Creation of a Chloroplast Microsatellite Reporter for Detection of Replication Slippage in *Chlamydomonas reinhardtii*\(^\text{†}^{\text{†}}\)

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Microsatellites are composed of short tandem direct repeats; deletions or duplications of those repeats through the process of replication slippage result in microsatellite instability relative to other genomic loci. Variation in repeat number occurs so frequently that microsatellites can be used for genotyping and forensic analysis. However, an accurate assessment of the rates of change can be difficult because the presence of many repeats makes it difficult to determine whether changes have occurred through single or multiple events. The current study was undertaken to experimentally assess the rates of replication slippage that occur in vivo in the chloroplast DNA of *Chlamydomonas reinhardtii*. A reporter construct was created in which a stretch of AAAG repeats was inserted into a functional gene to allow changes to be observed when they occurred at the synthetic microsatellite. Restoration of the reading frame occurred through replication slippage in 15 of every million viable cells. Since only one-third of the potential insertion/deletion events would restore the reading frame, the frequency of change could be deduced to be 4.5 \times 10^{-5}. Analysis of the slippage events showed that template slippage was the primary event, resulting in deletions rather than duplications. These findings contrasted with events observed in *Escherichia coli* during maintenance of the plasmid, where duplications were the rule.

Microsatellite sequences, also called simple sequence repeats (SSRs), are short tandem DNA repeats, 1 to 6 bases long, commonly found in the genomes of eukaryotes and some prokaryotes (4, 6, 12, 40). These repeats, which are present in both the noncoding and coding regions of genomes, are unstable, undergoing additions or deletions of one or more repeat units, leading to variations in the length of the microsatellite (17, 35, 38). In most cases, the insertions or deletions (indels) occur as a consequence of slippage of the template or daughter strand at the replication fork (16, 18). Although a similar indel could theoretically occur through intra- or intermolecular recombination, homologous recombination does not appear to be involved in microsatellite variability, since the variation is independent of homologous recombination factors, such as the RecA protein (18).

Because of their abundance and variability, microsatellite loci have been used extensively as genetic markers in evolutionary and ecological studies of natural populations in eukaryotes (14, 37) and also as highly polymorphic markers for forensics and genotyping of animals (39). Well-saturated microsatellite maps have been developed for nuclear genomes from a number of plants, including rice, maize, barley, *Arabidopsis*, and soybean (14, 23). In some cases, nuclear microsatellite instability is remarkably high: in chickpea, indels were found at an average rate of 2.9 \times 10^{-3} to 10 \times 10^{-3} per (TAA)\(_n\) locus per generation (36). An even higher rate of somatic instability was found for mononucleotide microsatellites in reporter genes in *Arabidopsis thaliana*, with individual leaves having many sectors (2, 3).

Microsatellites and larger tandem repeats have also been identified in all chloroplast genomes from which sequence data are available (reviewed in reference 25), including the unicellular green alga *Chlamydomonas reinhardtii* (20). In barley, rice, and pine, chloroplast microsatellites have been used to reveal much higher levels of diversity than can be observed through traditional chloroplast restriction fragment length polymorphism analysis (reviewed in reference 25). From their observations of sequence data from *Pinus* species, Provan et al. (24) concluded that SSR length polymorphisms occur in chloroplast DNA (cpDNA) at frequencies of 3 \times 10^{-5} to 8 \times 10^{-5} per site per year.

The aforementioned studies provide a useful starting point for assessing microsatellite variability, but interpretation of SSR data based solely on an evolutionary context is problematic, because single changes can easily involve multiple repeats. Because the repeats are identical, independent deletions or duplications can result in the same DNA variations. Although such “synapomorphies” occur in parallel, they would be scored as arising from a single event. With a goal of providing an accurate assessment of the rate of chloroplast microsatellite variability, we created an experimental system that would allow us to monitor change in a microsatellite reporter in the cpDNA of the green alga *C. reinhardtii*. Since such mutational analyses require data from a large number of individuals, the algal system was chosen over a higher plant due to the ease in handling large populations in a limited space. In addition, the easy transformability and selection procedures for *Chlamydomonas* cpDNA have made it a uniquely accessible system for such a study. To create the microsatellite slippage reporter, a stretch of repeats was introduced into a cpDNA gene essential for photosynthesis (*rbcL*) so that it created a disruption in the

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reading frame. Restoration of photosynthetic competence could be achieved only by duplication or deletion events.

**MATERIALS AND METHODS**

*Chlamydomonas* strains, media, and growth conditions. The *C. reinhardtii* strain used for biotic transformation and all replication slippage experiments was the wild-type CC-125 (mating type plus [mt+]). For crosses, the two strains CC-67 (mt-; cpDNA with erythromycin resistance) and CC-3455 (mt+; cpDNA with a dominant-negative mutation of the *Escherichia coli* recA) were used as parents at two different stages in the experiments. Strain 18-7G, which carries an *rbcL* mutation (34), was used for growth comparisons. All of the strains were obtained from the *Chlamydomonas* Culture Collection when it was located at Duke University. *C. reinhardtii* was grown at room temperature (22 to 25°C) in Tris-acetate-phosphate (TAP) medium (10), either on solid plates or in liquid medium. The liquid cultures were grown on a rotary shaker at a speed of ~200 rpm under 245-mol per m² per s photosynthetic photon flux (245-PPF) containing “high” light or at about 10 PPF for “low-light” conditions. Growth curves were plotted from hemocytometer counts (13) of aliquots removed from 50-ml cultures grown in TAP medium lacking acetate (TMP). When testing for photosynthetic competence on plates, either TMP or HS (10) medium was used, with cultures grown in TAP medium lacking acetate (either HS agar [10] or TMP agar) to test for photosynthetic competence. The plates were placed under lights (~40 PPF) on a shelf at room temperature.

**DNA isolation, PCR amplification, and sequencing.** *Chlamydomonas* total genomic DNA was prepared as described previously (11). The *rbcL* (5'-GGC CTTTCTATGCTGACTG-3') and *rbcLmid* (5'-CCGGAGATTTAATTTAGA-3') were used as the PCR amplification reactions as the forward and reverse primers, respectively, to amplify a 560-bp segment (including the microsatellite insertion site) from the *rbcL* gene of the wild-type, OF, IF, and revertant colonies. In some experiments, an ~180-bp PCR product was generated using a different set of primers, *rbcLfor2* (5'-CTAGGAAATCAAGTGTTAG-3') and *rbcLrev* (5'-CCGGAGATTTAATTTAGA-3'). Five-μl aliquots of each PCR product were used for direct sequencing. Amplification reactions were performed with a minicycler model PTC-150-16 (MR Research Inc., Watertown, MA). The PCR-amplified products were separated on 1.5% agarose gels in Tris-borate-EDTA buffer for the 560-bp and 180-bp products, respectively.

To check for the presence of the *E. coli* Δ*recA* gene, the *recA* locus was amplified using PCR primers, *rbcLfor2* and *rbcLrev* (5'-GGC AGATTTAATTTAGA-3'), in order to readily visualize changes in the repeat number (Fig. 2). DNA (1 to 5 ng) was added to individual 100-μl reaction mixtures containing 200 μl of each dideoxynucleotide, 2.5 units of Taq polymerase (Gibco BRL), 10 pmol of each primer, and 1× PCR buffer (Gibco BRL). Conditions for the 30 cycles of PCR were as follows: denaturation at 95°C for 2 min and 85°C for 5 min, followed by 94°C for 40 s, 30 s at 48°C to anneal the primers with the template, and 30 s at 72°C for DNA extension. A final extension was done for 10 min at 72°C. All of the PCR products showed a paired oligonucleotide band. A 2-kb fragment (including the microsatellite insertion site) from the *rbcL* gene of the wild-type, OF, IF, and revertant colonies was amplified using PCR primers, *rbcLfor2* and *rbcLrev* (5'-GGC AGATTTAATTTAGA-3'), in order to readily visualize changes in the repeat number (Fig. 2). DNA (1 to 5 ng) was added to individual 100-μl reaction mixtures containing 200 μl of each dideoxynucleotide, 2.5 units of Taq polymerase (Gibco BRL), 10 pmol of each primer, and 1× PCR buffer (Gibco BRL). Conditions for the 30 cycles of PCR were as follows: denaturation at 95°C for 2 min and 85°C for 5 min, followed by 94°C for 40 s, 30 s at 48°C to anneal the primers with the template, and 30 s at 72°C for DNA extension. A final extension was done for 10 min at 72°C. All of the PCR products showed a paired oligonucleotide band. A 2-kb fragment (including the microsatellite insertion site) from the *rbcL* gene of the wild-type, OF, IF, and revertant colonies was amplified using PCR primers, *rbcLfor2* and *rbcLrev* (5'-GGC AGATTTAATTTAGA-3'), in order to readily visualize changes in the repeat number (Fig. 2). DNA (1 to 5 ng) was added to individual 100-μl reaction mixtures containing 200 μl of each dideoxynucleotide, 2.5 units of Taq polymerase (Gibco BRL), 10 pmol of each primer, and 1× PCR buffer (Gibco BRL). Conditions for the 30 cycles of PCR were as follows: denaturation at 95°C for 2 min and 85°C for 5 min, followed by 94°C for 40 s, 30 s at 48°C to anneal the primers with the template, and 30 s at 72°C for DNA extension. A final extension was done for 10 min at 72°C. All of the PCR products showed a paired oligonucleotide band.

**Assay for changes in the microsatellite reporter in cpDNA of *C. reinhardtii*.** The cells with the out-of-frame microsatellite (OF28) were grown in liquid TAP cultures to late log phase (2 × 10^6 to 4 × 10^6 cells/ml) on a rotary shaker for about 7 days at room temperature in low light. Cell counts were determined with a hemocytometer. The cells were concentrated about 10-fold by centrifugation. An aliquot of the concentrated cells was used for serial dilution onto agar-solidified TAP and was kept on low-light shelves to determine the number of viable cells plated. The remaining cells were plated as 500-μl aliquots on medium lacking acetate (either HS agar [10] or TMP agar) to test for photosynthetic competence. The plates were placed under lights (~40 PPF) on a shelf at room temperature.

**FIG. 1.** Diagram of the cpDNA segment in the transforming plasmids pfF24 and pOF28. The shaded boxes represent the chloroplast-borne genes of *C. reinhardtii*, and the arrows indicate the directions of their transcription. Two triangles represent the insertions in the transforming plasmid construct: the aadA cassette is the selectable marker that was inserted between two chloroplast genes; the IF24 (24-bp) and OF28 (28-bp) SSRs were inserted at the sixth codon position of the *rbcL* gene in plasmids pfF24 and pOF28, respectively. The enzymes used for cloning are indicated. The size of the reporter construct was 8.1 kb, including the pUC8 vector.
template, and 2 min at 68°C for DNA extension. The final extension was as described above. The PCR products were run on 1% agarose gels.

PCR products from the rbcL5-rbcLmid amplification were submitted for sequencing by the Michigan State University Genomics Technology and Sequencing Facility (http://www.genomics.msu.edu) using the internal primer rbcLfor2. Sequence analyses used Megalign Software from the DNAStar Program (Madison, WI).

Mutation rate. The microsatellite mutation rate was determined by averaging the frequencies of reversion to photosynthetic competence on parallel plates in two separate experiments and dividing the number of revertants by the number of viable cells calculated to have been spread on each plate.

Reconstruction experiment. Logarithmically growing cultures of two nonphotosynthetic strains, the OF28 reporter construct and the 18-7G rbcL point mutant, were diluted to $1 \times 10^7$ cells/ml, as were two photosynthetically competent strains, CC-124 and the IF24 control line. All four strains were taken through a 10-fold dilution series for individual platings on TAP and TMP media. From the dilutions of CC-124 and IF24 that should have had 100, 1,000, and 10,000 cells/ml, 20 μl was added to 2-ml aliquots of the nonphotosynthetic cell lines ($1 \times 10^7$ cells/ml), and two 500-μl aliquots were plated on TMP medium and placed in the light.

RESULTS

Introduction of replication slippage substrates into the cpDNA of C. reinhardtii. Wild-type C. reinhardtii cells (CC-125) were transformed biolistically with the plasmids pIF24 and pOF28 (Fig. 1) containing the AAAG repeats in numbers appropriate to give an “in-frame” (24-bp) or “out-of-frame” (28-bp) insertion. The transformants were selected based on their resistance to spectinomycin conferred by the presence of the aadA cassette in the transforming plasmids. Several rounds of subcloning were required to obtain homoplasmic strains, as monitored by PCR amplification. The compositions of the original inserts were verified by sequencing (Fig. 3). In the OF28 line, a premature stop codon in the rbcL gene makes the cells nonphotosynthetic. The IF24 line has a six-codon insertion relative to the wild type. IF12 and IF36 are two photosynthetically competent lines recovered from the initial OF28 line.

Test for photosynthetic competence in the transformed cells containing the reporter constructs. To assess their photosynthetic competence, the in-frame and out-of-frame constructs were inoculated into liquid TAP medium and placed on shakers under low-light and high-light conditions. For comparison, wild-type CC-125 cells and the rbcL mutant 18-7G were grown as controls. As shown in Fig. 4, the wild-type CC-125 and IF24 cells grew well, showing that they were photosynthetically competent. In contrast, the cells of the 18-7G and OF28 strains were light sensitive and did not grow under these conditions. In low light, the cultures all showed similar growth properties (data not shown), growing steadily, although more slowly than the CC-125 and IF24 cultures did in high light.

Rates of mutation at the AAAG n microsatellites in the cpDNA of the OF28 cells. Procedures for determining the cell viability and mutation frequency are described in Materials and Methods. The number of viable cells plated was determined from dilution plating of the OF cells on a nonselective medium. In order to monitor the replication slippage events at the AAAG repeats in the rbcL gene, the OF cells were plated on media that selected for restored photosynthetic competence. In 3 to 5 days, the bright-green color of the cells faded and the lawn of cells appeared bleached. One would have

FIG. 3. Sequences and amino acid predictions of the rbcL replication slippage constructs. The wild-type rbcL gene is shown at the top, with boldface characters indicating the 4 bases of the 3’ overhang at the BspMI cut site. The OF28 line has a 28-bp insertion in its rbcL gene, creating a stop codon that renders the cells nonphotosynthetic. The IF24 line has a six-codon insertion relative to the wild type. IF12 and IF36 are two photosynthetically competent lines recovered from the initial OF28 line.

FIG. 4. Growth curves. Cultures were inoculated at $10^4$ cells/ml, and cell densities were determined over a 6-day period. The out-of-frame (OF28) and in-frame (IF24) constructs were present in each of two independent transformants. CC-125 is a wild-type control, and 18-7G is an rbcL mutant.
thought the cells were dead, but in 4 to 6 weeks, small green colonies appeared. As shown in Table 1, each plate of approximately 50 million viable cells gave rise to hundreds of photosynthetic revertants, with an average frequency of $1.5 \times 10^{-5}$. These colonies were transferred with sterile toothpicks to fresh HS-plus-spectinomycin plates for maintenance. As described below, all of the colonies arose due to indels in the (AAAG)$_n$ microsatellite region.

**Reconstruction experiment.** Because colonies were not seen on the HS medium until many weeks after being plated, we believed that the replication slippage events had not occurred during the growth of the liquid culture or at least had not sorted out prior to the plating. To test this interpretation, a mixing experiment was performed, with small numbers of photosynthetically competent cells (CC-124 and IF24) being added to nonphotosynthetic cells (OF28 and 18-7G) prior to plating them on medium lacking a carbon source. Control platings of the nonphotosynthetic cells alone were observed with a dissecting microscope over several days. Most cells divided into clusters of four before arresting. In 3 to 4 days, the cell lawn had bleached, but after 5 days, small colonies were visible from the wild-type CC-124 cells, and after 8 days, small colonies were visible on the plates with the IF24 cells. Table 2 shows data from the experiment, collected from two plates for each combination.

**Molecular analysis of slippage events at the (AAAG)$_n$ microsatellite site.** Since the OF28 microsatellite reporter construct consisted of a 28-bp insertion containing a stretch of (AAAG)$_n$, repeats, a loss of one repeat unit and an insertion of a 36-bp in-frame variant (Fig. 3, lower line). Like the deletion product (data not shown). One subsequent experiment yielded a 36-bp in-frame variant (Fig. 3, lower line). Like the deletion variants, it was photosynthetically competent but grew more slowly than the wild type.

**Rates of change at the microsatellite reporter in recombination-deficient chloroplasts.** In theory, microsatellites containing short direct repeats could undergo intra- or intermolecular events with restored photosynthetic competence was chosen from three independent experiments to observe the types of slippage that had occurred and also to compare their respective frequencies of occurrence using PCR to visualize the microsatellite. As shown in Fig. 5, the amplification product (141 bp) from the wild-type C. reinhardtii cells. Sequencing showed that the two classes of variants had experienced deletions of one or four copies of the 4-bp repeat (Fig. 3); there were 58 of each type of deletion. One variant had a product larger than the PCR fragment from the OF28 cells. In that cell line, the presence of two PCR products (Fig. 6, lane 3) indicated that the cells were heteroplasmic: the upper band shows a larger product than the OF28 PCR product, and the lower band shows a slightly smaller fragment than the 169-bp fragment seen in OF28 cells. Most likely, this condition reflects a recombination event, with the two products representing reciprocal recombinant molecules. In later experiments, a few other colonies were heteroplasmic, but they contained the original-size microsatellite tract, as well as a deleted product (data not shown). One subsequent experiment yielded a 36-bp in-frame variant (Fig. 3, lower line). Like the deletion variants, it was photosynthetically competent but grew more slowly than the wild type.

**FIG. 5. Slippage events at the cpDNA microsatellite reporter.** Shown are examples of the PCR-amplified rbcL segment from the original out-of-frame insertion strain (OF28 [OF]), the wild-type C. reinhardtii strain (WT), and several photosynthetically competent colonies derived from the OF28 strain (1 to 3), using primers rbcLfor2 and rbcLrev. Lane M contains a 123-bp DNA ladder (Invitrogen).

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>No. of plates</th>
<th>No. of viable cells/plate</th>
<th>No. of PS* colonies per plate</th>
<th>Mean no. of PS colonies per plate</th>
<th>Median no. of PS colonies per plate</th>
<th>SD for no. of colonies per plate</th>
<th>Mutation frequency (per 10$^7$ viable cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>$4.6 \times 10^7$</td>
<td>381–843</td>
<td>675</td>
<td>749</td>
<td>151</td>
<td>147</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>$7 \times 10^7$</td>
<td>958–1,215</td>
<td>1,057</td>
<td>1,050</td>
<td>76</td>
<td>151</td>
</tr>
</tbody>
</table>

* PS, photosynthetic.

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**TABLE 2. Mixing experiment**

<table>
<thead>
<tr>
<th>Expected no. of colonies of photosynthetic strain</th>
<th>Observed no. of green colonies in bleached lawn of cells from strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18-7G</td>
</tr>
<tr>
<td></td>
<td>CC-124  IF24</td>
</tr>
<tr>
<td>50</td>
<td>131 ± 7 53 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>16 ± 5 6 ± 1</td>
</tr>
<tr>
<td>0.5</td>
<td>3 ± 1 2 ± 2</td>
</tr>
</tbody>
</table>

* Aliquots from a 10-fold dilution series of the photosynthetically competent lines CC-124 (wild type) or IF24 (8-codon insertion in rbcL) were added to nonphotosynthetic cells ($1 \times 10^6$ cells/ml) carrying a point mutation in rbcL (18-7G) or an out-of-frame insertion (OF28). The expected colony numbers are based on hemocytometer cell counts. Photosynthetic colonies were scored after 5 to 8 days.

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**TABLE 1. Frequency of mutation of microsatellite reporter**

<table>
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<tr>
<th>Expt no.</th>
<th>No. of plates</th>
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<th>SD for no. of colonies per plate</th>
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<td>76</td>
<td>151</td>
</tr>
</tbody>
</table>

* PS, photosynthetic.
E. coli cpDNA microsatellite reporter and the combined the OF28 reporter construct with a microsatellite variability in this study. To test this deduction, we combined the OF28 construct with a ΔN recA construct (7) by classical crossing procedures. The E. coli ΔN recA gene has been shown to inhibit chloroplast recombination when inserted into cpDNA (7). Our first assessment tested the presence and activity of the ΔN recA gene in the original strain, CC-3455, by PCR amplification using appropriate primers to visualize the gene (described in Materials and Methods) and then by confirming the strain’s hypersensitivity to 5 mM 5-fluorodeoxyuridine relative to wild-type cells, as described previously (7). This line was crossed to an mt− cell line containing the slippage construct with the linked adaA cassette. The spectinomycin resistance conferred by the adaA gene was used to select biparental progeny from which the desired recombinant could be isolated. Initially, very few biparental progeny were recovered, so the mt− gametes were exposed to a handheld UV lamp for 2 min prior to mating, and we were able to obtain 5.8% biparental zygotes.

From dissected meiotic progeny, spectinomycin-resistant cells were selected and checked for the presence of both the ΔN recA gene and the slippage construct in the cpDNAs by PCR amplification using the recAΔN1/recAΔ3end and rbcLfor2/rbcLrev primer pairs, respectively. As represented by the meiotic progeny analyzed in Fig. 7B, about half of the biparental meiotic progeny (12 of 27) carried the ΔN recA gene. In contrast, all the progeny that showed resistance to spectinomycin also contained the 28-bp out-of-frame insertion in the closely linked rbcL gene (Fig. 7A). Two meiotic progeny that contained both the slippage construct and the ΔN recA gene were chosen for further experiments. Since the presence of ΔN recA should reduce chloroplast recombination, we tested the cell lines for the frequency of restoration of photosynthetic competence due to insertion/deletion events at the reporter gene. As shown in Table 3, the recovery of photosynthetically competent colonies from the slippage reporter in a recombination-deficient background occurred at a frequency of Φ.5 × 10−5, a rate that is approximately three times higher than that of the recombination-proficient lines.

**Table 3. Rates of spontaneous replication slippage events in the cpDNA of C. reinhardtii containing the OF28 construct in a recombination-deficient background.**

<table>
<thead>
<tr>
<th>ΔN recA</th>
<th>Total no. of viable cells plated (10^6)</th>
<th>No. of cells that showed photosynthetic competence</th>
<th>Rate of mutation to photosynthetic competence per 10^7 viable cells plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>3,360</td>
<td>517</td>
</tr>
<tr>
<td>2</td>
<td>104.2</td>
<td>5,640</td>
<td>541</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Although the existence of microsatellites and their variability have been well documented in the chloroplast genomes of many plant species, few studies have observed their rates of change and the mechanics of the process in vivo. Phylogenetic comparisons have determined the relative rates of indels at cpDNA microsatellites in an evolutionary context, and results have indicated that the chloroplast DNA has a lower mutation rate for microsatellites than does the nuclear DNA (24, 38). Some evolutionary studies have indicated that indels at cpDNA microsatellites occur at a much higher rate than do base substitutions (8, 24, 29), but other assessments have concluded that indels are less frequent (30, 32). In fact, several traits intrinsic to microsatellites make it difficult for biosystematic comparisons to accurately quantify their variability: deletion or duplication of the same number of repeats could occur independently in two plant lines, causing homoplasy; in addition, multiple sequential changes in the copy number of a repeat within one plant line could be scored as only a single change, thereby distorting their relative frequencies of occur-

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**FIG. 6.** Microsatellite changes in six photosynthetic colonies (1 to 6) isolated from the OF28 cell line. See the legend to Fig. 5 for abbreviations. The PCR products in lane 3 are 4 bp larger and 4 bp smaller than the original OF28 insert.

**FIG. 7.** PCR analysis to identify meiotic progeny carrying both the cpDNA microsatellite reporter and the E. coli ΔN42RecA gene. (A) PCR screening of spectinomycin-resistant progeny for the microsatellite reporter. PCR products from 13 progeny (1 to 13) and the wild type (WT) were amplified with the rbcL5 and rbcLmid primers to allow visualization of the region containing the microsatellite. (B) PCR products indicating the presence of the ΔN42RecA gene. The lanes are labeled as in panel A. The faint lower band is derived from excess oligonucleotides in the reaction mixture.
of the cells. A change in the microsatellite that reestablishes the reading frame should produce a dominant negative allele, some cells in the colony had become photosynthetically competent due to indels within the microsatellite. Hence, we were unable to quantify the rate of mononucleotide repeat variation, but it must be several orders of magnitude higher than for the 4-bp microsatellite.

In our PCR screens of a subset of the photosynthetic revertants of the AAAG microsatellite, only one colony appeared to contain reciprocal-recombination products (Fig. 5). Hence, replication slippage seemed to be responsible for the production of the microsatellite indels within the chloroplast, as is true for other biological systems. However, to more thoroughly assess the contribution of recombination to microsatellite variability, the microsatellite reporter was combined with a chloroplast-located dominant-negative allele of the E. coli recA gene. To our surprise, the frequency of variation resulting in photosynthetically competent colonies in the recombination-deficient strain was about three times higher than in the recombination-proficient lines (Table 3). Since slippage that restores the rbcL reading frame should produce a dominant allele, we believe that this result indicates that the majority of these mutations are eliminated through recombination (gene

![PCR products from E. coli subclones carrying the pOF28 plasmid. The subclones were randomly selected; DNA was extracted and amplified with oligonucleotides rbcLfor2 and rbcLrev. Lane M contains the 123-bp DNA ladder.](https://example.com/fig8.jpg)
conversion). Reducing recombination among the highly polyploid cpDNAs would enhance the survival and recovery of the revertant alleles.

With one exception, the slipped colonies that we recovered experienced deletion of one or four repeat units; the two deletion types occurred with equal frequencies. In one later experiment, we recovered a two-repeat-unit duplication that restored photosynthetic competence (IF36). Although photosynthetically competent, IF36 grows somewhat more slowly than the wild type, and hence, there may be some bias against the recovery of longer forms. Nonetheless, the fact that we were able to recognize and recover a duplication variant leads us to conclude that replication slippage in the chloroplast DNA of *Chlamydomonas* has a bias toward deletions rather than insertions. This is in marked contrast to what occurs to this same substrate when it is maintained in a plasmid in *E. coli*. As shown in Fig. 8, expansions rather than deletions typified the changes in the bacteria.

The observation that deletions are the prevalent form of replication slippage in the *Chlamydomonas* chloroplast suggests that the template strand of the cpDNA is more prone to slippage than is the daughter strand. A deletion bias during replication slippage has been reported for other organisms, including cpDNA of a number of plant species, such as petunia and alfalfa (1), mitochondrial microsatellites of yeast (31), and nuclear genomes of animals, such as snails (39). In the *Chlamydomonas* chloroplast genome, “short dispersed repeats” have been reported to have proliferated to a great abundance (20), and therefore, we were surprised to find a strong deletion bias. However, the short dispersed repeats described earlier (20) are significantly longer (30 to 36 bp) than those we analyzed and are rarely tandem direct repeats, so it is unlikely that they are substrates for replication slippage.

We have previously shown that base substitution at a particular target site in the cpDNA occurs at a rate of $1.5 \times 10^{-9}$ to $11 \times 10^{-9}$ cells in *Chlamydomonas reinhardtii* (11). This means that cpDNA replication slippage at a 4-base repeat occurs 1,000 to 100,000 times more frequently than base substitution. Although our results have the advantage of providing base substitution and replication slippage rates in the same organism, the values may not be directly comparable, since the base substitution rates were observed for the 16S rRNA gene within the cpDNA inverted repeat, while the slippage events occurred in a reporter construct in the single-copy region. Others have found that copy correction in the inverted repeats reduces the mutation rate by half (reviewed in reference 21).

In bacteria, the mismatch repair system defined by the MutHLS repair system is known to be the key surveillance system for correcting mismatches generated by replication slippage events (4). During replication through microsatellites, strand slippage results in the formation of a looped structure with unpaired bases on either the template or the daughter strand. Such a looped structure is recognized by the MutS protein of the mismatch repair pathway and targeted for excision (18). It has been hypothesized that in prokaryotes, a looped structure is more readily recognized and repaired than is a repeat of four or more nucleotides (18, 22). Our chloroplast data paint a different picture; variations due to slippage of the AAAG repeats were definitely recovered less frequently than changes in a mononucleotide repeat, which were so frequent that we could not quantify them. The observation that the same 4-bp-repeat microsatellite in *E. coli* experiences daughter strand slippage at an extremely high rate suggests that endo-oxidation may have benefited the chloroplast by providing its genetic system with repair enzymes that minimize replication slippage of the daughter strand.

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