HS (minimal) and HSHA (acetate) culture media (Sueoka, 1960).

Plasmids

Plasmid P-17 DNA containing the wild-type Bam10 chloroplast DNA restriction fragment in pBR313 and the 5.3-kb BamHI-EcoRI and 2.9-kb KpnI-EcoRI subclones in vectors pUC8 and pUC18, respectively, were obtained from Drs. John Boynton, Nicholas Gillham, and Elizabeth Harris, Duke University. To construct pCrcss18 and pCrcss19, the 5.3-kb BamHI-EcoRI restriction fragment was cloned into the BamHI and EcoRI sites of plasmids pTZ18R and pTZ19R (Pharmacia LKB Biotechnology Inc.), respectively. Single-strand DNA was prepared according to the manufacturer's instructions.

The 170-bp HincII fragment containing the maize chloroplast *rbc*L promoter region and transcription initiation site was isolated from pZmc460 DNA (Larrinua et al., 1983) and BglII linkers were added for cloning into the BglII site in the nontranslated region of the 1.5-kb BglII-BamHI NPT II fragment (Beck et al., 1982). EcoRI linkers were added for cloning into pUC19 DNA. A 1.2-kb *rbc*L-NPT II fragment was isolated following digestion with SmaI, which cuts within the polylinker region of pUC19 and within the 3′-

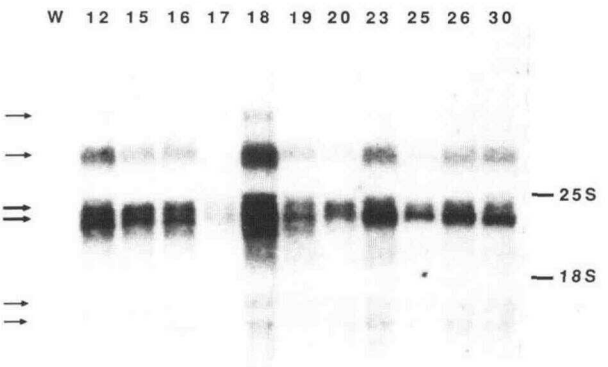

Figure 6. RNA Gel Blot Analysis of NPT II Transcripts in pCrc2 and pCrc3 Transformants.

Total RNA (10 μg) from wild-type cells (lane W), pCrc2 transformants (lanes 12, 15 to 20), and pCrc3 transformants (lanes 23, 25, 26, 30) was probed with NPT II DNA. The relative positions of the nuclear 25S and 18S rRNA bands are indicated on the right. The predominant mRNA transcripts are identified on the left by the thick black arrows and the less abundant RNA transcripts are indicated by the thin black arrows.

flanking region of the NPT II gene. This DNA was then cloned into the 5.3-kb BamHI-EcoRI fragment in pUC8, which had been digested partially with KpnI, and the protruding ends were removed by treatment with T4 DNA polymerase to produce plasmids pCrc1, pCrc2, and pCrc3.

Chlamydomonas Chloroplast Transformation Procedure

Bombardment of *Chlamydomonas* cells was carried out essentially as described by Boynton et al. (1988). Cells of the nonphotosynthetic mutant strain *ac-u-c-2-21* were grown at 26°C in permissive HSHA media (Sueoka, 1960) under dim light (3 $\mu E/m^2 \cdot sec^{-1}$) to late-log density, and used to innoculate 300-ml HSHA cultures that were grown under identical conditions with vigorous shaking for 4 d. Cells were harvested at a density of 1.5 to 2×10^6 cells/ml and resuspended in HSHA media at a density of 1×10^9 cells/ml. A 0.1-ml aliquot of cells was added to 0.9 ml of 0.11% soft agar (Difco) in HSHA media cooled to 45°C and mixed gently, and 0.2-ml aliquots containing 2×10^7 cells were plated onto 9 × 50 mm Petri dishes of 4-d-old HSHA media and swirled uniformly over the surface of the media. These cells were transported immediately to the New York State Agricultural Experiment Station for bombardment and were returned the next day. Bombardment was performed with 1.2-μm tungsten microparticles coated with the appropriate DNA and fired (gunpowder charge No. 1) onto a plate of cells at a distance of 15 cm. Upon return to our laboratory, bombarded cells were resuspended in 0.7 ml of HS media and spread onto 100-mm dishes of minimal HS media and incubated at 26°C under bright light (100 $\mu E/m^2 \cdot sec^{-1}$). Photosynthetically competent cells were picked and maintained on solid HS medium in bright light (250 $\mu E/m^2 \cdot sec^{-1}$). Transformation frequencies were based upon the average number of colonies observed per plate (seven to 10 replicates per transforming DNA species).

DNA and RNA Isolation

Total cellular DNA and RNA were prepared from 50-ml HS cultures shaken vigorously in bright light (250 $\mu E/m^2 \cdot sec^{-1}$). DNA was prepared essentially as described by Dellaporta et al. (1983), and total RNA was isolated as described by Merchant and Bogorad (1986).

DNA and RNA Hybridization Analyses

For DNA gel blot hybridizations (Southern, 1975), DNA was restricted and electrophoresed through 0.8% TBE-agarose gels (Maniatis et al., 1982) and transferred to Gene-Screen (Du Pont-New England Nuclear) nylon membranes. Gel-purified DNA restriction fragments were radioactively oligolabeled (Feinberg and Vogelstein, 1983) with ^{32}P (1 to 2×10^9 cpm/μg of DNA) and added to the hybridization buffer at a concentration of 1×10^6 cpm/ml. Hybridization and washing conditions are described elsewhere (Church and Gilbert, 1984). For RNA hybridizations, samples of total RNA were denatured and electrophoresed through a 4-morpholinepropanesulfonic-formaldehyde 1.2% agarose gel (Thomas, 1980) and transferred to Gene-Screen nylon membrane. Hybridization and washing were performed as described for DNA gel blot hybridizations. All filters were exposed to Kodak XAR-5 film with an intensifying screen (Dupont Cronex Lightning Plus II) at −80°C.

Enzymes and Chemicals

Restriction enzymes were from New England Biolabs and Pharmacia and T4 DNA ligase and T4 DNA polymerase were from New England Biolabs. Deoxynucleotide triphosphates were from PL Biochemicals and α-^{32}P-dCTP (~800Ci/mmol) was from New England Nuclear. Random primers for oligolabeling were from Pharmacia.

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