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CHLOROPLAST DNA MUTATIONS: AN EXPERIMENTAL EVALUATION

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By

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ABSTRACT

CHLOROPLAST DNA MUTATIONS: AN EXPERIMENTAL EVALUATION

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Spontaneous mutation events may give rise to base substitutions as well as insertions / deletions of bases. The rates at which these events occur can be considered to be indicators of the fidelity and efficiency of the replication and repair machinery. Mutation rates for the chloroplast DNA in plants have been obtained through evolutionary observations rather than by experimental approaches. One of the goals of this research was to experimentally assess the rates of different types of mutations occurring *in vivo* at selected target sites in the cpDNA of the unicellular green alga, *Chlamydomonas reinhardtii*. The rates of point mutations in the cpDNA were assessed for three different positions in the *16S rRNA* gene, where changes confer spectinomycin resistance accompanied by the loss of a restriction endonuclease cut site. Results showed that the rates of forward mutation in the cpDNA to spectinomycin resistance ranged from 3×10^{-11} to 1.1×10^{-8}, and among the mutations recovered, transversions occurred at an approximately four fold higher rate than transitions. Insertion/deletion events in the

cpDNA were examined by creating a reporter construct that carried a series of short direct repeats representative of naturally occurring microsatellites. The reporter construct contained an out-of-frame insertion of a $(GAAA)_6$ tract in the *rbcL* gene of the *Chlamydomonas* cpDNA which allowed the visualization and quantification of insertion/deletion events, that were thought to have resulted from replication slippage. Results showed that this site underwent slippage at frequencies that ranged from 1-70 X 10^{-6} and analyses of the slipped cells showed that only deletions had occurred at this site. Once the mutation rates were quantified, a plasmid-based insertional mutagenesis system was used to isolate putative mutator lines that showed high rates of cpDNA replication slippage. One of the tagged lines has been confirmed to act as a cpDNA mutator. This mutator line will be further analyzed to identify the gene disruption that has caused the phenotype.

In addition to studies with the mutators in *Chlamydomonas,* experiments utilized the *plastome mutator* of *Oenothera*. To examine whether the *pm*-activity repairs damage caused by the mutagen 9-aminoacridine hydrochloride (9AA), a mutant plant A-E was selected for molecular analyses. As a necessary prerequisite, the cpDNA was confirmed as the site of the mutation induced by 9AA, through demonstration that the yellow sectors in progeny derived from this mutant plant cosegregated with the cpDNA of the mutant parent, and not with a mitochondrial DNA marker. Although 9AA acted as a weak plastome mutagen, when applied to seeds with the plastome mutator genotype, it did not synergistically increase the mutation frequency. Hence, the *plastome mutator* product was not involved in repairing 9AA-induced mutations.

For my parents and Dipnath

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CHAPTER 1

Introduction

Chloroplasts: structure and origin

Land plants and green algae are similar in that they possess chlorophyll a/b-containing chloroplasts. Chloroplasts are double-membrane organelles that specialize in photosynthesis. Chloroplasts possess their own genomes that are referred to as chloroplast DNAs (cpDNAs) or plastomes. Chloroplasts have been proposed to be descendants of cyanobacterial endosymbionts and it has been hypothesized that the creation of functional plastids involved the migration of large numbers of genes from the prokaryotic ancestral genome to the plant nucleus (Cavalier-Smith 1992; Gray 1992; Wolfe et al. 1992). Gene transfer requires a series of steps, which include the integration of a copy of the plastid gene into the nucleus (duplication), acquiring of the proper gene expression and transit signals by the nuclear copy, followed by subsequent loss of the original genes from the chloroplast ancestor. The gene *tuf*A encoding the chloroplast translation factor is such an example, as it is found in the cpDNA of most green algae, but is absent from land plant cpDNAs. Molecular and phylogenetic analyses have shown that the nuclear genome of *Arabidopsis* contains a *tuf*A-like gene of a prokaryotic origin, whereas some lineages of green algae have *tuf*A sequences in both the genomes. The modern day chloroplast has transferred many of its genes to the nucleus and thus depends on it for many of its essential functions (Wolfe et al. 1992).

Chloroplast DNA: structure and gene content

The chloroplast genomes are circular in structure and the genome sizes vary from 50- 300 kb among different algal and plant species (Gillham 1994). Chloroplast genomes normally possess two unique sequence regions and two large inverted repeats (IRs). The

two repeat sequences are positioned on the circular molecule in an asymmetric fashion such that it divides the two unique sequences into a small and a large unique sequence region known as the small single copy (SSC) and the large single copy (LSC) regions respectively. However, in the unicellular green alga, *Chlamydomonas reinhardtii*, the two unique sequence regions are roughly the same size. The cpDNAs of some land plants (such as rice and tobacco) and the green alga (*C. reinhardtii*) have been completely sequenced and well characterized (Shinozaki et al. 1986; Hiratsuka et al. 1989; Maul et al. 2002). The plastid genomes contain genes that encode for essential components required for chloroplast structure, photosynthesis and gene expression. However, none of the plastid genomes sequenced so far contain genes that code for the enzymes required for the replication and repair of the chloroplast DNAs.

Since cpDNA sequence data from different plants and algae have shown that the genes for its replication and /or repair are absent from their respective plastid genomes, it can be concluded that those genes reside in the nucleus and would possess appropriate chloroplast targeting signals. The gene products in this case, however, would still retain prokaryotic features reminiscent of their cyanobacterial ancestry. Alternatively, it may also be possible that the genes responsible for chloroplast DNA replication and repair may have been totally lost during the evolutionary process and the cpDNA may share replication and repair enzymes with its eukaryotic nuclear counterpart. The nuclear enzymes in the latter case may have acquired transit peptides and could function in replication and/or repair of both the genomes.

Chloroplast DNA mutations

Mutations are heritable changes in DNA sequences that are acted upon by natural selection and contribute to genetic diversity. Mutations occur spontaneously or can be induced by mutagens. The two major types of mutations that are observed in organisms are base substitutions and insertions/deletions. Base substitutions occur when one base is exchanged for another in a DNA sequence. Base substitutions can arise naturally through intracellular metabolism such as spontaneous alkylation, deamination of bases or modification by reactive oxygen species. Base substitutions can also result from the incorrect insertion of nucleotides during DNA replication (Kunz et al. 1998). The second type of spontaneous mutation that occurs frequently in organisms is the duplication/deletion of bases in regions of the DNA that contain repeated sequences (microsatellites). Replication slippage has been implicated to be the mechanism by which such deletions or insertions are generated at these repeats (for a detailed description of replication slippage refer to Figure 4-1). Prokaryotes and eukaryotes have strategies to minimize both of these types of mutations. These include elimination of the DNA-damaging agents, a high accuracy of DNA replication, and repair of the damage after it has occurred.

A low spontaneous mutation rate can be regarded as an indicator of efficient replication and repair mechanisms in a given organism. *In vivo* mutation rates have been well documented in both prokaryotes and eukaryotes through the use of experimental analyses. In plants, the rates of mutations of nuclear and the chloroplast genomes have been studied through evolutionary comparisons. The highly conserved nature of

chloroplast DNA sequences indicates that it has a lower mutation rate than does the nucleus (Palmer 1985; Zurawski and Clegg 1987; Wolfe et al. 1987; Clegg et al. 1994). These observations have led to the suggestion that the enzymes responsible for the maintenance of chloroplast replication and DNA repair have high levels of fidelity. However such evolutionary comparisons do not always offer a true assessment of mutation rates: a comparison of the chloroplast DNA sequences from the degenerate cpDNAs of certain parasitic plants and a study of non-coding regions have both shown that certain segments of the chloroplast DNA molecule may evolve at a much higher rate (Wolfe et al. 1992; Gielly and Taberlet 1994). As a first step towards the understanding the mutational mechanisms that govern the chloroplast DNA, I created reporter systems to experimentally assess the rates of base substitution and insertion/deletion events. Furthermore, the experimental data obtained allowed me to compare the relative frequencies of occurrence of different types of base substitutions as well as replication slippage events that occurred in the cpDNA

Assessment of relative mutation rates

Evolutionary comparisons of the cpDNA microsatellites between different species of pine (*Pinus*) indicated that the length polymorphisms occur at rates ranging from 3.2 to 7.9 X 10^{-5} per site per year (Provan et al. 1999). In contrast, another evolutionary study has determined that nucleotide substitutions occur within a range of 1.1 to 3 X 10^{-9} substitutions per synonymous site per year in the cpDNA (Muse 2000). Studies with the *Oenothera plastome mutator* have shown that within a 450-bp intergenic spacer region of the cpDNA, variations in two chloroplast microsatellites occurred more frequently than

did base substitutions when different species of *Oenothera* were compared (Wolfson et al. 1991). These suggest that the cpDNA replication/repair machinery may be less efficient in handling insertion/ deletion events than base substitutions. However, these deductions were based on evolutionary data and therefore do not reflect a true comparison of mutation rates. The experimental data obtained from my studies allowed me to directly compare the rates of spontaneous insertion/deletion mutations to the base substitution events that occur *in vivo* in the cpDNA and thereby provided an accurate assessment of the relative rates of the two mutation events.

Overall goal for determining the spontaneous mutation rates for the cpDNA

In summary, the first part of my research project involved the creation of reporter constructs to monitor and quantify the rates of different types of mutations that occurr in the cpDNA of *Chlamydomonas reinhardtii*. Specifically, I compared the relative rates of *in vivo* base substitutions to replication slippage events for the cpDNA. In addition, the analyses of the different mutants obtained from my study enabled me to assess the individual frequencies of transition and transversion mutations in the cpDNA as well as to quantify the frequencies of occurrence of duplication and deletion events occurring at the microsatellites. These results were then compared to the conclusions from phylogenetic analyses in order to see if my experimental data were consistent with the evolutionary evidence.

Mutation avoidance in the cpDNA

Studies in *Escherichia coli* and *Saccharomyces cerevisiae* have shown that in order to achieve a low rate of mutation, organisms must employ numerous mutation avoidance strategies such as a high accuracy of DNA replication, with maintenance of DNA in an error free state either through the elimination of DNA damaging agents or by repairing the DNA damage after it has occurred (Schaaper and Dunn 1991; Kunz et al. 1998). The understanding of such mechanisms for the chloroplast DNA is still at a very rudimentary stage and most of the studies have used *in vitro* enzyme assays. The following is a brief summary of what is known regarding the mutation avoidance machinery of the chloroplast.

Removal of DNA damaging agents

Oxygen radicals are highly reactive and are generated in cells exposed to radiation or chemicals. They also arise during the normal metabolic processes such as respiration or photosynthesis in plants (Ohtsubo et al. 1998). Intracellular reactions producing active oxygen species have been identified as major sources of DNA damage and as contributors to spontaneous mutation (Schaaper and Dunn 1991). One of the deleterious outcomes is the production of 8-oxoguanine (8-oxoG) which has ambivalent base pairing properties due to its ability to pair effectively with both A and C during DNA synthesis. Consequently it is an intrinsically highly mutagenic base analog either as a constituent of DNA (8-oxodG) or when present as a deoxynucleoside triphosphate (8-oxodGTP) (Fowler et al. 2003). Specialized systems that are aimed at protecting the cell against the effects of such oxidative stress exist: these are the MutT, MutY and the MutM systems of

E. coli that carry out related but separate aspects of 8-oxoG metabolism. The eukaryotic homologs have also been identified for these genes. Figure 1-1 outlines the different pathways that exist in bacteria for the prevention and repair of 8-oxoG mediated mutagenesis. As seen in the figure, the MutT gene product hydrolyzes 8-oxodGTP to prevent its use as a substrate by DNA polymerase; mut*T* deficient strains show an increase in A/T → C/G transversions resulting from the misincorporation of 8-oxodGTP opposite adenine in the template (Fowler et al. 2003). Nuclear and chloroplast *MutT* genes have not yet been identified in plants.

Maintenance of replication fidelity

The low cpDNA mutation rates indicated by evolutionary studies suggest that the chloroplast possesses efficient DNA replication machinery. This section outlines certain features of cpDNA replication that have been compiled from studies with different plants. Aspects of replication of the cpDNA have been documented by microscopy, genetic analyses and *in-vitro* replication assays in a range of plants including *C. reinhardtii*, pea, soybean and *Oenothera* (Kolodner and Tewari 1975a and b; Wadell et al. 1984; Chiu and Sears 1992; Heinhorst and Canon 1993). However, none of the genes have been isolated or characterized from the respective nuclear genomes of any of the plant species mentioned above. Based on prokaryotic and eukaryotic systems it is known that a basic set of enzymes is necessary for the efficient replication of the cpDNA molecules. These include the DNA polymerases, helicases, primases, topoisomerases, single stranded DNA binding protein (SSB), and other accessory factors to the polymerases.

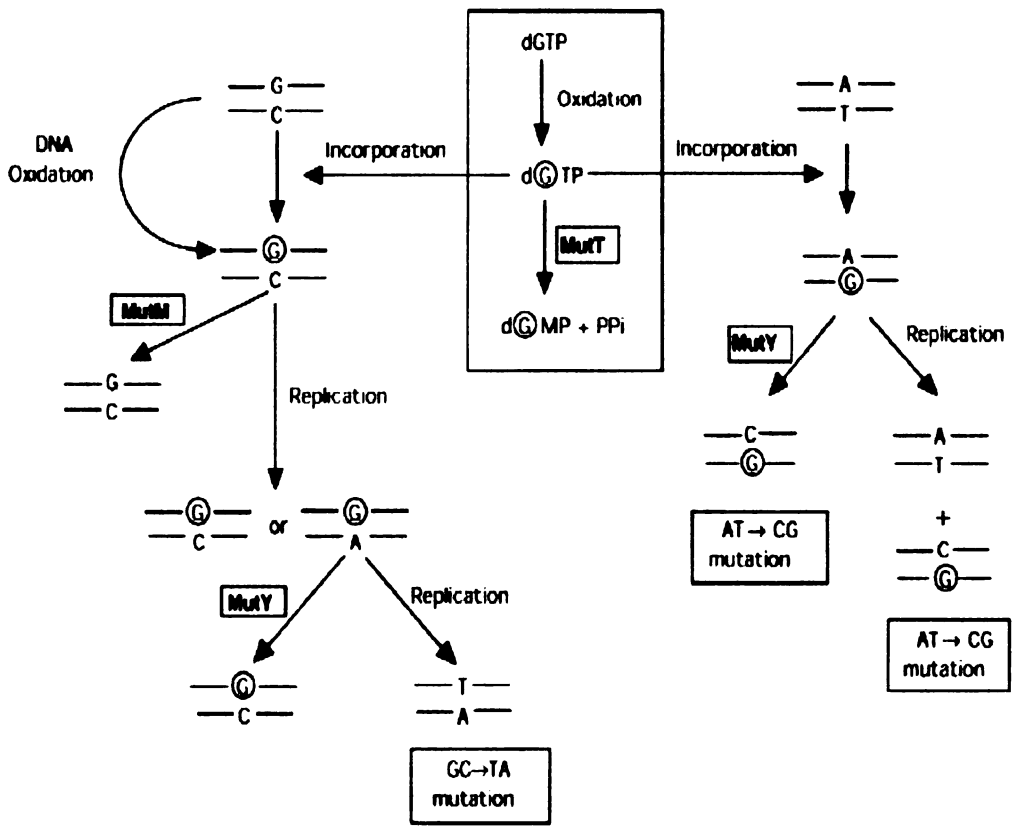

Figure 1-1. Consequences of incorporation of 8-oxodGTP opposite template C and A, and the roles of the mutT, mutM and mutY gene products in 8-oxoG metabolism. The boxed part of the pathway highlights the role of mutT in removal of the 8-oxodGTP from the nucleotide pool and the non-boxed regions show the roles of mutY and mutM gene products in the repair of the damage caused due to incorporation of 8-oxodGTP. (Adapted from Fowler et al. 2003)

The DNA polymerases from pea, spinach, soybean and *Chlamydomonas* chloroplasts have been purified: all the chloroplast polymerases have an estimated molecular mass between 90,000 and 120,000, belong to the γ-class of DNA polymerases and are resistant to the DNA polymerase α inhibitor, aphidicolin (Sala et al. 1980; McKown and Tewari 1984; Heinhorst et al. 1990; Wang et al. 1991). The DNA polymerases from chloroplasts of spinach and pea have moderate fidelity due to the presence of associated 3'→5'- exonucleases (Keim and Mosbaugh 1991; Gaikwad et al. 2002).

Both DNA topoisomerase I and II activities have been observed from the chloroplasts of algae and plants such as spinach, pea and cauliflower and *Chlamydomonas* (Fukata et al. 1991; Heinhorst and Cannon 1993; Woefle et al. 1993; Mukherjee et al. 1994; Nielsen and Tewari 1998; Reddy et al. 1998). A DNA primase activity has been reported from the chloroplasts of pea (Nielsen et al. 1991) and a DNA-dependent helicase was purified from soybean chloroplasts (Cannon and Heinhorst 1990). Although the presence of these different replicative enzymes has been demonstrated from chloroplasts, their direct roles in chloroplast DNA replication have not been proven yet.

DNA repair pathways

One of the other aspects of the mutation avoidance machinery of the cpDNA is the presence of efficient DNA repair pathways that eliminate damage that would otherwise result in high mutation frequencies. The types of DNA damage for which one

would anticipate that repair enzymes should exist include: mismatched bases, double-strand breaks, and chemically modified bases. It is expected that plants possess protection against all these kinds of damage, and since they possess three separate genomes, repair enzymes are required for all of them. The cpDNA lesions must be corrected by distinct repair pathways whose components are encoded in the nucleus, since plastid genomes that have been sequenced lack genes for repair enzymes (Britt 1996). Unlike the replicative enzymes, however, few repair enzymes have been isolated from chloroplasts of plants.

One of the few repair enzymes whose activity has been detected in the chloroplasts of plants is the RecA protein. This protein is essential for both recombination and repair activities in prokaryotes and eukaryotes. A homolog of the *E. coli Rec*A gene, which has been cloned from *Arabidopsis,* has been hypothesized to play a role in the repair of the cpDNA (Cerutti et al. 1992). Its role has been shown in *C. reinhardtii* by transforming the chloroplasts of *Chlamydomonas* with a wild-type copy of the *E. coli* gene and a dominant negative variant and then monitoring the recombination and repair abilities in these different transformants (Cerutti et al. 1995). Results showed that the transformant with the dominant negative gene had reduced efficiencies of repair of cpDNA damage, thereby indicating a role for the native chloroplast RecA gene product in repair of chloroplast DNA.

Plants require sunlight for photosynthesis but at the same time they are exposed to the harmful effects of solar UV radiation (Gallego et al. 2000). UV-light results in

oxidative lesions as well as in the creation of cross-links in DNA (cyclobutane pyrimidine dimers and 6-4-photoproducts), the accumulation of which affects cell viability (Gallego et al. 2000; Waterworth et al. 2002). A light-dependent repair pathway exists for the repair of the above pyrimidine dimers: this process is called photoreactivation, and is mediated by photolyase enzymes that directly reverse DNA damage in an error-free manner (Waterworth et al. 2002). Chloroplast DNAs of algae and plants are not immune to the impact of UV-irradiation, accumulating pyrimidine dimers and 6-4 photoproducts. However, surprisingly, little or no repair of the pyrimidine dimers of the chloroplast DNAs in *Arabidopsis thaliana* has been observed (Chen et al. 1996). In contrast *C. reinhardtii* has been shown to possess photolyase activity in both the chloroplast and the nucleus; and a gene (*PHR2*) encoding the chloroplast photolyase has been identified (Small and Greimann 1977; Small 1987; Petersen and Small 2001).

Specialized systems that are aimed at protecting the cell against the effects of reactive oxygen species are represented by the MutT, the MutY and MutM systems of *E. coli* (outlined in Figure 1-1). While MutT acts to remove the mutagenic 8-oxoG, the MutY and MutM gene products act to repair the damage caused due to the incorporation of 8-oxodGTP opposite template C and A and subsequent replication events. The MutM and MutY proteins are glycosylases: MutM acts upon several modified purines including 8-oxoG and also initiates base excision repair of 8-oxoG when paired with C. If unrepaired, subsequent mispairing of 8-oxoG with dATP would lead to G/C → T/A transversions (Carbera et al. 1988; Boiteux et al. 2002). MutY is also a glycosylase that removes adenine from A-G, A-8-oxoG and A-C mispairings, and thus a defective *mutY*

allele results in enhanced production of G/C → T/A mutations (Fowler et al. 2003). Therefore, analysis of a collection of spontaneously arising base substitution mutations can provide an insight into the relative efficiencies of different mutation avoidance pathways that exist in chloroplasts.

Both the prokaryotic and eukaryotic types of *MutM* gene have been isolated in *Arabidopsis*: a structural homolog of the bacterial *MutM* named *AtMMH* has been cloned (Ohtsubo et al. 1998) and the homolog to the eukaryotic gene *OGG1* has also been found in *Arabidopsis* (Dany and Tissier 2001). The presence of both types of DNA glycosylases has led to the hypothesis that the prokaryotic-type protein, AtMMH, may be translocated into the organelles, most possibly to the chloroplast, although it lacks any obvious localization signals for the organelle. However, it should be noted that the existing programs (such as chloroP) that are used to predict the transit peptide sequences for the chloroplast are not 100% accurate and therefore it is possible that the prokaryotic homolog of the *Arabidopsis* MutM may possess a chloroplast transit sequence that was not detected by the existing programs.

The mismatch repair system (MMR) is one of the major DNA repair systems that is responsible for recognizing and correcting mismatched bases that are incorporated during replication. In addition, the mismatch repair proteins recognize numerous types of DNA damage and either initiate or directly participate in the cellular responses to such damage (Harfe and Jinks-Robertson 2000). The mismatch repair system is thus critical for maintaining the overall integrity of the genetic material. Proteins involved in the

MMR system have been identified in both prokaryotes and eukaryotes, and the basic features of the mismatch system have been highly conserved during the process of evolution. The bacterial MMR system involves three proteins: MutS, MutL and MutH, of which MutS and MutL protein are active as homodimers (Modrich and Lahue 1996). Most eukaryotic organisms characterized to date possess multiple MutS homologs (MSH proteins) and multiple MutL homologs (MLH proteins). A MutH functional homolog has been found in humans (Bellacosa et al. 1999; Harfe and Jinks-Robertson 2000).

In plants, the investigation of the MMR system has only recently begun and both MutS and MutL homologs have been identified and partially characterized from some plant species. Six genes encoding MutS homologs have been identified in the *Arabidopsis thaliana* genome (Culligan and Hays 2000); while two MutS homologs have been identified from *Zea mays* (Horwath et al. 2002). In addition, partial cDNAs of putative MMR genes were isolated from *Triticum aestivum* (Korzun et al. 1999), rice, *Brassica napus* and soybean (Horwath et al 2002). Three MutL homologs are also encoded by the *A. thaliana* genome, although none has been characterized functionally (Jean et al. 1999; Harfe and Jinks-Robertson 2000). None of the isolated MutS or MutL homologs from plants has a known chloroplast transit peptide sequence, and thus they may be responsible for the mismatch repair of nuclear genomes only. However, as stated earlier, the programs that predict chloroplast transit peptide sequences are not completely accurate in identifying all types of transit sequences and hence it is also highly possible that one of the MutS and MutL homologs identified in plants could be translocated to the chloroplasts. Genes for components of the mismatch repair system are of particular

interest because they contribute to the fidelity of maintenance of repeat lengths at microsatellites.

The understanding of the molecular mechanisms of chloroplast DNA replication or repair is still at a very rudimentary stage, and very little is known about the nuclear genes that encode for these proteins. Since the cpDNA sequence data from *C. reinhardtii* and land plants have shown that the genes required for its replication and repair are absent in their respective plastid genomes, one of the goals for my research project was to establish a system for identifying the nuclear genes that contribute to the maintenance of cpDNA replication fidelity and or participate in DNA repair.

Use of mutators in the isolation of genes required for DNA maintenance

One of the strategies to dissect the mechanisms that are responsible for the maintenance of the *E. coli* and yeast genomes has been through the isolation and characterization of strains that have enhanced spontaneous mutation rates (also known as mutators). The mutator phenotypes arise from defects in genes that act to minimize genetic instability (Kunz et al. 1998). In this way, *E. coli* and yeast strains have been isolated that show high rates of replication errors or defects in DNA repair. Characterizations of the defective gene(s) have shown that these loci define crucial genes for the mutation avoidance pathways.

In a review of all the different isolated mutators in *E. coli,* Miller (1998) points out that genes involved in mismatch repair (*mutH, mutL, mutS*), excision repair (*ung*

encoding an uracil DNA glycosylase) and oxidative repair (*mutY, mutT, mutM*) of the bacterial genome were defined using the mutator approach. In addition, the mutator loci also identified genes that are responsible for the maintenance of replication fidelity in bacterial cells, such as those that code for the proofreading subunit of the polymerases or for factors that are associated with polymerases and contribute their processivity such as the genes for β-sliding clamp protein and the clamp loader, γ complex (Krishnaswamy et al. 1993).

Mutator alleles have also been defined from yeasts: these include genes that are involved in proofreading, mismatch correction, base excision and nucleotide excision repair activities. Exonucleolytic proofreading-deficient mutants of the two replicative polymerases (δ and ε) exhibit a mutator phenotype with a mutation rate up to 130-fold higher over the wild type (Kunz et al. 1998). Mismatch repair is defective in the mutator strains due to the inactivation of any one of the *PMS1, MSH2, MSH3, MSH6* and *MLH1* genes. However, some of the mutators produced characteristic mutation patterns that did not overlap, suggesting that the substrates may be different for each mismatch repair complex. For example, more insertion/deletion mutations were produced in the *msh2* strain in contrast to the *msh6* strain that produced mainly base pair substitutions (Marsischky et al. 1996). Inactivation of the genes coding for the nucleotide excision repair (*RAD1*), recombinational repair (*RAD52*) or base excision repair (*APN1*) also resulted in enhanced rates of specific kinds of mutations when compared to the mutation rates of wild-type cells. In addition to the mutators that affect nuclear genomic stability in yeast, a yeast mutator strain with a defect in the exonucleolytic proofreading domain of

the mitochondrial polymerase was also isolated which showed increased levels of spontaneous mutation rates of mitochondrial DNA (Foury and Vanderstraeten 1992).

Previous studies with chloroplast mutators

Nuclear genes that affect chloroplast mutation rates (plastome mutators) should also include loci that play a role in mutation avoidance and replication fidelity of the cpDNA. To date, plastome mutators have been isolated and characterized from three plant species. Most of these mutations that occur in those strains appear as chlorophyll-deficient sectors (reviewed by Börner and Sears 1986). The plastome mutators dramatically increase the rates of spontaneous mutation in the cpDNA without inducing any nuclear gene mutations. The plastome mutators can be divided into two categories: they either cause the same type of mutational phenotype repeatedly (such as the *albostrians* mutator of barley and *iojap* mutator of maize) or they can induce a wide variety of mutations such as the *plastome mutator* in *Oenothera* (Epp 1973; Epp and Parthasarathy 1987) and *chloroplast mutator* in barley (Prina 1992).

Among the first class of plastome mutators, the *iojap* gene (*Ij*) from maize has been isolated, cloned and sequenced, but the sequence has not illuminated the function of the gene product (Bryne and Taylor 1996; Han et al. 1992). Thus it is still unknown how a defect in the nuclear gene (*Ij*) produces striped plants with poorly developed chloroplasts in the white sectors and lacking chloroplast ribosomes. The *albostrians* mutant in barley is well characterized physiologically: white seedlings contain rudimentary plastids which are photosynthetically inactive, have only trace amounts of

chlorophylls and lack ribosomes (Hess et al. 1992; Hedtke et al. 1999). Molecular characterization of the *albostrians* gene has not yet been reported.

The second class of plastome mutators produces a wide range of phenotypic change (such as chlorophyll deficiencies, morphological abnormalities, sterility, resistance to atrazine and ultrastructural irregularities of the chloroplasts). The range of mutational phenotypes obtained indicates that multiple loci in the chloroplast DNA are susceptible to mutations (Chiu et al. 1990; Sears and Sokalski 1991). The chloroplast mutator locus of barley (*cpm),* which belongs to this second class of mutators, has been characterized for cpDNA alterations. In one instance, it was shown that *cpm* caused a nucleotide substitution in the chloroplast-encoded *psb*A gene (Rios et al. 2003). However, the *cpm* locus has not been isolated or further characterized, and hence its mode of action in inducing cpDNA mutation is still unknown.

At the molecular level, the *plastome mutator* (*pm*) allele of *Oenothera* is the best characterized among all the known plastome mutators. The *plastome mutator* was originally isolated by Mel Epp in 1973, and *Oenothera* plants homozygous for the *pm* allele exhibit a 1000-fold higher frequency of cpDNA mutations in comparison to the wild-type plants (Epp 1973; Sears and Sokalski 1991). Short direct repeats and stretches of adenines were observed to be the preferential substrates for *plastome mutator* (Blasko et al. 1988; Chiu et al. 1990; Chang et al. 1996; Stoike and Sears 1998). The *plastome mutator* locus was thus hypothesized to code for a protein that is responsible for preventing elevated slippage of the cpDNA during replication, and thus play a role in the

avoidance of insertion/deletion mutations of the cpDNA molecules. However, the *plastome mutator* locus has not been identified nor has the genetic defect been characterized biochemically.

Mutator targets and assessment of mutation rates at these sites

Because microbial systems are much more tractable for establishing correlations between mutations and their phenotypes in comparison to higher plants, my experimental assay was designed to be similar to assays in *E. coli* and yeast that have been successful in isolating loci that are involved in mutation avoidance pathways. From such studies it has been observed that specific mutators in most cases accelerate particular types of mutation events. For example, analysis of mutations in a mutator for the exonucleolytic proofreading domain of polymerases in yeast shows increases only in single base pair events (substitutions, insertions and deletions), whereas a mutation of the yeast gene encoding the proliferating cell nuclear antigen (PCNA) destabilizes mostly short direct repeat stretches or microsatellite sequences (Kokoska et al. 1999; Kunz et al. 1998). Thus the choice of a mutator target would define the types of mutators that the assay would generate. With this in mind, one of my research objectives was to isolate mutators in *C. reinhardtii* that would display replication slippage rates that are higher than those observed in the wild-type cells.

Studies with the *Oenothera plastome mutator* in our lab have identified potential target sites for the *pm* gene: these included regions of the cpDNA that contained stretches of short direct repeats, with the smallest repeat unit involved being a single base (in a

stretch of tandemly repeated adenines) (Chang et al. 1996; Stoike and Sears 1998). Therefore as a starting point, I looked for mutators that specifically caused increases in the rates of changes in the repeat lengths of microsatellites resulting from insertion/deletion events. For this purpose, the naturally occurring microsatellites could not be used as they occur mostly in the non-coding regions of the genome, and changes in repeat lengths at those sites would not result in a visible phenotype. Thus, a reporter construct containing a microsatellite as a slippage substrate was first created and then introduced into an essential chloroplast encoded gene for my assay, the details of which are explained in Chapters 4 and 5. The type of slippage substrate that was chosen had been known to vary in the *Oenothera plastome mutator* lines (Wolfson et al. 1991; Sears et al. 1994) and similar sequences are found in the cpDNA of other plants.

Expected mutator loci

Several factors affect the frequency of replication slippage in bacteria and yeast during the replication process: these include the presence of stable hairpin structures in the DNA that lead to increased stalling of the replication fork, defects in proteins that ensure the processivity of polymerases such as the single-stranded binding protein (SSB) or the sliding clamp and clamp loader for polymerase, helicases, etc. In *Salmonella typhimurium*, deletion rates of short direct repeat increased in a line with a mutant *ssb* allele (Mukaihara and Enomoto 1997). Similar observations have been made in *E. coli* and also in the case of a replication helicase mutant, *hep*, suggesting that replication slippage is facilitated by the pausing of the replication complex (Bierne et al. 1997). Following replication, the newly replicated DNA is scrutinized by a mismatch repair

system that recognizes and corrects mismatches resulting from the replication errors (Schaaper 1993). Inactivation of the general mismatch correction system in bacteria and yeast has been shown to be associated predominantly with an increase in insertion/deletion mutagenesis, indicating that the mismatch repair system is crucial to the maintenance of stability of the microsatellites in the respective genomes (Schaaper and Dunn 1987; Yang et al. 1999). Using the mutator assay I hope to identify similar gene(s) involved in DNA replication and/or repair that act to maintain stability of the microsatellites of the cpDNA.

Overall objective of my thesis project

In order to understand the molecular mechanisms of chloroplast DNA mutations, I decided to experimentally assess the rates of base substitutions and replication slippage in the cpDNA of the unicellular eukaryote, *Chlamydomonas reinhardtii.* Since studies in bacteria and yeast have shown the potential of isolating mutators for the understanding of replication and repair processes, a subsequent goal was to isolate a library of chloroplast DNA mutators. Characterization of these mutator strains would be very useful in the elucidation of different genes that are involved in mutation avoidance and maintenance of the chloroplast genome. Traits of *Chlamydomonas,* outlined in the following section, made it particularly ideal for this research project.

Chlamydomonas as the experimental organism

In recent years the green unicellular biflagellated alga *Chlamydomonas reinhardtii* has emerged as a powerful model system for studying a wide range of topics

in cellular and molecular biology: genetics and biogenesis of organelles, especially chloroplasts, photosynthesis, flagellar assembly and functions, cell wall synthesis, mating reactions and carbon, sulfur, nitrogen metabolism (Rochaix 1995). For these reasons, it is often referred to as the photosynthetic yeast. For my research, *Chlamydomonas* also appeared to be an ideal experimental organism: firstly, the introduction of slippage constructs into the cpDNA of *C. reinhardtii* can be accomplished because its chloroplast DNA can be successfully transformed (Boynton et al. 1988). The transformation of cpDNA through biolistics occurs through homologous gene replacement and several markers are available for selection of the transformants (Goldschmidt-Clermont 1991). Secondly, the reporter construct that will be used to monitor slippage events in the cpDNA (details described in Chapter 4) offers a strong phenotypic selection, and this would allow the slipped cells to be readily distinguishable from the non-slipped cells.

Determining the rates of spontaneous mutation frequencies for slippage events and base substitutions requires the growing and handling of large numbers of cells. Therefore, *C. reinhardtii* was ideal for this purpose as large numbers of cells could be plated on few plates, allowing me to screen millions of cells in search of the rare mutation event. Once the mutation rates were established in the appropriate reporter strains, plasmid-based nuclear gene disruptions were performed to create the chloroplast mutator phenotype. Procedures for the nuclear transformation of *Chlamydomonas* needed for this part are well established (Kindle 1990), and dominant selectable markers are also available for this purpose (Stevens et al. 1996). The isolated mutator phenotype was confirmed through crosses and tetrad analysis, and future experiments using plasmid

rescue are being done to isolate the disrupted nuclear gene that caused the mutator phenotype. The completed sequence of the nuclear genome of *C. reinhardtii* and the EST database will then allow the recovery of the wild-type gene.

In summary, my studies provided the first experimental assessment of cpDNA mutation rates that occur spontaneously *in vivo* in *C. reinhardtii*. The frequencies of different types of base substitution mutations obtained from analyzing the mutants provided an insight into the relative frequencies of transition and transversion events that occur in the cpDNA. In addition, the assessment of relative rates of base substitutions and replication slippage events allowed us to make deductions about the efficiencies by which they are generated and/or repaired in the cpDNA. Furthermore, I was able to establish a mutator assay that was successful in the isolation of a chloroplast mutator in *C. reinhardtii* that shows elevated levels of replication slippage. Characterization of the disrupted gene in this mutator line will hopefully lead us to the identification of the first gene involved in mutation avoidance of the cpDNA in *C. reinhardtii*.

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CHAPTER 2

Chloroplast mutations induced by 9-aminoacridine hydrochloride are independent of the *plastome mutator* in *Oenothera*

Note. The content of this chapter has been accepted for publication in the *Theoretical and Applied Genetics* as Guha Majumdar M, Baldwin S, and Sears BB 2003.

I would like to acknowledge that I have contributed to the molecular data presented in this chapter, while the initial mutagenesis and phenotypic characterizations were performed by Sue Baldwin. In specific, I have done all the DNA isolations, PCR amplifications and Southern hybridizations that are outlined in the Chapter and also have contributed fully to the writing and compilation of all figures and tables of the manuscript.

Abstract

Oenothera plants homozygous for the recessive *plastome mutator* allele (*pm*) show chloroplast DNA (cpDNA) mutation frequencies that are about 1000-fold higher than spontaneous levels. The *pm*-encoded gene product has been hypothesized to have a function in cpDNA replication, repair and/or mutation avoidance. Chemical mutagenesis studies with the alkylating agent nitroso-methyl urea (NMU) showed a synergistic effect of NMU on the induction of mutations in the *pm* line, suggesting an interaction between the *pm*-encoded gene product and one of the repair systems that corrects alkylation damage. To examine whether the *pm*-activity extends to the repair of damage caused by non-alkylating mutagens, 9-aminoacridine hydrochloride (9AA) was tested for synergism with the *plastome mutator*. A statistical analysis of the data reported here indicates that the *pm*-encoded gene product is not involved in the repair of the 9AA-induced mutations. However, the recovery of chlorotic sectors in plants derived from the mutagenized seeds shows that 9AA can act as a mutagen of the chloroplast genome.

Introduction

Mutators serve as genetic tools for the study of mutation avoidance and repair pathways (Miller 1998). Bacterial mutator genes encode proteins that contribute to the fidelity of DNA replication (e.g. *mut D* and *pol A* in *E. coli*) as well as those that are involved in DNA repair (such as genes of the mismatch repair system: *mut H, L* and *S* in *E. coli*). Studies with chloroplast mutator systems have sought similar characterizations of loci involved in chloroplast DNA (cpDNA) replication and/or repair pathways. One

such mutator locus is defined by the nuclear-encoded *plastome mutator* (*pm*) allele in *Oenothera*.

The *plastome mutator* induces a high frequency of non-Mendelian chloroplast mutations when the *pm* allele is homozygous (Epp 1973; Sears and Sokalski 1991). Due to the high frequency of cpDNA mutations by the *plastome mutator*, the *pm*-encoded gene product has been hypothesized to have a function in cpDNA replication and/or repair. Studies by Blasko et al. (1988) and Chiu et al. (1990) showed restriction fragment length polymorphisms (RFLPs) in the chloroplast DNA (cpDNA) specific to the *pm* line. Subsequently, mutations caused by the *plastome mutator* were shown to occur at cpDNA microsatellite regions, resulting in the deletion or duplication of repeat(s) probably through replication slippage (Chang et al. 1996; Stoike and Sears 1998).

In order to test whether a general defect in chloroplast DNA repair or mutation avoidance is responsible for the *plastome mutator* activity, Sears and Sokalski (1991) looked for synergistic effects of ultraviolet light (UV-light) and the chemical mutagen, nitroso-methyl urea (NMU) in several *pm* lines. No synergism in mutation induction was noted when the *pm* lines were irradiated with UV-light, allowing the conclusion that the *pm*-encoded gene product is not involved in the repair of UV damage. In contrast, when the potent plastome mutagen NMU is applied to the *pm* lines, extremely high frequencies of mutation result, indicating a synergistic effect. Thus the alkylation damage caused by NMU is probably repaired by a pathway that has the *pm*-encoded gene product as a component (Sears and Sokalski 1991).

To examine whether the *plastome mutator* activity extends to the repair of damage caused by non-alkylating mutagens, we chose to test 9-aminoacridine hydrochloride (9AA) for synergism with the *plastome mutator*. 9AA intercalates between the stacked nitrogen bases at the core of the DNA double helix (Nasim and Brychcy 1979). The intercalated 9AA mimics base pairs and thus causes deletions and additions of bases upon replication. In *Salmonella* (Hoffman et al. 1989; Kopsidas and MacPhee 1994) and *E. coli* (Thomas and MacPhee 1985; Gordon et al. 1991), 9AA causes single base-pair deletions or duplications that result in frameshift mutations, with preference for runs of mononucleotides (Skopek and Hutchinson 1984). Since an oligo-A stretch has been found to be a *plastome mutator* target in *Oenothera* (Chang et. al. 1996), 9AA seemed to be a good choice to test for potential synergism with the *plastome mutator*.

In plants such as barley and onion, the application of 9AA has been reported to cause chlorotic mutations and growth retardation (D'Amato 1950; D'Amato 1952). Therefore, as a first step in this study, our goal was to determine whether 9AA could cause similar mutations in *Oenothera* and/or could act specifically as a cpDNA mutagen. Once it was established that 9AA could cause mutations in the cpDNA, the next goal was to determine whether it could act synergistically with the *plastome mutator*.

Materials and Methods

Plant material

Homozygous plants of *Oenothera hookeri* strain *Johansen* containing plastomes I, II and IV were constructed originally by Professor W. Stubbe (University of Düsseldorf), and the lines were maintained by self-pollination. The *Johansen* strain containing either plastome I or II was used as the green parent in crosses with the variegated mutant A-E. Self-pollinations of the *pm/pm* line result in the accumulation of mutations (Chiu et al. 1990). Hence, efforts were taken to obtain a *pm/pm* line with a low background mutation rate. These seeds were produced by crossing a +/*pm* line as the female parent with a *pm/pm* line as the pollen donor which should result in an equal mixture of *pm*/+ and *pm/pm* seeds, with most plastids transmitted from the female parent.

9-aminoacridine hydrochloride mutagenesis

After a four-hour imbibition period, seeds were surface-sterilized by incubating them in a solution of 50% bleach and 0.1% SDS for 30 minutes, followed by one rinse with 0.01 N HCl and several rinses with sterile water. To establish a dose curve, 9AA (Sigma) was added at specified final concentrations (see Results) for 0.5 hours, 8 hours, 16 hours and 32 hours. The seeds were swirled gently throughout the incubation period, and rinsed several times before placing them in a beaker of sterile water where they were allowed to germinate. Control seeds were swirled for corresponding time periods in water.

Maintenance and scoring of plants

All the lines were maintained by leaf tip cultures and by self pollinations as described by Stubbe and Herrmann (1982). Seed germination medium was made according to Chiu et al. (1990). For the mutagenesis experiments, seedlings were planted in multipots (Hummert Seed Co.); the trays were placed under aquarium lights (GE) and were checked periodically for chlorotic sectors and other developmental abnormalities.

Transmission Electron Microscopy

Preparation, sectioning, and microscopy of leaf samples were performed by the MSU Center for Electron Optics.

Statistical Analyses

To discern whether the 9AA treatment significantly altered the mutation frequency in the mixed *pm/pm, pm*/+ line, the data contained in Table 2-3 were analyzed by applying the Chi-Square goodness-of-fit test to the numbers of observed and expected mutations. If the chemical and the genetic condition independently cause mutations, then the following formula should predict the expected number of mutations:

[(CN) + (NP)] - [CP (½ N)] = Expected

where N is the population size of the *pm/pm, pm*/+ seeds, C is the frequency of mutations in the chemically treated wild-type plants, and P is the observed frequency of mutations of *pm/pm, pm*/+ alone without any chemical treatment. CP would give the expected value

of both causing a mutation in the same plant, but is applicable to only half of the population, as half of the seeds are *pm/pm*.

Total DNA isolation

Total DNA was isolated from *Oenothera* leaves using a protocol adapted from David Jarrell (unpublished results) and also from Stoike and Sears (1998).

Isolation of DNA from a mitochondria-enriched fraction

For Southern analysis, DNA was isolated from a mitochondria-enriched fraction using a protocol adapted from Sears et al. (1989). The leaf tissue from the plants was first ground using a 10-fold vol/wt ratio of homogenization medium (50 mM Tris, 6% Sorbitol, 6 mM EDTA, 0.1% BSA, 0.3% polyvinyl polypyrrolidone, 1 mM Ascorbic Acid and 3 mM Cysteine) to leaf tissue. The mixture was filtered through two layers of cheese cloth and one layer of miracloth, and the filtrate was centrifuged in a Sorvall centrifuge using a GSA rotor at 5900g for 6 minutes to remove chloroplasts and starch. The supernatant was centrifuged at 25,000g to pellet the mitochondria. The mitochondrial pellet was then resuspended in 3 ml CTAB buffer and DNA was extracted using the protocol described above.

PCR amplification

The *oriB* primers and conditions for amplifying the *16S* rRNA - *trnI* region of the cpDNA of *Oenothera* are described by Stoike and Sears (1998), except for the annealing temperature, which was set at 48°C. The PCR products were run on a 1% agarose gel along with a 123-bp DNA ladder (GIBCO BRL) and photographed using a Polaroid camera. The gel picture was digitally scanned using a Hewlett Packard ScanJet 4C and displayed using the Adobe Photoshop software (San Jose, CA).

Southern analysis

The DNA from the mitochondria-enriched fraction was digested with *Bam* HI (GIBCO BRL) and then electrophoretically separated on a 0.8% agarose gel at 60 V for approximately 8 hours. The DNA was transferred overnight to a nylon membrane according to the method of Sambrook et al. (1989). The membrane was probed with a radioactively labeled CMS-Sprite cosmid clone 651-12C2 (containing 12 random mitochondrial DNA fragments from *Phaseolus vulgaris*) using the standard nick translation method described by Sambrook et al. (1989); the ^{32}P-dATP used for the labeling was obtained from Andotek Sciences Co. The filter was washed according to the method described by Sambrook et al. (1989), and then exposed to film (Kodak X-O-Mat) at -80°C for 17 hours.

Results

9-aminoacridine hydrochloride dosage trials on wild type line

In *Oenothera*, different plastid DNA (plastome) types (I, II, III and IV) are transmitted differentially in crosses and also have specific RFLPs associated with them (Chiu et al. 1988; Chiu et al. 1990). Previous studies have shown that the different plastome types are equivalent targets for the *plastome mutator* (Sears and Sokalski 1991; Chang et al. 1996). The different plastome types also responded similarly to preliminary 9AA dosage trials to determine the concentration and the time interval that would result in the highest frequency of mutations. Complete data from the dosage trials from only plastome II are reported here (Table 2-1) since the mutant plant (A-E) used for further characterizations was isolated from one of these trials.

In two sets of experiments, 500 seeds were treated with 0, 2 or 10 μg/ml concentrations of 9AA for 0.5 – 32 hours (Table 2-1). Mutant sectors were observed at both the 2 and 10 μg/ml concentrations when the seeds were exposed for 16.5 hours. In the preliminary trials, mutations were observed in seeds incubated for other periods of time, but their most frequent occurrence was always after a 16-hour treatment.

Table 2-1. Sector frequencies for wild type seedlings containing plastome II treated with 0, 2 and 10 µg/ml concentrations of 9AA for different exposure times. Each trial contained 500 seeds, except for one treatment[a], which contained 512 seeds.

9AA (µg/ml)	Exposure time (hours)	% Germination	% Viability	Mutation frequency[b]
Experiment 1				
0	32	41.8	40.2	0
10	1[a]	100	55.9	0
10	6.5	72.6	53.4	0
10	16.5	93.2	68.2	3/318 (0.9%)
10	32	80.6	59.3	0
Experiment 2				
0	32	92.0	63.9	0
2	1	90.4	83.8	0
2	6	90.2	83.3	0
2	16.5	75.2	93.6	3/352 (0.9%)
2	32	92.6	86.2	0

[b]Mutation frequency = $\frac{\text{Number of seedlings with sectors}}{\text{Number of viable seedlings}}$

Mutant phenotypes from acridine treatment

The 9AA treatment produced a spectrum of pigment mutations in *Oenothera*, with white, yellow and light green mutations being observed as both solid and mottled sectors in leaves. Developmental anomalies such as abnormal leaves with fused midribs and plants without trichomes were also observed in the 9AA treated seedlings, but these were transient and never seen beyond the second set of leaves. Because so few mutants were recovered, only one segregated into the germline and could be analyzed by crossing. This mutant was labeled A-E and had pale yellow sectors.

Transmission electron microscopy

The mutant A-E was examined by transmission electron microscopy (TEM) to characterize organelle ultrastructure. The plastids in the mutant sectors contained swollen vesicles along with either no (Figure 2-1 A) or few grana stacks (Figure 2-1 B). In contrast, the mitochondria in the mutant sector appeared normal (Figures 2-1 A and B).

Inheritance of the acridine-induced mutation

Initial inheritance studies showed non-Mendelian inheritance and vegetative segregation of the A-E trait, indicating that the lesions caused by 9AA could reside either in the cpDNA or mitochondrial DNA (mtDNA). In *Oenothera*, the differential pattern of transmission of an organelle mutation through a cross can be used to determine whether the mutation lies in the cpDNA or mtDNA. The mtDNA is inherited only from the maternal parent (Brennicke and Schwemmle 1984), whereas the cpDNA can be inherited

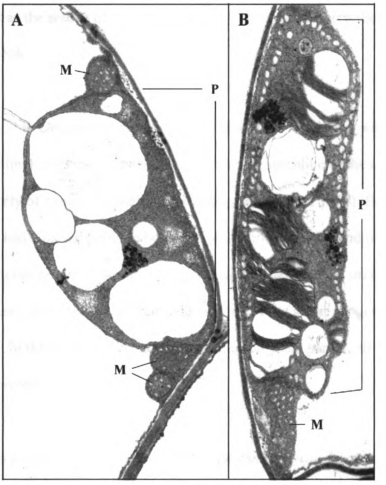

Figure 2-1. A-B Electron micrographs of plastids (**P**) with aberrant thylakoid structures and mitochondria (**M**) from the mutant yellow sectors of A-E.

from both parents (Stubbe and Herrmann, 1982). Therefore, when the mutant plant is used as the source of pollen, the progeny should not inherit the mutation if it is in the mtDNA.

In *Oenothera* plants with non-Mendelian mutations, when a leaf containing a full periclinal chimera occurs at a floral node, the germline of the adjacent flower consists entirely of mutant organelles (Kutzelnigg and Stubbe 1974). However, the mutant plant A-E had a partial periclinal chimera with both wild-type and mutant organelles present in the germline. In crosses using mutant A-E as the maternal parent, 103 out of the 205 progeny inherited the mutant organelle, and most of the progeny were variegated (Table 2-2). In the reciprocal crosses with A-E as the male parent, no variegated progeny were recovered.

Co-segregation of the mutant phenotype analyses using molecular markers

Data from the TEM studies had indicated that the chloroplast was the affected organelle in the 9AA treated mutant plant A-E, whereas the transmission patterns of the mutation in reciprocal crosses suggested that the 9AA-induced mutation could be in the mtDNA. However, in *Oenothera*, when a weak plastome type is used as the male parent in a cross, the plastids may be transmitted only or predominantly from the maternal parent (Chiu et al. 1988). Therefore it could not be determined with confidence which organelle genome carried the 9AA-induced mutation. In order to establish whether the mutation in A-E co-segregated with the cpDNA, the green and yellow sectors from

Table 2-2. Crosses to establish the inheritance pattern of the 9AA-induced organelle mutation in A-E. The table lists the number of seeds obtained and the fraction that germinated. The F_1 progeny showed 100% viability, but varied in the inheritance of the A-E mutation.

Parents[a]		# of seeds	% Germ. F_1	Progeny phenotypes			
				% Progeny with mutant tissue			
Maternal	Paternal			Mutant	Variegated	Green	N
Variegated (II) ½ mutant ½ green	Green (I)	396	37.6	2	68	79	149
				46.5 %			
Variegated (II) ½ mutant ½ green	Green (IV)	100	32	2	17	13	32
				59.4 %			
Variegated (II) ½ mutant ½ green	Green (IV)	24	100	5	9	10	24
				58.3 %			
Green (I)	Variegated (II) < ½ mutant > ½ green	390	87.7	0	0	342	342
				0 %			
Green (IV)	Variegated (II) ½ mutant ½ green	109	91.7	0	0	100	100
				0 %			

[a] A variegated plant with a heteroplasmic germline transmitted the A-E organelle mutation; the other parent contributed the green wild-type plastids, with Roman numerals indicating the plastome types.

the variegated progeny were dissected and DNA was extracted separately, so that a co-segregation analysis could be conducted.

Chloroplast DNA marker: To determine whether the yellow sectors in the progeny from the cross (A-E X wild type) co-segregated with the physical markers on the cpDNA, PCR amplification using primers flanking the *oriB* region was performed with DNAs from the green and yellow sectors of the progeny as well as from the wild type and the mutant parent (A-E). Figure 2-2 shows that the cpDNA marker in the yellow tissue in the progeny plant (lanes 5 and 6) is identical to the band of plastome type (II) from the mutant parent A-E plant (lane 2). The green tissue from the progeny plant (lanes 3 and 4) showed heteroplasmy as indicated by the presence of PCR products similar to those from both the wild type and the mutant parent (lanes 1 and 2 respectively). A middle band was also seen in the DNAs amplified from the green sectors; this third band is an amplification artifact of the PCR reaction, also observed from a mixing experiment using two different plastome types (not shown).

Mitochondrial DNA (mtDNA) marker: Southern analysis was conducted to test whether the unique mtDNA band of the mutant parent A-E (indicated by an arrow in Figure 2-3) cosegregated with the mtDNA of the yellow sectors in the progeny plants. For this purpose, DNAs from the mitochondrial-enriched fractions of the same samples used for the cpDNA analysis were extracted. These DNAs were digested and then blotted onto a membrane that was hybridized to a labeled mitochondrial cosmid probe (see Materials and Methods). Southern blot analysis showed no difference between the yellow and the

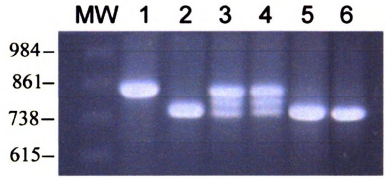

Figure 2-2. PCR analysis of *16S rRNA-trnI* spacer region. From left to right on the gel, primers amplified through the *16S rRNA-trnI* region from the following *Oenothera* cpDNAs: *lane 1* green wild-type (wt) parent with plastome type I; *lane 2* mutant variegated parent A-E, with plastome type II; *lanes 3 and 4* replicate samples of green tissue from a progeny of the cross, A-E X wt; *lanes 5 and 6* replicate samples of yellow sectors from a progeny of the cross, A-E X wt. The *lane MW* shows the DNA size markers (in bp) from the GIBCO-BRL 123-bp DNA ladder.

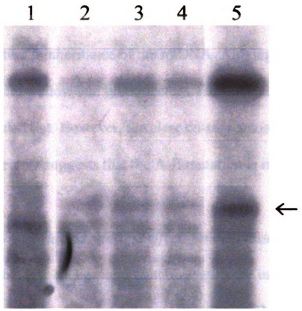

Figure 2-3. Southern hybridization analysis using DNA from the mitochondria-enriched fraction, extracted from the green and yellow sectors of the progeny and also from the parents of the A-E X wt cross. The numbered lanes contain *Bam* HI-digested DNAs from the following plants: *lane 1* green wild-type parent; *lanes 2 and 3* green sectors of the progeny from the cross; *lane 4* yellow sectors from the progeny and *lane 5* the mutant variegated parent, A-E (5). The mtDNA of the two parents used for the cross is distinguished by a single band (indicated by an arrow) that is exclusive to the mutant parent A-E.

green sectors from the progeny plant (lanes 2, 3 and 4), as all contained the band exclusive to the mtDNA of the maternal parent, A-E (lane 5); a result which is consistent with the strict maternal inheritance of the mtDNA. Although there was no RFLP between the mtDNA from the yellow and green sectors, the existence of a point mutation between them cannot be ruled out. However, the clear co-segregation of the cpDNA PCR product with the yellow sectors suggests that the A-E mutation is most likely on the cpDNA.

9-aminoacridine hydrochloride dosage effects on the plastome mutator line

Seeds with a low background mutation rate were used to determine whether 9AA acts in synergism with *pm* to induce mutations in the cpDNA, (see Materials and Methods for details on how a *pm* line with low background mutation rate was produced). As a control, wild type seeds containing the same plastome type as the *pm* seeds were also treated with the chemical for comparison. Mutant sectors were observed in both the 9AA-treated wild type and the mixed *pm* seedlings (Table 2-3). Since the untreated *pm*-line produces a high background of sectors, statistical methods were employed to assess whether the higher number of seedlings with sectors in the 9AA-treated *pm*-line reflected an additive or synergistic interaction of the two conditions (see Materials and Methods). These analyses showed that the differences in the final mutation frequencies of the treated and control *pm/pm* plants were not statistically significant ($0.05 < p < 0.10$).

Table 2-3. Effect of *plastome mutator* on the sector frequency in seedlings containing plastome I treated with 9AA. Each trial contained 500 seeds with an exposure time of 16 hours. Sectors were scored by the four-leaf stage.

Nuclear background	µg/ml of 9AA	% Germination	% Viability	No. of seedlings with sectors (% mutation frequency[a])
Wild type	0	49.0	54.6	0
Wild type	2	47.2	66.5	2/157 (1.3 %)
Mixed +/*pm* and *pm/pm*	0	93.6	70.9	13/332 (3.9%)
Mixed +/*pm* and *pm/pm*	2	93.4	62.5	22/292 (7.5%)

[a]% Mutation frequency determined using the formula described in Table 2-1.

Discussion

The study described in this paper was undertaken to determine whether the *pm*-encoded gene product is involved in repairing the damage caused by frameshift mutagens, represented by 9AA, in order to delineate the role of the *plastome mutator* in the replication and /or repair of cpDNA. To determine whether 9AA acts as a plastome mutagen in *Oenothera*, initial mutagenesis studies tested for conditions that would produce the maximum number of mutations, identified by the appearance of chlorotic sectors. Incubation of seeds in 9AA caused a low frequency of chlorotic sectors in the seedlings. Dosage trials established that increased exposure times or concentrations of the mutagen did not affect the germination or viability of the treated seedlings (Table 2-1), nor did those conditions produce a higher frequency of chlorotic sectors. We are perplexed about why the 32-hour treatment did not result in seedlings with sectors. Perhaps, the slight drop in seedling viability reflects death of the affected plants. Under any circumstance however, the low mutation rate indicates that the mutagen lacks good penetration or potency.

The inheritance studies produced progeny with sectors indicating both vegetative segregation and non-Mendelian inheritance of the mutation. Therefore the mutation was located in either the chloroplast or mitochondrial genome. In order to clarify which genome was targeted by the mutagen, several approaches were taken. TEM studies showed that the ultrastructure of the mitochondria in the mutated tissue was normal whereas the plastids were affected in the yellow sectors (Figure 2-1). These observations indicated that the chloroplast was the likely target organelle of 9AA, although instances

are known, where mtDNA mutations affect plastid development and ultrastructure (for example, the NCS mutations in maize: Hunt and Newton 1991; Gu et al. 1993).

It is possible to distinguish between chloroplast and mitochondrial mutations in *Oenothera* by crosses: mutations that are carried by the cpDNA can be inherited from both parents while mtDNA shows purely maternal inheritance (Brennicke and Schwemmle 1984). Initial data from crosses of one mutant (A-E) indicated maternal inheritance of the 9AA-induced mutation (Table 2-2); however, since the mutation only occupied a portion of the germ line, the data from the crosses remained inconclusive.

Because the crossing data were inadequate for establishing the target organelle of 9AA, co-segregation analysis using molecular markers was performed. As shown in Figures 2-2 and 2-3, the mtDNA is inherited uniformly from the maternal parent, whereas in contrast, the yellow sectors co-segregated with the cpDNA from the mutant parent (A-E). The green sectors on the other hand contained chloroplasts from both the maternal and paternal parents. These data are most consistent with the plastome as the site of the mutation.

Since the data suggested that 9AA targets the cpDNA of *Oenothera*, its ability to act synergistically with the *plastome mutator* was explored. In the wild-type line, 1.3% of the seedlings showed mutant sectors after exposure to 9AA, whereas in the seeds that were half *pm/pm* and half heterozygote, a six-fold higher mutation frequency was observed (Table 2-3). However, most of these mutations can be credited to the *plastome*

mutator, and our chi square analyses using the adjusted expected number of mutations (See Materials and Methods), indicate no statistical significance. This lack of synergistic effect implies that the *pm*-encoded gene product does not act upon the DNA lesions caused by 9AA. Because studies with prokaryotes have shown that the methyl-directed mismatch repair system (MMR) is involved in correcting 9AA-provoked mismatches (Miller 1998; Skopek and Hutchinson 1984), we conclude that the *pm*-encoded gene product is not likely a component of the cpDNA mismatch repair system.

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CHAPTER 3

Experimental assays of chloroplast DNA base substitutions in *Chlamydomonas reinhardtii*

Abstract

Base substitutions are minimized in organisms through error-free DNA replication, removal of DNA damaging agents as well by removal of damaged nucleotides by the DNA repair machinery. The absence of either of these mutation avoidance pathways results in an increase of base substitution rates and occurrence of specific mutational patterns. Comparisons of spontaneous base substitution rates and the spectra of mutations between wild type and mutator strains have helped to identify the role of each type of pathway in mutation avoidance. Our investigation provides the first experimental assessment of spontaneous base substitution mutations occurring at a target site in the chloroplast DNA of the green alga *Chlamydomonas reinhardtii*. In *Chlamydomonas* mutations at two sites within a conserved region of the chloroplast-encoded *16S rRNA* gene have been reported to confer spectinomycin resistance; they also result in the loss of an *Aat* II restriction endonuclease cut site. Rates for base substitution events in the chloroplast DNA were obtained for the chloroplast DNA at this target site. Our data showed that these mutations occur in the chloroplast DNA at a range of 3-1800 per 10^{11} viable cells plated. Analysis of the different types of base changes that occurred in the spectinomycin-resistant mutants also allowed us to quantify the relative frequencies of transition and transversion mutations that occurred in the chloroplast DNA. We report the isolation of four new mutations in *Chlamydomonas reinhardtii* at the above conserved residues of the chloroplast *16S rRNA* gene, two of which are novel and have not been reported from other organisms. In addition, all three bases underwent changes that are predicted to disrupt a conserved stem structure in the 16S rRNA

molecule, thereby reinforcing the importance of this stem structure in the binding of spectinomycin.

Introduction

Base substitutions can arise naturally through intracellular metabolism such as spontaneous alkylation, deamination of bases or modification by reactive oxygen species. Base substitutions can also result from the incorrect insertion of nucleotides during DNA replication (Kunz et al. 1998). Base substitutions are minimized in several ways that result in the low rates of occurrence of these mutations in organisms ranging from prokaryotes to eukaryotes. These include a high accuracy of DNA replication, elimination of DNA-damaging agents and the repair of DNA damage after it has occurred. Replication fidelity is achieved at two separate levels: accurate base selection during the insertion of nucleotides and the editing or proofreading by the 3' → 5' exonuclease that is associated with the DNA polymerase. A small fraction of replication errors may evade the correction machinery; these errors are then rectified by the mismatch correction systems that repair the newly synthesized DNA molecules (Yang et al. 1999). If DNA replication errors still escape all the above-mentioned error avoidance pathways, they give rise to spontaneous base substitution mutations.

Spontaneous mutations may also result from unrepaired DNA damage caused by different agents. Intracellular reactions producing active oxygen species have been identified as major sources of DNA damage and as contributors to spontaneous mutation

(Schaaper and Dunn 1991). Specialized systems that are aimed at repairing a single type of base substitutions exist, such as the MutT system, the MutY and the MutM systems of *E. coli,* which protect the cell against the effects of such oxidative stress. Characteristic types of point mutations have been associated with the absence of each type of repair pathway in *E. coli* (Schaaper and Dunn 1991; Fowler et al. 2002 and Boiteux et al. 2002). The *MutT* gene product hydrolyzes 8-oxodGTP to prevent its use as a substrate by DNA polymerases and *mutT* deficient strains show an increase in A/T → C/G transversions resulting from the misincorporation of 8-oxodGTP opposite template A (Fowler et al. 2002). The MutM and MutY proteins are glycosylases; MutM acts upon several modified purines including 8-oxoG, and also initiates base excision repair of 8-oxoG when paired with C. If unrepaired, subsequent mispairing of 8-oxoG with dATP would lead to G/C → T/A transversions (Carbera et al. 1988; Boiteux et al. 2002). MutY is also a glycosylase that removes adenine from A-G, A-8-oxoG and A-C mispairings, and thus a defective mutY allele results in enhanced production of G/C → T/A mutations (Fowler et al. 2003). Since different types of base substitutions may be produced when specific repair pathways are absent, an analysis of a collection of mutants that arise spontaneously can provide insight into the relative efficiencies of different mutation avoidance pathways that exist in different organisms.

One of the general strategies that has been used to better understand the mechanisms responsible for the generation of spontaneous mutations is by comparing the mutation rates and patterns between wild type strains and mutator strains that have enhanced spontaneous mutation rates. The rationale for this approach is that the mutator

phenotypes are caused by defects in genes whose products act to minimize DNA alterations. Mutations that arise spontaneously in wild-type cells represent those which occur normally even in the presence of all the mutation avoidance machinery. To date, spontaneous mutations have been extensively studied using experimental assays in both prokaryotic systems such as *E. coli* (Schaaper and Dunn 1987) and eukaryotic systems such as *Saccharomyces cerevisiae* (Yang et al. 1999). In plants, conclusions about the spontaneous mutation rates for the nuclear and chloroplast genes have been deduced from evolutionary comparisons rather than from experimental investigations (Wolfe et al. 1997, Clegg et al. 1994). Molecular evolutionary studies using DNA sequence data were used to estimate the rates of nucleotide substitution in the chloroplast DNA (cpDNA) of plants: ranging from 1.1 to 3 X 10^{-9} substitutions per synonymous site per year (Muse 2000). The purpose of the current study was to quantify the rates of nucleotide substitution in the cpDNA using an experimental approach. For this purpose, the rates and types of base substitutions were observed at a target region in the cpDNA of a model system, *Chlamydomonas reinhardtii*.

For the mutation assay, a particular region in the chloroplast encoded *16S rRNA* gene was chosen because mutations at two sites (three different base changes in total) in that gene were known to confer resistance to spectinomycin in *C. reinhardtii*. These changes also eliminate an existing restriction endonuclease (*Aat* II) cut site, (Harris et al. 1994) and are therefore easily detectable both at the phenotypic and molecular levels (Figure 3-1 A). This forward system was preferable for the study of base substitutions in the cpDNA as it allowed us to detect multiple base substitution events within one

particular target site without a bias towards any particular base substitution. For a detailed description of the assay refer to Figure 3-1 B and the Materials and Methods section.

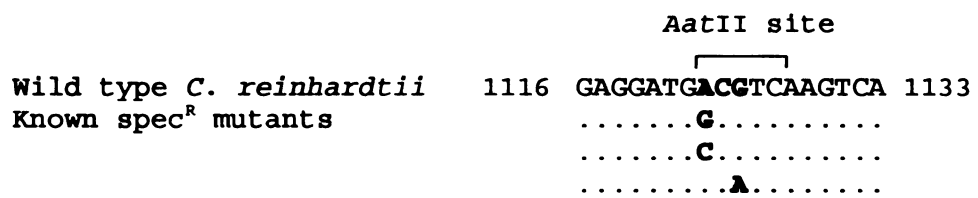
Analysis of the different mutants obtained from the above assay would allow us to establish the baseline spectrum of base substitutions that naturally occur in the cpDNA of *C. reinhardtii*. Since no prior studies exist for plants, our results will be compared to similar mutational spectra obtained from both prokaryotic and eukaryotic systems. These comparisons may provide us with an insight of whether the DNA replication machinery of chloroplasts resembles that of prokaryotic or eukaryotic systems. Since chloroplasts have an endosymbiotic derivation from cyanobacteria (Gray 1993), it is expected that the types of mutations and their abundance seen in the chloroplasts would be similar to that observed in prokaryotes. However, all of the components of the chloroplast DNA replication and repair machinery are encoded by the nucleus and therefore it may also have acquired characteristics from the eukaryotic nucleus.

The relative abundance of the two different types of base substitutions (transitions and transversions) that occur has been shown to differ among prokaryotes and eukaryotes: for example, wild-type *E. coli* and yeasts have shown some differences in the ratios of transition to transversion mutations (Schaaper and Dunn 1991; Kunz et al. 1998; Yang et al. 1999). A study using an *in vitro* assay with the chloroplast DNA polymerase from pea has reported that transversions were generated at a slightly higher frequency than transitions (28 and 26 respectively) (Gaikwad et al. 2002). Analysis of the different base substitutions generated from our assay would provide an insight into the relative

frequencies of transitions and transversions that occur *in vivo* in the cpDNA and could also be compared to similar ratios from the *E. coli* and yeast studies.

Different base substitutions arise due to different types of mismatches that are generated during DNA replication or due to DNA damage. Furthermore, mismatches are recognized and repaired at different efficiencies by the exonucleases (Keim and Mosbaugh 1991), mismatch repair proteins (reviewed in Harfe and Jinks-Robertson 2000) and other repair pathways, such as the oxidative repair pathway (Fowler et al. 2002). Different base changes were reported to occur at different frequencies in *E .coli* and yeasts (Schaaper and Dunn 1987; Yang et al. 1999) due to differences in efficiencies of their mutation avoidance systems. Analysis of the different types of base substitutions generated in our assay would allow us compare the types and frequencies of mismatches that result in the different base substitutions in the cpDNA and other organisms. In addition, we hoped that the mutation spectrum observed in the wild-type cells would allow us to deduce the presence or absence of various chloroplast DNA replication and repair components in *C. reinhardtii*.

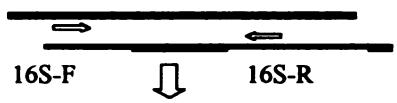
A



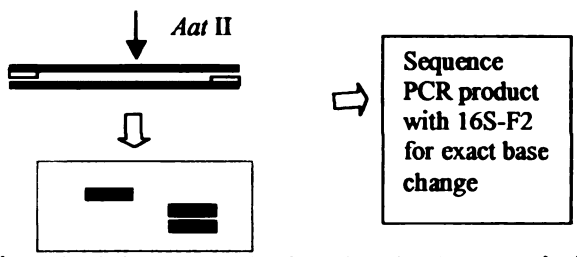
A. Nucleotide sequence of wild-type *C. reinhardtii* (top line) and three spectinomycin-resistant mutants (lower lines. The *Aat* II recognition site is indicated. (Adapted from Harris et al. 1994)

B

PCR amplification of DNA from spectinomycin-resistant mutants with primers flanking the *Aat II* target site in the *16S rRNA* gene



Digestion of the PCR product with *Aat II* restriction endonculease



Agarose gel electrophoresis to check for mutants that have lost the *Aat* II cut site in the target region of the *16S rRNA* gene

B. Experimental procedure to identify spectinomcyin-resistant mutants of *Chlamydomonas reinhardtii* that have undergone specific base changes at the target site in the *16S rRNA* gene. Oligonucleotides that flank the *Aat* II cut site are named 16S-F, 16S-F2 and 16S-R.

Figure 3-1. Base substitutions at a particular region in the gene coding for *16S rRNA* in *Chlamydomonas reinhardtii* confer resistance to spectinomycin.

Materials and Methods

Strains and media

The *Chlamydomonas reinhardtii* strain used for all experiments was the wild-type CC125 (mt+) strain, which was obtained from the *Chlamydomonas* Genetics Center at Duke University (Harris 1989). *C. reinhardtii* was grown in tris-acetate phosphate (TAP) media (Gorman and Levine 1965) on either solid plates or liquid media when required. The cultures were incubated under growth lights at room temperature (22 – 25 °C). The liquid cultures were put on a rotary shaker under constant light at a speed of ~200 r.p.m. The spectinomycin-resistant transformants were grown on TAP media supplemented with 100 μg/ml spectinomycin dihydrochloride (Sigma Chemical Co.).

Assay for base substitution mutations to spectinomycin resistance

The wild type (CC125, mt+) cells were grown in liquid TAP culture to late log phase (2-4 X 10^6 cells/ml) for about 7 days at room temperature under constant white lights on a rotary shaker at a speed of ~200 r.p.m. Cell counts were determined by using the compound microscope and the standard hemocytometer method as described in the *Chlamydomonas* sourcebook (Harris 1989). 250 ml of the cells was harvested by centrifugation at 8000 X g in a Sorvall centrifuge using a GSA rotor at 4°C for 10 mins under sterile conditions. The supernatant was discarded and the cells were resuspended in a volume of TAP medium that would result in a final concentration of 2 X 10^8 cells/ml.

An aliquot of these cells were used for dilution platings to determine the number of viable cells plated. 100 µl aliquots from the 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated on TAP plates and then grown under lights at room temperature. The number of colonies that grew on these plates was then counted to assess the total number of viable cells that were plated for the experiment.

The remaining volumes of the concentrated CC125 cells were then plated as 500 µl aliquots on TAP agar supplemented with 100 µg/ml of spectinomycin. The TAP + spectinomycin plates were put under growth lights on a shelf at room temperature. The total number of spectinomycin resistant colonies that grew on these plates were counted, and then transferred using sterile toothpicks and replated on TAP + spectinomycin plates for further DNA analyses.

Total DNA isolation

Chlamydomonas total genomic DNA was prepared using a modified protocol adapted from the 'miniprep' method described by Rochaix et al. (1988). The cells were scraped from a plate using yellow pipette tips and resuspended into a 1.5 ml microfuge tube that contained 0.5 ml of 1X TEN buffer (1M TRIS, 0.5 M EDTA and 5 M NaCl). The cells were then spun in a microcentrifuge (13,000 r.p.m.) and the supernatant discarded; the pellet was then resuspended by vortexing in 150 µl of sterile water. To these resuspended cells, 300 µl of SDS-EB buffer was added (10% SDS, 5M NaCl, 0.5M EDTA and 1M TRIS-HCl, pH 8.0). The cells were incubated on ice for 5 min and then one phenol:chloroform:isoamyl alcohol (25:24:1) extraction was done, with centrifugation for

10 min. The aqueous phase was transferred to a fresh microfuge tube and the DNA isolations were then done according to the protocol described by Rochaix et al. (1988).

PCR amplification

DNAs extracted from all the spectinomycin-resistant colonies and also the wild type CC125 cells were then used for PCR amplification. The 16S-F (5'-CCGCACAAGCGGTGGATT-3') and the 16S-R (5'-CCTTCCAGTAGCCCTACC-3') primers were used in the PCR amplification reactions as the forward and reverse primers respectively to amplify a 580-bp fragment from the 16S *rRNA* gene. DNA (1-5ng) was added to individual 100 µl reactions containing 200 µmol of each dideoxynucleotide, 2.5 units of Taq polymerase (GIBCO BRL), 10 pmoles of each primer and 1 X PCR reaction buffer (GIBCO BRL). Conditions for the 30 cycles of PCR were as follows: denaturation at 95°C for 2mins, 85°C for 5 mins, followed by 94°C for 40 secs; 30 secs at 48°C to anneal the primers with the template and 30 secs at 72°C for DNA extension. The final extension after all the cycles was done for 10 mins at 72°C. All the PCR reactions were performed with a minicycler model PTC-150-16 (MJ Research Inc., Watertown, Mass.).

Restriction digestion and Sequencing

The 580-bp PCR products were digested with the restriction enzyme *Aat* II (New England Biolabs) at 37°C according to the manufacturer's instructions. The digested products were then run on a 1.5% Agarose gel with a 100-bp DNA ladder (Invitrogen Life Technologies, CA). The gel was photographed using the BIORAD Alpha imager and the image was displayed using the Quantity One software. The PCR products that had

lost the restriction endonuclease cut site were then further sequenced to determine the exact base change that had occurred resulting in the loss of the restriction cut site. For sequencing, the PCR products were cleaned by removing the excess primers, enzyme, dNTPs using the Qiaquick PCR purification kit (Qiagen Inc.,CA) following the manufacturer's instructions. The concentrations of the purified PCR products were then determined by running them on an agarose gel along with a standard (0.5 μg/μl) uncut lambda DNA marker (GIBCO BRL). The purified PCR products were then sent for sequencing to the Michigan State University Genomics Facility, East Lansing, MI (www.genomics.msu.edu), with an internal primer (16S-F2) provided for sequencing (5'-CGTCAGCTCGTGCTGTG-3').

Computer Analyses

The sequence data obtained from the sequencing facility were analyzed using the Megalign Software from the DNAStar Program (Wisconsin, Madison). Using this program, the wild-type sequence was used to align the data from the different spectinomycin mutants lacking the *Aat* II cut site to determine the exact base substitution that had occurred.

Mutation Rate

To obtain the cpDNA base substitution rate, the total numbers of spectinomycin-resistant colonies that had lost the *Aat* II cut site were determined for each experiment, and then using the following formula, the mutation rate (μ) was determined:

$$\frac{\text{Number of spectinomycin resistant colonies with loss of } \textit{Aat} \text{ II site}}{\text{Total number of viable cells plated (obtained from dilution plates)}}$$

Results

Estimation of rates of base substitution mutations at positions 1123 to 1125 in the 16S rRNA gene

Specific base changes in the chloroplast encoded *16S rRNA* gene of *C. reinhardtii* confer spectinomycin resistance and also eliminate the *Aat* II endonuclease recognition site. In order to determine the rates of spontaneous mutations in the cpDNA at the *Aat* II target site, DNAs from the spectinomycin-resistant mutants were analyzed by PCR amplification followed by restriction digestion of the PCR product. Since changes in other genes coding for ribosomal proteins can also result in spectinomycin resistance (Harris et al. 1994), the *Aat* II assay allows a quick determination of whether the target site in the *16S rRNA* gene contains the mutation. 64 out of the total 121 spectinomycin-resistant mutants isolated from three independent trials were altered at the *Aat* II target site (Table 3-1). Figure 3-2 shows a subset of the spectinomycin resistant mutants assayed. Among the seven mutants shown, two (m6 and m7) have lost the *Aat* II cut site. Among the five spectinomycin-resistant mutants that had retained the cut site, two of them (m3 and m4) have a small deletion in the 331-bp fragment. The rates of spontaneous mutations *at the target site* of the cpDNA ranged from 3-1800 per 10^{-11} viable cells plated in the three independent experiments (Table 3-1).

Experiment #	Total number of cells plated (X 10^9)	Total number of spectinomycin resistant colonies	Number of spectinomycin resistant colonies that lost the *Aat* II cut site	Rate of mutation at the *Aat* II target site
1	1.1	20	20	**1.8×10^{-8}**
2	33.9	2	1	**2.9×10^{-11}**
3	41.9	99	43	**1.1×10^{-10}**

Table 3-1. Frequencies of spontaneous base substitutions at the *Aat* II target site in the chloroplast DNA of *Chlamydomonas reinhardtii.*

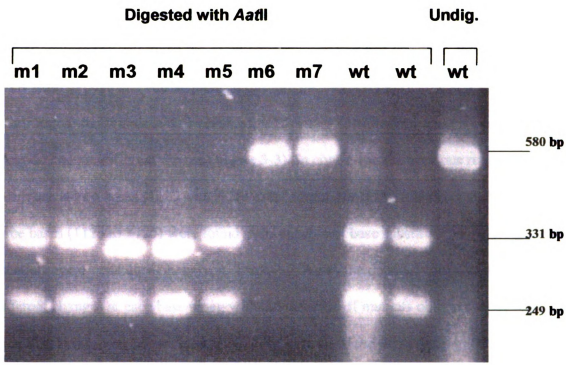


Figure 3-2. PCR amplification using the 16S-F and 16S-R primers followed by restriction digestion with *Aat* II. Agarose gel showing digested and undigested PCR products from the wild-type (wt) and digested PCR products from the spectinomycin mutants (m1 to m7).

Analysis of base substitutions for each base in the Aat II target site

The 64 spectinomycin mutants that were obtained from the above three trials were further analyzed to determine the exact base changes that had occurred. For this purpose, DNA from the mutants was sequenced through the target region of the *16S rDNA*. According to the sequence data obtained, all the base substitution changes could be grouped into six different classes (Figure 3-3). In contrast to the previous report of mutations at two bases (1123 and 1125) in the target *Aat* II site (Harris et al. 1994), all three bases (1123 to 1125) were found to be capable of base substitutions resulting in the spectinomycin-resistance phenotype. Table 3-2 shows the detailed compilation of the different changes at each base along with the numbers of mutants obtained from each trial. Since each trial did not produce mutations in all of the three bases in the *Aat II* target site (see Table 3-2), a range of mutation frequencies has been reported for each base of the target site in Table 3-3.

Spectrum of base substitution mutations in the cpDNA of C. reinhardtii

DNA sequence analyses of the 64 mutants showed six different types of base substitutions among which an A/T → C/G change was the most frequent (Table 3-3). However, the G/C → C/G type of base substitution occurred in each trial (Table 3-2) and was the second most frequent type of base substitution overall.

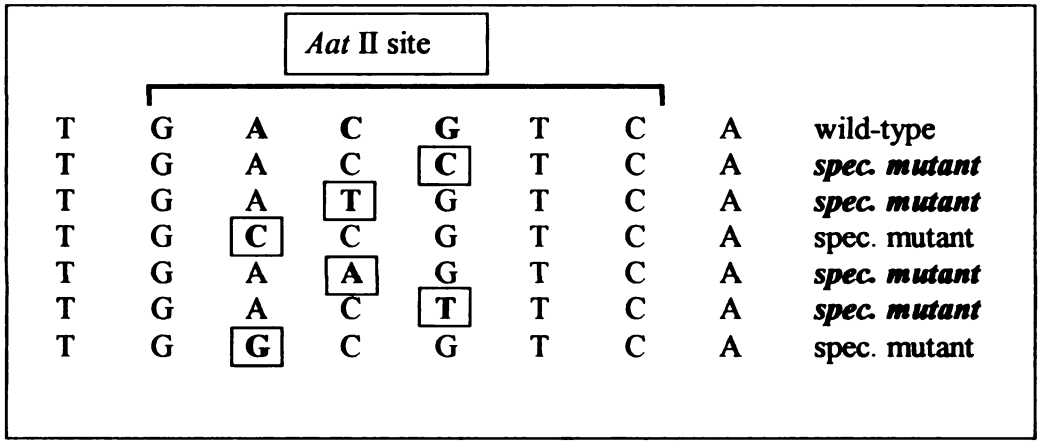


Figure 3-3. Sequence alignment of wild-type and spectinomycin resistant colonies at the *Aat* II target site showing the six different base changes, compiled from all three trials. The new mutants are represented in ***bold italics***.

	A → C	A → G	C → T	C → A	G → C	G → T
Expt.-1			8 / 20		12 / 20	
Expt.-2					1 / 1	
Expt.-3	30 / 43	1 / 43	4 / 43	2 / 43	1 / 43	5 / 43

Table 3-2. Total numbers of the different types of base substitutions observed in each experiment.

Ratios of transition to transversion mutations

Both transitions and transversions occurred at the target *Aat* II site. However, analysis of the base substitutions at the above cpDNA target site showed that in all three trials transversions occurred more frequently than transitions (Tables 3-2 and 3-3). In the first experiment, transversions occurred at a slightly higher rate than transitions (12:8), but in the third experiment transversions occurred eight times more frequently than transitions (38 transversions in contrast to 5 transitions). Overall, transversion mutations occurred nearly four times more frequently than transitions.

Comparison of the base changes in the 16S rRNA target site from spectinomycin-resistant mutants in C. reinhardtii and other organisms

The chloroplast genes for the *16S rRNAs* are highly conserved at the sequence level and most closely related to eubacterial sequences (Harris et al. 1994). Figure 3-4 shows a comparison of the different types of changes at the above region of the *16S rDNA* observed in spectinomycin-resistant mutants from different organisms. Among the six types of base substitutions reported in this study (Figure 3-3), four are new for *C. reinhardtii*. Two of those are known to give spectinomycin-resistance in *E. coli* and tobacco (Svab and Maliga 1991; Harris 1994). Two of the six mutations are novel, and their isolation underscores the importance of all three bases in the binding of the antibiotic to the *16S rRNA* molecule.

Wild type base	G	A	C	G	T
Changes[1] Number of mutants Frequency of mutations ($/10^{-8}$)		C (tv) 30/64 0.072	T (ts) 12/64 0.73	C (tv) 14/64 1.085	
Changes[1] Number of mutants Frequency of mutations ($/10^{-8}$)		G (ts) 1/64 0.002	A (tv) 2/64 0.005	T (tv) 5/64 0.012	
Range of mutation frequencies in all three experiments		<1 in $2.9X\ 10^{-11}$ to $7.2\ X\ 10^{-10}$	<1 in $2.9\ X\ 10^{-11}$ to $7.2\ X\ 10^{-9}$	$2.9\ X\ 10^{-11}$ to $1.1\ X\ 10^{-8}$	

Table 3-3. Comparison between the types, numbers and frequencies of base substitutions among the three bases at the *Aat* II target site. [1]Base changes are represented as transitions (ts) or transversions (tv).

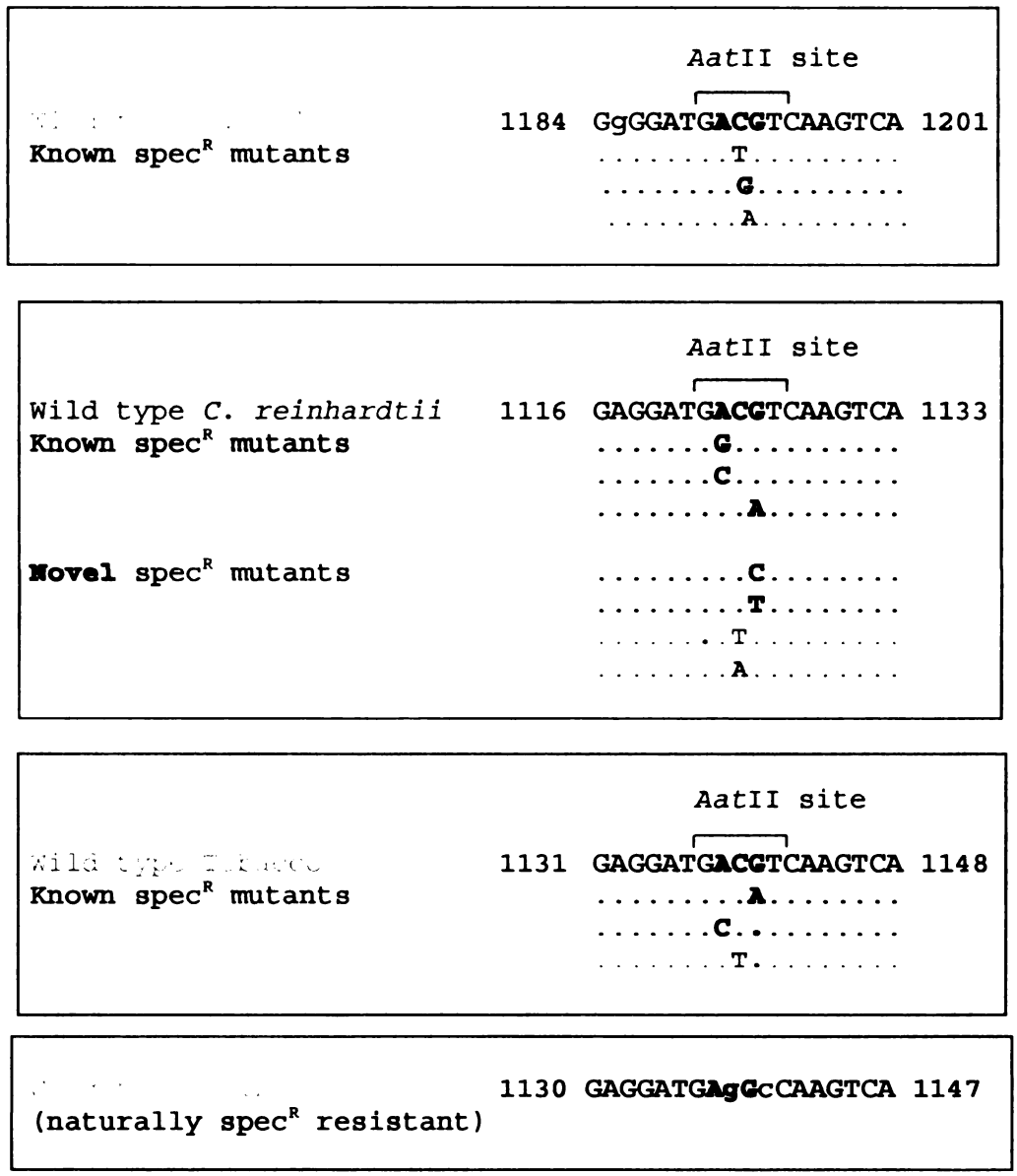


Figure 3-4. Alterations in the gene encoding *16S rRNA* compiled from spectinomycin-resistant mutants of different organisms (Adapted from Harris et al. Genetics: 123: 281-292, 1989.)

Discussion

Mutations in the 16S rRNA gene that confer spectinomycin resistance

Spectinomycin is an aminoglycoside antibiotic that blocks protein synthesis on prokaryotic-like ribosomes (Fromm et al. 1987). Evidence exists for the binding of this antibiotic to the RNA component of the small ribosomal subunit and several mutations in the 16SrRNA molecule that disrupt the binding of the antibiotic have been obtained from a wide range of organisms (Mark et al. 1983; Harris et al. 1989). Isolation of the spectinomycin-resistant mutants that have changes in the *16S rRNA* gene has therefore helped to pinpoint the molecular basis of resistance to spectinomycin. Studies have shown that a conserved stem structure in the 3' end of the 16SrRNA molecule is involved in the binding of the antibiotic because mutations that disrupt the right arm of the stem confer resistance in *E. coli*, as well as chloroplasts of *Nicotiana* and *Chlamydomonas* (De Stasio et al. 1989; Harris et al. 1989). The three nucleotides that were assayed for mutations in our study (1123 to 1125) are located in the right arm of the above-mentioned stem structure that is conserved among organisms (Schwarz and Kossel 1980). Previous studies with *Chlamydomonas* had identified mutations in two of the three bases (Figure 3-4), and our results provide additional evidence regarding the importance of all three bases in the binding of the antibiotic to the stem loop. In addition, some of the spectinomycin-resistant mutants that had retained the *Aat* II cut site showed deletions in the 331-bp fragment (bases 1455 to 1124) of the PCR product (Figure 3-2) indicating that deletions at the 3'end of the molecule are also able to confer resistance. Experiments are currently being performed in the Sears laboratory to isolate more of these deletion mutants in an effort to understand the basis of their resistance to spectinomycin.

Rates of cpDNA base substitutions

The primary objective of this study was to experimentally assess the rate of base substitutions at a region of the *16S rDNA* in *C. reinhardtii*. Results obtained from this study indicate that base substitutions occur at low rates between 2.9 in 10^{-11} to 1.8 in 10^{-8} viable cells plated. This range of low nucleotide substitution rates provides evidence for the existence of a high fidelity of cpDNA replication and efficient repair machinery, even though the genes for chloroplast DNA replication or repair have not yet been identified through genomic analyses. *In vitro* studies with the pea cpDNA polymerase including its 3' → 5' exonuclease had indicated that the error rate for the polymerase was approximately 1 X 10^{-6} (Gaikwad et al. 2002). Therefore, we can hypothesize that the lower rates of spontaneous base substitution mutations seen *in vivo* are due to the involvement of post-replication repair systems that act to maximize cpDNA fidelity.

Mutation rates can either be measured by reversion of known mutations, as in the Ames test, or by screening for forward mutations: the latter strategy was used in our case and mutations were detected by the appearance of spectinomycin-resistant *C. reinhardtii* cells. Since the single chloroplast of *C. reinhardtii* contains around 100 copies of the cpDNA, a cell will not be counted as a mutant until the number of mutant alleles has increased to the point where the cell is phenotypically mutant (Birky and Walsh 1992). Chloroplasts also have relaxed rather than stringent control of replication, such that DNA molecules are randomly chosen for replication and are also partitioned randomly to the daughter cells at cell division. In addition, they also undergo intracellular selection when some cpDNA genomes replicate faster than some others in the same chloroplast

depending on their genotypes (Birky and Walsh 1992; Birky 1994; Birky 2001). Thus, the mutation frequency is a function of all of the above complex mechanisms. Depending on how early the mutation reached fixation, how fast the mutant alleles replicated and were partitioned; the mutation frequency to spectinomycin resistance could vary in each experiment. Since the mutation frequency varied among the different trials (3-1800 per 10^{11} viable cells plated), it remained to be seen whether the rates could go even higher if more trials were done. However, similar experiments in the Sears laboratory (unpublished results) using different substrates and different genes, to assay for frequencies of base substitution mutations in the cpDNA failed to produce mutants even when 10^9 viable cells were plated. These results provide confidence in my observations that the spontaneous frequency of base substitution mutations for the cpDNA perhaps does not occur at rates higher than 2 in 10^8 viable cells in *C. reinhardtii*.

None of the genes responsible for the cpDNA mutation avoidance systems has been isolated. One of the ways to identify them would be by isolating mutator lines that show elevated frequencies of base substitutions over spontaneous levels. Our study provides the first experimental assessment of spontaneous rates of nucleotide substitutions in the cpDNA and therefore, the next goal is to isolate *C. reinhardtii* lines that will show base substitution rates higher than the observed spontaneous levels. Characterization of these mutator genes would help us to identify the components required for cpDNA replication fidelity and repair.

Are there any differences in mutation frequencies among the three bases at the Aat II target site?

Since experiments 1 and 2 (Table 3-2) did not produce mutations at all three nucleotides in the *Aat* II target site, data from trial 3 were examined to compare the relative rates of base substitutions among the three nucleotide positions. Among the 43 mutations observed in the trial, 31 occurred at position 1123, while positions 1124 and 1125 each had six mutations. Therefore it appears that the adenine at position 1123 may be prone to more mutations than the other bases. However, due to the relaxed replication and partitioning of the chloroplast genomes, this difference in numbers may have been skewed if the predominant mutation (A to C) at base 1123 was fixed early as a mutation, or if the cpDNA with this mutation was preferentially replicated and partitioned to the daughter chloroplasts compared to the mutations in bases 1124 and 1125.

Rates of transition and transversion mutations in the cpDNA

Transition and transversion mutations are generated at different frequencies in prokaryotes and eukaryotes: a study in *E. coli* (Schaaper and Dunn 1991) has shown that transition mutations are generated at higher frequencies than transversions (175 and 118 respectively), whereas a similar study in yeast (Kunz et al 1998; Yang et al 1999) indicated that transitions occur at a slightly lower frequency than transversions (117 and 175 respectively). Our results show that among the cpDNA base substitutions obtained in each trial, transversions occurred more frequently than transitions, and in overall there were 51 transversions to 13 transitions. Relevant to this, an *in vitro* assay with the pea chloroplast DNA polymerase also showed that transversions were generated at a slightly

higher frequency than transitions by the polymerase (Gaikwad et al. 2002). In this respect, the mutational pattern of the cpDNA was more similar to that seen in eukaryotes. This was surprising as chloroplasts share a common ancestry with cyanobacteria (Gray 1993) and therefore we had expected that its mutational patterns would be similar to prokaryotes.

Effect of neighboring base composition on transition/transversion bias at bases

Evolutionary studies have shown that base substitutions are highly dependent upon the composition of the flanking bases in both the coding and non-coding regions of the chloroplast DNA (Morton 1995; Morton and Clegg 1995; Morton 1997; Morton et al. 1997). The types of substitutions were dependent on the composition of the two flanking bases: substitutions in G+C rich environments showed a strong bias towards transitions, while in A+T rich environments, transversions outnumbered transitions (Morton 1997). Only one of the three bases in the target site fits the criterion of being in an A+T rich or G+C rich context: the first of the three bases, wild type base "A" is surrounded by G+C, but in contrast to the observations by Morton, this site showed a stronger bias towards transversions than transitions (Table 3-3). However, since all trials did not produce changes in the target base 1123 (Table 3-2), the results were interpreted from only one trial and therefore, more data need to be gathered for this base as well as for other bases showing similar G+C rich environments, in order to establish a general trend for cpDNA mutations. Since evolutionary comparisons do not always reflect *in vivo* cpDNA conditions our experimental assay may provide a better tool to analyze whether the G+C rich environments can bias the type of base substitution at a target site.

Spectrum of cpDNA base substitutions in wild-type C. reinhardtii

One of the purposes of this study was to establish the different types of spontaneous base substitution that occur in a particular region of the cpDNA of *C. reinhardtii*. The spectrum presented here (Table 3-4) represents a first essential step towards the understanding of the types of spontaneous mutations that are generated *in vivo* in the cpDNA. A variety of base substitution events were recovered at the three nucleotides in the target site. This forward mutational assay had a distinct advantage as a mutational spectrum could be obtained without any bias towards a single base change in contrast to reversion analyses (Kunz et al. 1998). As summarized in the introduction, the roles of different repair pathways in minimizing specific base mutations have been deduced in both prokaryotes and eukaryotes by comparing the mutational spectrum in wild-type cells and cell lines lacking the particular repair pathway in question (Table 3-4). Similar mutational analyses have not been done for chloroplasts, and therefore the spectrum presented in Table 3-4 from our assay is the first representation of the different types of mutations that can occur at a target site in the cpDNA. As mentioned earlier, our intention is to isolate *C. reinhardtii* mutator lines and compare their mutation spectra, thus providing a direct function for each mutator gene in the generation of specific base substitutions.

Table 3-4 shows the number of different classes of mutants obtained from our experiments. The predominance of the A/T → C/G type of mutations recovered could mean that this type of mutation may be generated at relatively higher rates in the cpDNA

Original basepair	**Mutation**	**Class**	**Occurrences (Out of 64)**	**Repair pathway (genes involved)**
A:T	G:C	Transition	1	Mismatch Repair (*mut H,L,S* or homologs)
	C:G	Transversion	30	Oxidative Repair (*mut T*)
C:G	T:A	Transition	12	Mismatch Repair (*mut H, L, S* or *MLH1*, in eukaryotes) (Baross-Franscis et al. 2001)
	A:T	Transversion	2	Not Known
G:C	C:G	Transversion	14	Novel pathway in mice *MutY/MutM* in bacteria? (Shin et al. 2002)
	T:A	Transversion	5	Oxidative Repair (*mutY / mutM*)

Table 3-4. Spectrum of cpDNA base substitutions in wild type *Chlamydomonas reinhardtii* and the possible pathways by which each type is known to be repaired in different organisms.

or that it may also be repaired with a lower efficiency than any other type of base substitution. One of the major mutagenic base lesions in DNA caused by exposure to reactive oxygen species is 8-oxoguanine (8-oxoG), which has ambivalent base pairing properties and is capable of pairing effectively with both A and C during DNA synthesis (Dany and Tissier 2001; Fowler et al. 2002). The *mutT, mutY* and *mutM* error-prevention systems in *E. coli* and their homologues in eukaryotes protect cells against the effects of 8-oxoG (Boiteux et al. 2002; Fowler et al. 2002).The *mutT* mutator strains display a specific increase in A/T → C/G transversions (Fowler et al. 2002). In plants, chloroplasts have an abundance of reactive oxygen species and hence their DNA may undergo damage by 8-oxoG at high rates. Since the A/T → C/G type of transversion was observed at a higher frequency than the other base substitutions, we believe that this may indicate that the functional homolog of *mutT* gene product present in chloroplasts may be insufficient to efficiently handle the oxidative damage that occurs spontaneously. It is also possible that the chloroplasts may lack any *mutT* encoded repair enzyme, since no *mutT* homolog has been isolated in plants, even for the nucleus. No *mutT* homolog has been identified yet among the annotated genes in the Cyanobase (database for the cyanobacterium, *Synechocystis* PCC6803) indicating that it may be absent in the ancestor to the modern day chloroplasts. In contrast the nuclear homologs for the *mutY* and *mutM* repair pathway have been isolated in plants.

In conclusion, our study presents the first experimental assay for determining the rates of specific spontaneous base substitutions for the cpDNA. The mutational spectrum observed at a target site in the cpDNA of *C. reinhardtii* has also been reported. In

addition, our studies have provided new information about the role of all three bases in the *Aat* II target site in *16S rRNA* for the binding of spectinomycin.

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CHAPTER 4

Creation of a reporter construct to assay for chloroplast DNA replication slippage events

Abstract

Insertion or deletion of one or more bases occurs spontaneously at a higher frequency in regions of DNA that contain repeated sequences (microsatellites). Replication slippage has been implicated to be the mechanism by which these deletions and insertions occur at the repeats. The few estimates that are available for rates of replication slippage in the chloroplast DNA are based solely on evolutionary studies. This study was undertaken to experimentally assess the rates of replication slippage that occur *in vivo* in the cpDNA of *Chlamydomonas reinhardtii*. Since microsatellites occur primarily in the non-coding regions of the chloroplast genome, their changes cannot be measured experimentally as they do not produce any changes in the phenotype. Hence, a reporter construct was created in the cpDNA of *C. reinhardtii*, in which a stretch of GAAA repeats was inserted into a functional gene to monitor changes occurring at this microsatellite target site. Results showed that this site underwent slippage at frequencies that ranged from 1 to 70 per 10^6 viable cells. Analysis of these slippage mutants showed that deletions were the only type of slippage event observed, with deletion of one repeat being the most common event. These observations lead us to believe that during cpDNA replication in *C. reinhardtii*, the template strand is prone to more slippage than is the daughter strand.

Introduction

Microsatellites

Microsatellite sequences, also called simple sequence repeats (SSR) are short tandem repeats of DNA motifs, one to five bases long, commonly found in the genomes of eukaryotes and some prokaryotes (Broun and Tanksley 1996; Zhu et al. 2000). These repeats, which are present in both the non-coding and coding regions of genomes (Toth et al. 2000), exhibit a strong level of instability, undergoing additions or deletions of the repeat units leading to variations in the length of the repeated stretches (Viguera et al. 2001a). Because of their variability and abundance, microsatellite loci have been used extensively as genetic markers in evolutionary and ecological studies of natural populations in eukaryotes (Vasquez et al. 2000; Ishii and McCouch 2000). Genome rearrangements involving unstable microsatellites have been associated with human diseases: for example, a global microsatellite instability resulting from a defect in DNA mismatch repair has been linked to hereditary nonpolyposis colorectal cancer (Marra and Boland 1995) and instability of the mitochondrial DNA (mtDNA) has been associated with several neuromuscular diseases (Brown and Wallace 1994; Pinder et al. 1998).

Replication slippage

The mechanism by which the number of repeats increases or decreases appears to involve slippage during DNA replication (Zhu et al. 2000). Replication slippage is a recombination-independent process that occurs during synthesis of a daughter strand of DNA and involves the following steps: (i) arrest of DNA synthesis within a direct repeat;

(ii) dissociation of the replicative polymerase from the template followed by transient dissociation of the template and the nascent daughter DNA strands; (iii) incorrect re-association of the DNA strands; and (iv) resumption of DNA synthesis (Sia et al. 2000; Bzymek and Lovett 2001; Viguera et al. 2001b). This results in the production of one or more unpaired repeat units on either the template or the nascent strand, and if these unpaired loops are not repaired, a second round of replication would result in a tract that is shorter (if the unpaired repeats are on the template strand) or longer (if the unpaired repeats are on the nascent strand) than the original tract (Sia et al. 2000). Figure 4-1 describes the above process of replication slippage through which alterations are generated in the length of simple repetitive DNA sequences.

Factors that contribute to replication slippage

Error avoidance and correction are essential for reducing rates of replication slippage at short direct repeats (Kirchner et al. 2000). Replication slippage has been shown to be stimulated by blocking the progress of replication, and several DNA secondary structures have been shown to contribute to replication slippage at repeats (Pinder et al. 1998). At the level of DNA replication, several factors operate to reduce replication slippage by preventing the formation of unpaired repeat loops on the template or nascent strand (Xie et al. 1999). Proteins, such as the single-stranded DNA-binding protein (SSB), bind specifically to ssDNA thereby melting hairpin barriers to polymerase progression (reviewed in Chase 1984; Meyer and Laine 1990; Lohman and Ferrari 1994). The interaction of SSB with the ssDNA increases the processivity and thus prevents

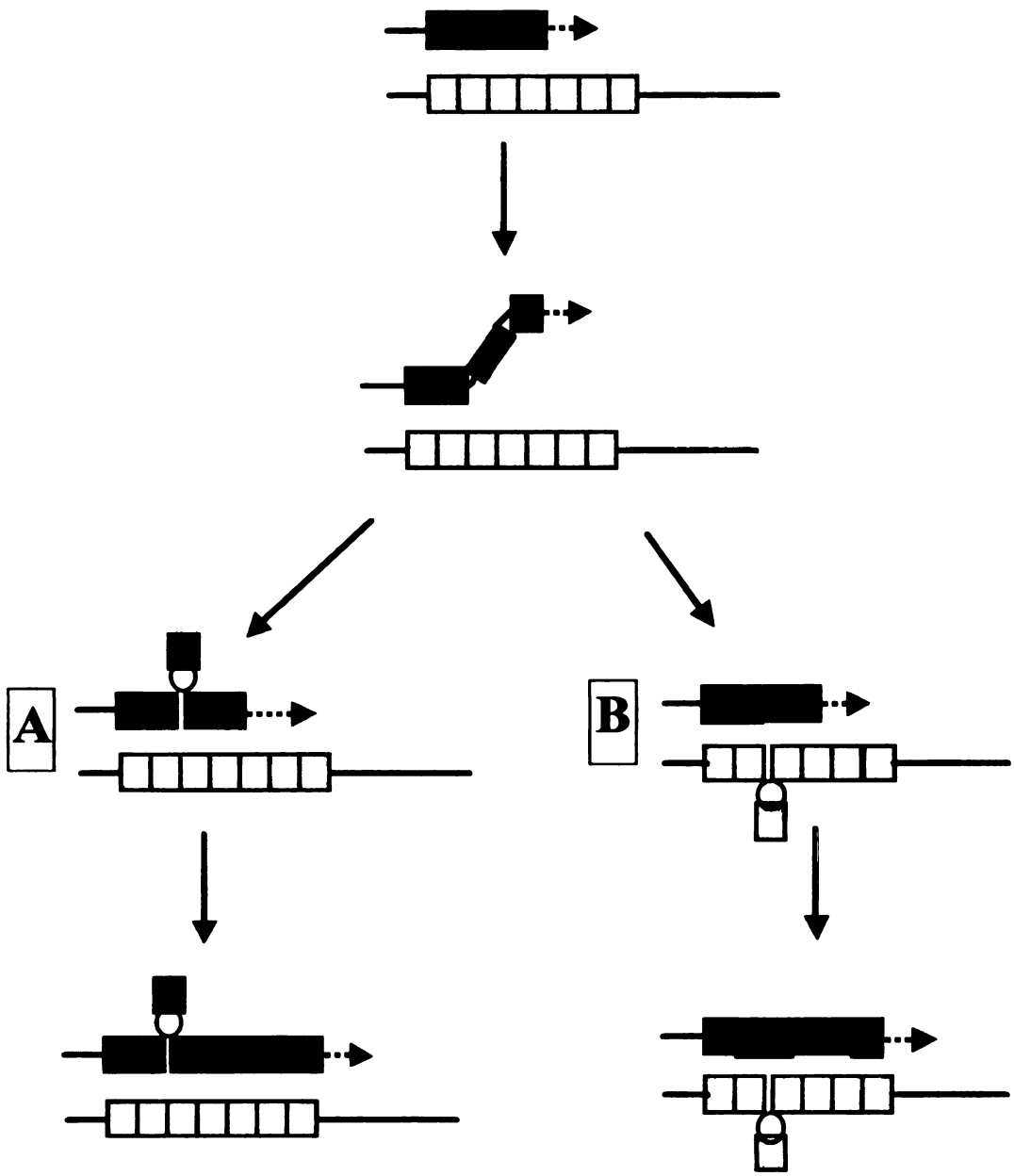


Figure 4-1. Model for replication slippage. ☐ Refers to the repeat units in the template strand, whereas ■ indicates the same repeat units on the daughter strand. Slippage of the daughter strand results in a duplication event (A), while slippage of the template strand during replication results in a deletion event (B).

Adapted from Hancock et al. 1996 and Kokoska et al. 1999)

replication slippage (Kelman et al. 1998; Viguera et al. 2001a). Mutations in the replication fork SSB increase the frequency of replication slippage events. Several other mutations have been reported that induce repeat tract instability by disrupting the processivity of the polymerase causing disassociation of the replication complex from the DNA. In *E. coli* these include mutations in the genes coding for the β clamp and the clamp-loading/recycling machinery of the DNA polymerase III (Saveson and Lovett 1997), while in yeast, mutations in DNA replication genes coding for the polymerase processivity factor PCNA, the flap endonuclease (*RTH1/RAD27*) and the large subunit of the yeast clamp loader (*RFC1*) were shown to confer repeat tract instability (Xie et al. 1999).

In addition to the above mentioned factors regulating the rates of slippage during DNA replication, organisms possess mismatch repair (MMR) systems that help to correct post-replication errors after slippage has occurred. The major system directed to repair loops containing one or a few unpaired nucleotides is the bacterial *mutHLS* pathway and a related but more complex pathway in eukaryotes (Xie et al. 1999). All eukaryotic organisms possess multiple MutS homologs (MSH proteins) and multiple MutL homologs (MLH proteins) with the active forms being heterodimers composed of two different MSH proteins or two different MLH proteins (reviewed in Harfe and Jinks-Roberston 2000). Mutations in these repair genes would therefore result in a phenotype with increased repeat tract instability.

Analysis of microsatellite mutations in the chloroplast DNA

Chloroplast DNA microsatellites

Microsatellites have become established as highly polymorphic markers for genetic studies of plants (Provan et al. 1997). Well-saturated microsatellite maps have been developed from a number of plant species such as rice, maize, barley, *Arabidopsis*, soybean (Ishii and McCouch 2000), and they have targeted mostly the plant nuclear genomes. Recently however, microsatellites have also been identified in chloroplast genomes and have been found in all completely sequenced plant chloroplast genomes to date as well as in hundreds of partial chloroplast sequences from plants such as *Nicotiana tabacum, Glycine soja, Oryza sativa, and Pinus thunbergii* (reviewed by Provan et al. 2001). Chloroplast microsatellites have also been reported recently from the plastid genome of *Chlamydomonas reinhardtii,* a unicellular green alga that is widely used as a model system for chloroplast DNA studies (Maul et al. 2002). Studies with barley, rice and pine have all shown that chloroplast microsatellites can be used to reveal much higher levels of diversity than can be observed through chloroplast RFLPs (Provan et al. 2001). In addition, high levels of length variations have been reported between chloroplast microsatellites from different species or populations of plants such as pine (Powell et al. 1995), rice (Provan et al. 1996; Ishii and McCouch 2000), the evening primrose (Blasko et al. 1988; Wolfson et al. 1991) and maize (Doebley et al. 1987).

Replication slippage rates in the chloroplast DNA (cpDNA) and comparisons with other genomes and organisms

The high allelic variability at the cpDNA microsatellites has been interpreted to be due to the occurrence of high rates of replication slippage resulting in changes in the length of the repeats. This could be due to the fact that the chloroplast DNA replication machinery is prone to "slippage" or that its post-replication repair machinery is unable to repair all the unpaired tracts created by replication slippage at the microsatellites. However, evolutionary comparisons of cpSSRs among different *Pinus* species indicated that SSR length polymorphisms occur in cpDNA at rates ranging from 3.2 to 7.9 X 10^{-5} per site per year (Provan et al. 1999). As a comparison, an experimental study using the nuclear microsatellites in the chickpea plant (*Cicer arrietinum* L), showed that the mutation rates at fifteen $(TAA)_n$ microsatellite loci in inbred populations occurred at much higher frequencies, ranging from 1.0 X 10^{-2} to 8.9 X 10^{-3} per locus in one species and 2.9 X 10^{-3} to 5.0 X 10^{-3} per locus in another species Udupa and Baum 2001). This suggests that relative to the nucleus, chloroplasts may have more efficient mutation avoidance machinery especially for handling replication slippage. However, since slippage rates are known to depend on the type of slippage substrate assayed as well as the length of the repeats in question, these studies may not necessarily be comparable.

An experimental study in *Saccharomyces cerevisiae* examined the relative frequency of mutation of similar microsatellites introduced into the mitochondrial DNA (mtDNA) and the nuclear DNA (Sia et al. 2000). Results obtained from this study have shown that slippage events at nuclear microsatellites occurred at a range between 1.2 X

10^{-5} to 1.3 X 10^{-5} per cell division. In contrast, the mitochondrial rates ranged from 4 X 10^{-6} to 2 X 10^{-10} per cell division. This comparison shows that the mtDNA microsatellites had 10 to 10,000 times lower rates of mutations than did the nuclear microsatellites. Thus, the mitochondria of yeast may possess more efficient DNA replication and repair systems that work to reduce the replication slippage rates at mtDNA SSRs than the respective nuclear counterparts. However, the same conclusion cannot be made for the plants because nuclear SSR mutation rates were based using an experimental system with inbred populations of chickpea (Udupa and Baum 2001) whereas the cpDNA SSR rates were obtained through evolutionary comparisons in pine (Provan et al. 1999).

Comparisons of the available data suggest that the nuclear genomes in plants have 100-fold higher slippage rates when compared to yeast. In humans, the rates for SSR length polymorphisms at nuclear microsatellites ranged from 6.2 X 10^{-4} to 1.2 X 10^{-3} per locus per gamete per generation (Weber and Wong 1993; Dib et al. 1996) while in mice they occurred at a range of 10^{-3} to 10^{-5} (Dallas 1992). Similar studies with cultured mammalian cell lines have shown that the rates of slippage at tetranucleotide repeats $(GAAA)_{17}$ occurred at a range of 7.5 X 10^{-6} to 1.3 X 10^{-5} per cell per generation (Lee et al. 1999). Thus a wide range of mutation rates is seen in nuclear SSRs in animals, but in general they occurred at a much lower frequency when compared to plant nuclear slippage rates. It should be noted that the mutation rates for SSRs from plant nuclear DNAs , yeast and animal nuclear DNA were based on experimental evaluations, whereas the cpDNA rates were based on evolutionary comparisons. Since the cpDNA repeats usually occurred in the non-coding regions of the genome, the mutational constraints

were different when compared to the nuclear repeats that were looked at in experimental assays. In addition, the directionality of the length mutations is often not clear in evolutionary comparison as the starting repeat units are not always well established. (Olmstead and Palmer 1994).

An experimental assay for assessing the rates of mutations for cpDNA SSRs

With the goal of establishing an experimental system to determine the mutation rates for cpDNA microsatellites, we created a reporter construct that allows us to monitor the rates of replication slippage in the cpDNA of *Chlamydomonas reinhardtii.* This unicellular green alga has been used as a model system for the studies of photosynthesis and photoprotection (Dent et al. 2001), and it is ideal for this study because assessment of mutation rates requires growing large numbers of individuals. Due to its size and unicellular nature, large numbers of cells can be grown and placed under selection, while occupying very little space.

An appropriate microsatellite locus had to be selected so that changes in length of the repeat units could be easily monitored. For this purpose, the existing cpDNA microsatellites in *Chlamydomonas* were not ideal as they occur mostly in the non-coding regions of the genome and hence a change in their length would not produce a visible phenotype. Thus, a stretch of repeats was introduced into a cpDNA gene essential for photosynthesis (*rbcL*) such that it created a disruption in its reading frame. Restoration of photosynthetic competence could only be achieved by replication slippage events as outlined in Figure 4-2. The cell lines that were thought to have undergone replication

slippage could be further verified by molecular techniques as well as the exact "type" of slippage event (insertion or deletion) at the microsatellites ascertained.

Recombination at the microsatellites

Since replication slippage occurs at regions of short direct repeats, it would be possible that homologous recombination (mediated by RecA) could give rise to the insertion/deletion events that restore the photosynthetic competence in the lines containing the out-of-frame *rbcL* insertions. An intermolecular recombination between repeat units located on different cpDNA molecules would result in an unequal crossing over, resulting in the formation of two different types of cpDNA molecules: one with deletions of the original repeat tract, whereas another molecule would contain additional repeat units. PCR screening of the cell lines at an early stage should be able to distinguish between recombination and slippage events: a mixture of different sized PCR products with both deletions and the reciprocal additions will be produced by recombination whereas replication slippage would result in the formation of only one sized product.

Choice of slippage substrate

Studies of insertion/deletion events at cpDNA microsatellites of *Oenothera* have shown that several microsatellites contain or are bordered GAAA repeat units (Wolfson et al. 1991; Sears et al. 1994). More importantly, this repeat is also one that has been found to vary in the cpDNA of the *Oenothera plastome mutator* lines.

Grow OF cells to log phase

↓

Plate OF cells on media selective for photosynthetic competence

↓

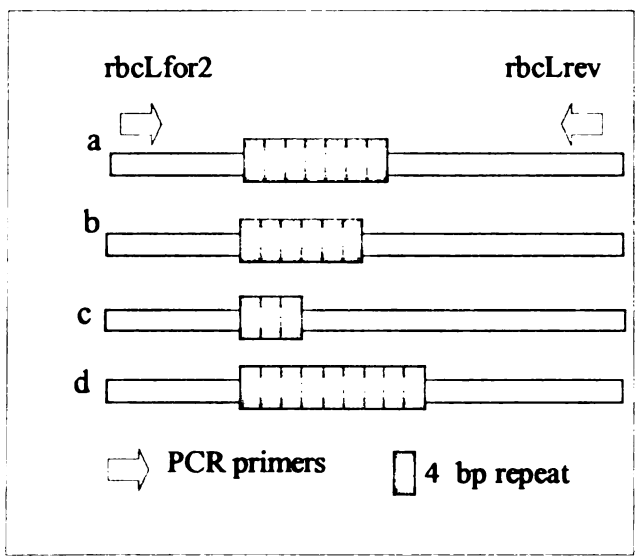
Dilution plating of OF cells on non-selective media for viable cell counts

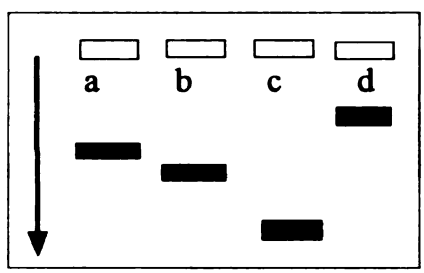
↓

Select "slipped" cells by their ability to grow on non-selective media

↓

PCR amplification using specific primers followed by agarose gel electrophoresis to allow these DNA differences to be readily observed





The PCR products would then be verified by sequencing.

Figure 4-2. Overview of assay for detection of replication slippage in the chloroplast DNA of the OF cells.

A survey of microsatellites from different eukaryotes has indicated that among the tetranucleotide repeats observed in the eukaryotic genomes, the GAAA-type was highly represented (Toth et al. 2000). Studies of nuclear genomes of tomato, swallows and humans have also shown that the GAAA repeats are highly polymorphic (Broun and Tanksley 1996; Lee et al. 1999; Twerdi et al. 1999). Therefore a 28-bp stretch made of GAAA repeats was chosen to be introduced into the *rbcL* gene of *C. reinhardtii* for our slippage assay. This insertion would create a premature stop codon in the reading frame of the *rbcL* gene (Figure 4-3) and hence *Chlamydomonas* cells containing this out-of-frame (OF) construct would be photosynthetically incompetent. Replication slippage events would restore the photosynthetic ability in these cells and thus a screenable phenotype could be obtained for direct visualization of replication slippage events.

In summary, our objective was to provide the first experimental estimate of the rates of replication slippage for a particular cpDNA microsatellite region of *Chlamydomonas reinhardtii*. Using the OF cell line with the microsatellite reporter construct, we could monitor the rates of changes at the (GAAA) repeat tract by screening for photosynthetic competence. Recovery of the photosynthetic revertants provided us with cells that had experienced insertions /deletions at the repeat tracts through replication slippage. The exact change could be determined in these revertants by PCR and sequencing. The rates for each type of slippage event obtained in our study could then be compared with the experimentally established rates of microsatellite mutations of plant nuclear as well as with data from other organisms.

Wild type

```
ATG GTT CCA CAA ACA GAA ACT AAA GCA GGT GCT GGA TTC AAA GCC GGT
 M   V   P   Q   T   E   T   K   A   G   A   G   F   K   A   G
```

An out-of-frame construct with SEVEN REPEATS inserted (6 GAAA and one CAAA)

```
ATG GTT CCA CAA ACA GAA ACA AAG AAA GAA AGA AAG AAA GAA AGA AAC TAA
 M   V   P   Q   T   E   T   K   K   E   R   K   K   E   R   N  STOP

AGCAGGTGCTGGATTCAAAGCCGGT
```

An in-frame construct and a slipped cell line with ONE (GAAA) REPEAT deleted

```
ATG GTT CCA CAA ACA GAA ACA AAG AAA GAA AGA AAG AAA GAA ACT AAA GCA GGT
 M   V   P   Q   T   E   T   K   K   E   R   K   K   E   T   K   A   G

GCT GGA TTC AAA
A   G   F   K
```

A slipped cell line with FOUR (GAAA) REPEATS deleted

```
ATG GTT CCA CAA ACA GAA ACA AAG AAA GAA ACT AAA GCA GGT GCT GGA TTC AAA
 M   V   P   Q   T   E   T   K   K   E   T   K   A   G   A   G   F   K

GCC GGT
A   G
```

Figure 4-3. Sequences and amino acid predictions of a part of the *rbcL* coding region in several *Chlamydomonas* lines.

The out of-frame line has a 28-bp insertion in its *rbcL* gene, creating a stop codon that renders the cells non-photosynthetic. The two types of slipped colonies are photosynthetically competent due to restoration of the *rbcL* reading frame due to deletion of one or four GAAA repeat units. The comparable sequence of the wild-type *rbcL* gene is shown at the top.

Materials and Methods

Chlamydomonas strains, media and growth conditions

The *Chlamydomonas reinhardtii* strain used for biolistic transformation and all replication slippage experiments was the wild-type CC125 (mt+). For crosses, the two strains, CC67 (mt-, cpDNA with erythromycin resistance and wild type *rbcL*) and CC3455 (mt+, cpDNA with wild-type *rbcL* and a dominant negative mutation of the *E. coli RecA*) were used as parents at two different stages in the experiments. All of these above strains were obtained from the *Chlamydomonas* Culture Collection at Duke University (Harris 1989). *C. reinhardtii* was grown in tris-acetate phosphate (TAP) media (Gorman and Levine 1965) on either agar plates or liquid media when required. The cultures were incubated under growth lights at room temperature (22 – 25 °C). The liquid cultures were grown on a rotary shaker under constant light at a speed of ~200 r.p.m. The spectinomycin-resistant biolistic transformants were grown on TAP media supplemented with 100 μg/ml spectinomycin dihydrochloride (Sigma Chemical Co.). The in-frame transformants (IF) were grown on TAP plates with agar and spectinomycin, under high light conditions; whereas the out-of-frame cells (OF) were grown on low-light shelves. The photosynthetic revertants were selected on HS minimal medium (Sueoka high salt medium, Harris 1988) under high light conditions.

Construction of transforming plasmids

The transforming plasmids pIF6 and pOF7 were constructed by Barbara Sears at Duke University (at the Gillham and Boynton laboratory). All manipulations for the creation of

the slippage substrates were carried out with a small3.9 kb subclone (*Bam*HI-*Eco*RI) of the p67 *Eco*RI clone, containing the wild-type *rbcL* gene obtained from the *Chlamydomonas* Genetics Center at Duke University (Harris 1988). A 24-bp insert of GAAA repeats at the *Bsp*MI cut site in the 6th codon position created an in-frame insertion of the *rbcL* gene, and this construct was carried in a plasmid called pIF6. A 28-bp insertion at the same site with an additional GAAA repeat is carried by a plasmid named pOF7. The oligonucletotide pairs used to generate the 24bp and the 28-bp insertions were as follows:

```
IF-F:      5'-GTTTCTTTCTTTCTTTCTTTCTTT-3'
IF-R:      3'-     GAAAGAAAGAAAGAAAGAAACAAA-5'

OF-F:      5'-GTTTCTTTCTTTCTTTCTTTCTTTCTTT-3'
OF-R:      3'-     GAAAGAAAGAAAGAAAGAAAGAAACAAA-5'
```

An even smaller subclone containing the 1.2 kb *Eco*RV-*Eco*RI fragment of the *Bam*HI-*Eco*RI subclone of p67 was used for inserting the slippage substrate, while the *aadA* expression cassette for the cpDNA was inserted into the Mfe I cut site of the *Bam*HI-*Eco*RI subclone of p67. Figure 4-4 shows the diagrammatic representations of pIF6 and pOF7 plasmids that were then transformed into the GM 2163 bacterial strain.

Chloroplast transformations

The chloroplast transformations of *Chlamydomonas* were done using the biolistic transformation method as described by Kindle et al. (1991) and Boynton and Gillham (1993). The wild type cc125 strain was used as the recipient strain and the plasmids pIF6 and pOF7 were used to transform the cells. For the transformations, the cc125 cells were grown under high light on a shaker at 200 r.p.m. to a cell density of about 2 X 10^6

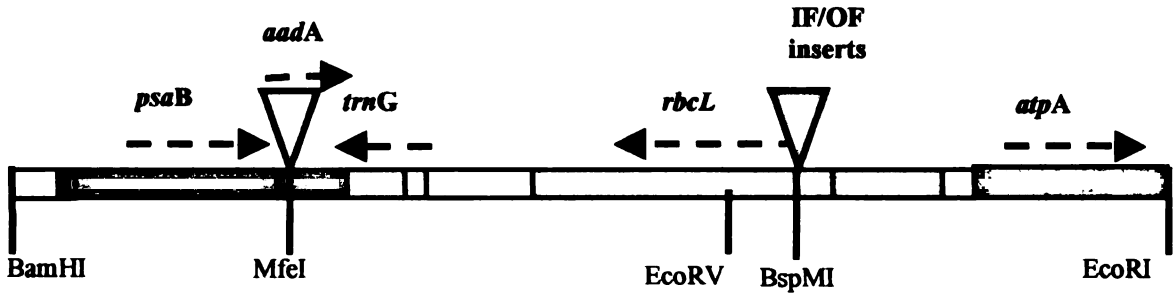


Figure 4-4. Diagrammatic representation of the transforming plasmids (pIF6 and pOF7). The colored boxes represent the chloroplast encoded genes of *C. reinhardtii*, and the arrows indicate the direction of their transcription. The two triangles represent the insertions in the transforming plasmid construct: the *aadA* cassette was inserted between two chloroplast genes and is the selectable marker; the IF and OF insertions were put in plasmids pIF6 and pOF7 respectively. IF contains a 24-bp insertion at the 6^{th} codon position of the *rbcL* gene whereas OF has a 28-bp insertion in the same position. The enzymes used for cloning are also indicated in the figure. The size of the *Bam*HI-*Eco*RI with the *aadA* gene is 5.5 kb. The insert was cloned into the *Bam*HI-*Eco*RI site of the pUC8 vector (size of the transforming plasmid = 8.1 kb).

cells/ml. The cells were then harvested by centrifugation and resuspended in TAP to a final concentration of 2 X 10^8 cells/ml. A 1 ml aliquot of the cells was added to 1 ml of melted sterile agar (42°C) and the two aliquots of 0.7 ml of this mixture were spread on TAP plates. The M10 tungsten particles for bombardment were coated with the transforming plasmid DNA (1 μg/ml) along with sterile aliquots of 50μl of 2.5M $CaCl_2$ and 20 μl of 0.1M spermidine. The particle bombardments were carried out using 1300 psi rupture membranes and each plate was exposed to a vacuum followed by a helium-propelled shot. From each plate the cells were resuspended in 1.5 ml of TAP and then spread on two plates of TAP and agar media supplemented with spectinomycin for direct selection of the transformants. The plates were put under high light when the pIF6 plasmid was used for transformation, while the pOF7 transformants were placed under low light conditions.

Tests for photosynthetic competence

The in-frame (IF), out-of-frame transformants (OF) and wild type cells were grown on different types of media and under two different light conditions to compare their photosynthetic competence. Cells were streaked on solid TAP (Gorman and Levine 1965) or HS media (Harris 1988) and then placed on low light and high light shelves to assay for photosynthetic competence. Similar tests were also performed with liquid media.

Assay for rates of replication slippage in the OF cells

The OF cells were grown in liquid TAP culture to late log phase (2-4 X 10^6 cells/ml) for about 7 days at room temperature under low light on a rotary shaker at a speed of ~200

r.p.m. Cell counts were determined by using the compound microscope and the standard hemocytometer method as described in the Chlamydomonas sourcebook (Harris 1988). 250 ml of the cells was sterilely harvested by centrifugation at 8000 X g in a Sorvall centrifuge using a GSA rotor at 4°C for 10 mins. The supernatant was discarded and the cells were resuspended in a volume of TAP medium that would result in a final concentration of 2 X 10^8 cells/ml.

An aliquot of these cells were used for dilution platings to determine the number of viable cells plated. 100 µl aliquots from the 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated on TAP plates and then grown under lights at room temperature. The number of colonies that grew on these plates was then counted to assess the total number of viable cells that were plated for the experiment.

The remaining volumes of the concentrated OF cells were then plated as 500 µl aliquots on HS minimal media with agar and supplemented with 100 µg/ml of spectinomycin. These plates were put under high lights on a shelf at room temperature. The total number of photosynthetically competent colonies that grew on these plates were counted, and then transferred using sterile toothpicks to HS + spectinomycin plates for further DNA analyses.

Total DNA isolation

Chlamydomonas total genomic DNA was prepared using as described in Chapter 3 of the thesis.

PCR amplification

DNAs were extracted from a sample of thecolonies that had reverted to photosynthetic competence and were used for PCR amplifications, along with the DNAs from the original OF and IF transformants and wild type cc125 cells. The rbcL 5 (5'-GGCCCTTTCTATGCTCGACTG -3') and rbcL mid (5'-CCGAATACGTTACCTAC -3') primers were used in the PCR amplification reactions as the forward and reverse primers respectively to amplify ~560-bp fragments (+24/28)bp from the *rbcL* genes of the wild type, OF, IF and revertant colonies. In some experiments in order to visualize the size differences among the slipped colonies, a smaller PCR fragment was generated (~180 bp) using a different set of primers, rbcL for2 (5'-CTACGTAATCAGGTGTGTAG-3') and rbcL rev (5'-CCGGACAGATTAATTTTAGGA-3'). DNA (1-5ng) was added to individual 100µl reactions containing 200 µmol of each dideoxynucleotide, 2.5 units of Taq polymerase (GIBCO BRL), 10 pmoles of each primer and 1 X PCR reaction buffer (GIBCO BRL). Conditions for the 30 cycles of PCR were as follows: denaturation at 95°C for 2mins, 85°C for 5 mins, followed by 94°C for 40 secs; 30 secs at 48°C to anneal the primers with the template and 30 secs at 72°C for DNA extension. After all the cycles the final extension was done for 10 mins at 72°C. All the PCR reactions were performed with a minicycler model PTC-150-16 (MJ Research Inc., Watertown, Mass.).

The PCR amplified products were run on a 1.5% agarose gel (for the 560-bp fragments) or a 2% agarose gel (for the 180-bp fragments).

A different set of primers was used to check for the presence of the *deltaN RecA* gene in the *Chlamydomonas* strains obtained in crosses, generating a 1kb PCR product. The forward primer was (5'-GTGCCATGGGTTCGCTTTCACTGGATA-3') and the reverse primer was (5'-CTGGCATGCTTAAAAATCTTCGTTAGTTTC-3'. The PCR program used for this reaction is as follows: denaturation at 95°C for 2 min, 85°C for 5 min, followed by 94°C for 1 min; 2 min at 36°C to anneal the primers with the template and 2 min at 68°C for DNA extension. The final extension after all the cycles was done for 10 mins at 72°C. The PCR products were run on a 1% agarose gel.

Sequencing and Computer Analyses

The 560-bp PCR products using the rbcL5 and rbcL mid primers from several samples were sequenced to determine the size of the rbcL segment in the region where the repeats were inserted. The purified PCR products (Chapter 3 for details) were sent for sequencing to the Michigan State University Genomics Facility, East Lansing, MI (www.genomics.msu.edu) and the internal primer rbcL for2 was used to prime the sequencing reaction. Computer analyses were done as described in Chapter 3.

Mutation Rate

To obtain the replication slippage rate, the total numbers of colonies that had reverted to photosynthetic competence were determined for each experiment, and then using the following formula, the mutation rate (μ) was determined:

$$\frac{\text{Number of photosynthetic revertant colonies}}{\text{Total number of viable cells plated (value obtained from dilution plates)}}$$

Chlamydomonas crosses

Crosses were done as described by Harris (1988) and in order to increase the biparental transmission of the cpDNA in the zygotes, a brief (2 min) UV treatment of the mt^+ parent was done before the crosses (Sager and Ramanis 1967). The meiotic progeny from the crosses were dissected by Barbara Sears according to Harris (1988) and the resulting lines were crossed to both mt^+ and mt^- gametes in microtiter plates in order to determine their mating types.

Results

Introduction of replication slippage substrates into the cpDNA of *C. reinhardtii*

Wild-type *C. reinhardtii* cells (CC125) were transformed using the biolistic transformation method for cpDNA (Kindle et al. 1991) with the plasmids pIF6 and pOF7 (Figure 4-4) that contained $(GAAA)_n$ repeat stretches in the "in-frame" and "out-of-frame" versions respectively. 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 cpDNA molecules all containing the repeat insertions in their *rbcL* genes. PCR amplification confirmed the insertion of the slippage constructs in the *rbcL* gene of the transformants. The PCR amplified products using the rbcL5 and rbcLmid primers from the IF and OF strains were visualized on an agarose gel (Figure 4-5), and they were 24-bp and 28-bp larger than the PCR product from wild-type cells (~580-bp). Thus, the

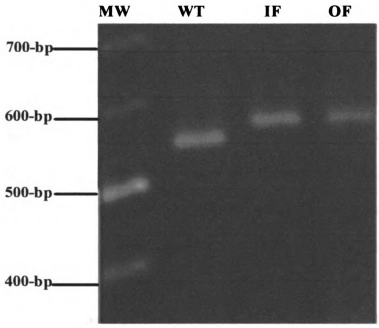


Figure 4-5. Agarose gel showing the PCR amplified products from the wild-type (WT), in-frame (IF) and out-of-frame (OF) cells. The primers used for amplification were the rbcL5 and rbcLmid primers, which generate 560-bp sized fragment for the wild-type cells and 584-bp or 588-bp sized products for the IF and OF cells respectively.

wild-type *rbcL* gene in the IF and OF strains was replaced by the in-frame and out-of frame constructs through homologous gene replacement. Since the OF product was only 4 bases larger than the IF product, the size differences were not clear on the agarose gel. Therefore the IF and the OF lines were sequenced through the insertions in their respective *rbcL* genes and the sequence alignments are shown in Figure 4-3. As seen in the figure, the OF line had a 4-bp larger insertion than the IF line, and based on the sequence information, it can be predicted that a premature stop codon is present in the *rbcL* gene of the OF line that would make the cells non-photosynthetic.

Test for photosynthetic competence in the wild-type and transformed cells

The IF (in-frame) and the out-of-frame (OF) transformants were then grown on media with acetate under low light (non-selective condition), and without acetate under high light (selective condition) to test for their photosynthetic competence. As a control, wild type CC125 cells were grown under similar conditions. As shown in Table 4-1, the IF and the CC125 cells were photosynthetically competent as they were able to grow on media without acetate and also under high light conditions. In contrast, the OF cells were not photosynthetically competent and died on media without acetate and also displayed a high-light sensitive phenotype, similar to other non-photosynthetic mutants of *C. reinhardtii*.

acetate	+	–	+	–
light	**high**	**high**	**low**	**low**
Wild-type cells (cc125)	Growth	Growth	Growth	Growth
In-frame cells (IF)	Growth	Growth	Growth	Growth
Out-of-frame cells (OF)	No growth	No growth	Growth	No Growth

Table 4-1. Phenotypes of the wild-type (cc125) and the transformed cell lines (IF and OF) of *C. reinhardtii* under different conditions.

The presence of acetate in the media provides an additional carbon source and thus enables both the photosynthetically competent and non-competent cell lines to grow on them.

Rates of mutation at the $(GAAA)_n$ microsatellites in the cpDNA of the OF cells

To phenotypically monitor the replication slippage event at the $(GAAA)_n$ repeats in the *rbcL* gene, the OF cells were plated on media that would select for the restoration of photosynthetic competence. To accurately calculate mutation rates, the number of viable cells plated was determined from dilution plating of the OF cells on a non-selective medium. Procedures for determining the cell viability and mutation frequency are described in the materials and methods section. Since the reading frame for the *rbcL* gene in the OF line could only be restored through the elimination or addition of the repeat units, the numbers of cells growing on the selective media would represent the number of cells that had "slipped". Replication slippage at the $(GAAA)_n$ microsatellite region in the three different experiments ranged from 1-70 in 10^6 viable cells plated (Table 4-2). A subset of the slipped cells from each experiment was then randomly chosen for characterization of the changes at the cpDNA microsatellite locus.

Molecular analysis of slippage events at the $(GAAA)_n$ microsatellite site

Since the microsatellite reporter construct in the OF cells consisted of a 28-bp insertion composed of a stretch of $(GAAA)_n$ repeats, a loss of one repeat unit or an insertion of two repeat units were two of the expected slippage events that could restore

Experiment #	Rates of reverison to photosynthetic competence (per 10^6 viable cells)	# of photosynthetic revertants checked by PCR	# of colonies with deletions due to replication slippage	Frequency of each deletion event	
				-1 repeat	-4 repeats
1	1.09	29	29	15	14
2	10.69	16	15*	9	6
3	69.75	62	62	34	38

Table 4-2. Rates of spontaneous mutations to photosynthetic competence in the OF strain from three different trials.

* Out of the 16 colonies checked by PCR amplification for the type of slippage event, one had undergone recombination while all others showed deletions of the repeats.

the reading frame of the disrupted *rbcL* gene. In addition, a deletion of 4 repeats (16 bp of the original 28bp construct) or an insertion of 5 repeats (creating a 48 bp insert at *rbcL*) could theoretically also restore photosynthetic competence in the OF cells. A subset of the "slipped" cells with restored photosynthetic competence was chosen from each of the three experiments to observe the types of slippage that had occurred and also to analyze their respective frequencies of occurrence.

PCR amplification of the DNA from the slipped cells, using *rbcL*-specific primers (rbcL for2 and rbcL rev) showed that among all the "slipped" colonies demonstrating a restoration of photosynthetic competence, deletions were the only type of slippage event that was observed (Figures 4-6 and 4-7), except in one cell line (Line 3 of Figure 4-7). In both the figures, the lane OF shows the PCR amplified product from the out-of-frame cells that is 169-bp long including the 28-bp insertion at the sixth codon position in the *rbcL* gene. Two different variants of the PCR amplified products were observed in the slipped cell lines: both were smaller than the 169-bp fragment found in the OF cells, but larger than the amplification product (141-bp) from the wild-type *C. reinhardtii* cells (Lane WT). The slipped lines labeled 1 and 2 in Figure 4-6 have a PCR product that is slightly lower than the band obtained from the OF cells, indicating that it had probably undergone a deletion of a single 4-bp repeat unit. The PCR product in lane 3 in Figure 4-6 is smaller than the ones that show a loss of one repeat unit (165-bp) but is larger than the wild-type fragment. Since the only other deletion event that could have given rise to the restoration of the photosynthetic competent phenotype was the loss of four repeat units, the size of the PCR product was postulated to be 153-bp.

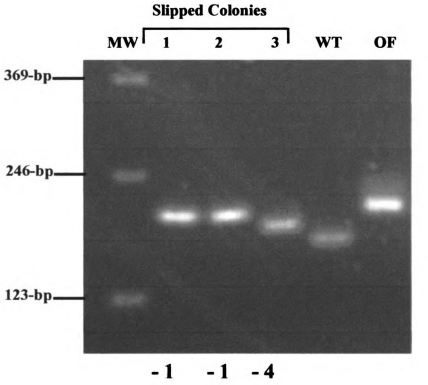


Figure 4-6. Agarose gel electrophoresis showing the PCR amplified products from the slipped colonies with one or four repeat units deleted. The slipped colonies are numbered from 1 through 3. (–1) and (–4) refers to the number of repeats deleted in each line with respect to the OF strain (which contained 7 repeats). MW indicates the 123-bp DNA ladder, WT refers to the wild-type *C. reinhardtii* strain, and OF is the original transformant with the 28-bp out-of-frame insertion in the *rbcL* gene.

From more than 100 revertant lines that were checked by PCR, only one showed a product size higher than the PCR fragment from the OF cells. In that cell line, two PCR products were seen in one lane, indicating the cells were heteroplasmic. In Figure 4-7, Line 3 has an upper band, which is a higher sized product than the OF PCR product, and a lower band that is slightly smaller sized than the 169-bp fragment seen in OF cells. Most likely, this condition reflects a recombination event, with the two products representing the reciprocal recombinant molecules generated due to recombination between two cpDNA molecules with the GAAA repeats.

To verify the interpretation of events based on the size of the PCR products, sequencing was performed on representatives of the two main size classes from the slipped lines. As controls, sequencing was also done on the wild-type (cc125) and the OF lines. PCR amplification was performed using primers that give a larger product (rbcL 5 and rbcL mid) from all the selected lines. An internal primer rbcL rev, was used to prime sequencing reactions from the PCR products. Sequence comparisons confirmed my interpretations of the PCR data. As predicted, of the two slipped colonies sequenced, one showed a loss of a GAAA repeat unit whereas the other cell line had restored photosynthetic competence due to the loss of four GAAA repeats.

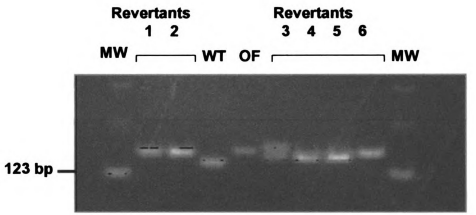


Figure 4-7. Agarose gel showing the PCR amplified products from a subset of the revertant colonies along with the wild-type (WT) and the out-of-frame (OF) cells MW represents the molecular weight marker (123-bp DNA ladder).

Slippage rates in a *C. reinhardtii* strain with a recombination-deficient background

A *C. reinhardtii* strain was constructed in which the chloroplast genome carried both the *rbcL* gene with the 28-bp microsatellite insertion and a *deltaN RecA* gene (by crosses described in Figure 4-8). Cross 1 was performed to move the chloroplast transgenes into a mt^- strain. Since the mating type locus is a nuclear gene, half of the progeny of cross 1 were mt^- cells. Almost all of the progeny of cross 1 inherited the *aadA* and the *rbcLOF* genes since chloroplast markers are transmitted predominantly from the maternal (mt^+) parent. Cross 2 was designed so that a recombinant could be selected that would have both the *rbcLOF* marker and a *deltaN RecA* gene in its cpDNA. The *deltaN RecA* gene which is a dominant-negative mutation was introduced by Cerutti et al. (1995) into the cpDNA of a *C. reinhardtii* strain. In the new strain, cpDNA recombination is reduced five-fold and the cells are sensitive to chloroplast-targeted mutagens, indicating that the *E. coli* gene is able to have a negative impact on the endogenous chloroplast recombination and repair system.

The *deltaN RecA* strain used in the cross was first verified for the presence of the *RecA* gene by PCR amplification using appropriate primers (described in Materials and Methods). Since these cells also show reduced survival rates in the presence of a mutagen, 5mM 5-fluorodeoxyuridine (FdUrd) relative to wild-type cells (Cerutti et al. 1995), experiments were done to verify the high sensitivity of the *deltaN RecA* strain to the above mutagen (data not shown). In *C. reinhardtii* about 5% of the zygotes produce colonies have biparental inheritance of cpDNA. The *deltaN RecA* gene was carried in the cpDNA of the mt^+ parent, whereas the slippage construct was contained in the cpDNA of

Cross1: Transfer of the cpDNA slip construct of the OF line mt^+ into a mt^- background.

(mt^+) OF (cpDNA: spec-resistant, *rbcL* -OF) (*aadA*)

X

(mt^-) cc67 (cpDNA: spec-sensitive, wild type *rbcL*)

↓

Select cell line that is mt^- containing the *aadA* gene and *rbcL*-OF.

Cross 2. Introduction of the cpDNA slippage reporter construct into a strain with a chloroplast recombination-deficiency (containing a dominant negative *E. coli RecA* gene that lacks the first 42 amino acids).

(mt^+) cc3455 (cpDNA: *deltaN RecA*, spec-sensitive) (wild type *rbcL*)

X

(mt^-) OF (cpDNA: spec-resistant, *rbcL*-OF) (*aadA*)

↓

Select a line containing the *aadA* gene, *rbcL*-OF and the *deltaN RecA* gene.

Figure 4-8. Crosses to introduce the slippage construct (GAAA repeats) into a chloroplast DNA subject to reduced homologous recombination.

mt^- parent. The slippage construct included the *aadA* cassette that confers spectinomycin-resistance to cells, and thus biparental progeny could be selected based on their resistance to spectinomycin.

An initial cross in which the zygote progeny were immediately selected for spectinomcyin resistance failed to produce progeny with the desired combination of markers, so the mt^+ parent was UV-irradiated immediately prior to mating to increase the frequency of biparental progeny (Harris 1989). The 2-minute exposure of the mt^+ gametes to UV-irradiation from a hand-held lamp resulted in 15.3 % biparental zygotes (data not shown) and tetrad analysis was performed to obtain individual meiotic progeny. The dissected meiotic progeny were allowed to form colonies on non-selective media and then spectinomycin-resistant cells were selected and checked for the presence of both the slippage construct and the *deltaN RecA* gene in the cpDNAs by PCR amplification as described in Materials and Methods.

The cpDNAs from the progeny were amplified using the *rbcL*-specific primers to check for the presence of the 28-bp insert in their *rbcL* gene. Figure 4-9 shows that all the progeny from Cross 2 (a through q) that showed resistance to spectinomycin, also contained the 28-bp out-of-frame insertion in their *rbcL* gene. Figure 4-10 shows the PCR amplified products from a subset of these progenies from Cross 2 (a-m) using the RecA primers. Among the tetrad progenies showing resistance to spectinomycin, only progeny c, g, f, j, k and m had the *deltaN RecA* gene in their cpDNAs. Combining the results from figures 4-9 and 4-10 two colonies from progeny "f" that contained both the

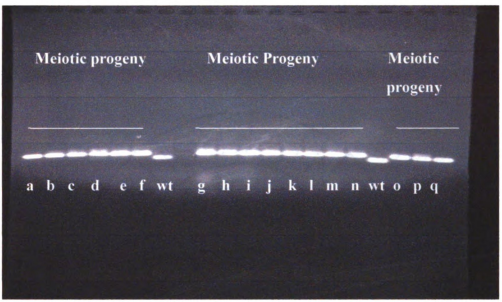


Figure 4-9. Agarose gel showing PCR amplified products from the meiotic progeny of the cross CC3455 X OF, to check for the slippage insert. The lane wt refers to the PCR product from the wild-type *Chlamydomonas* (CC125).

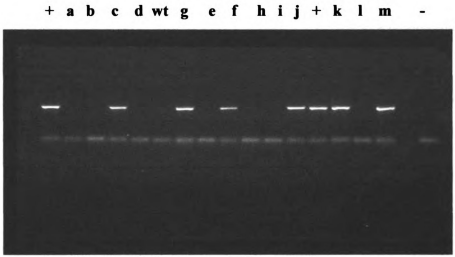


Figure 4-10. Agarose gel showing PCR amplified products of the meiotic progeny from the cross CC3455 X OF, to test for the presence of the delta N RecA gene. (+) represents the parent CC3455 which is the positive control containing the delta N *RecA* gene, whereas wild type CC125 is the negative control (-) .

slippage construct and the d*eltaN RecA* gene were chosen for future replication slippage experiments. Since these cell lines have a chloroplast recombination-deficiency, the data obtained should allow us to determine the impact of recombination on insertion/deletion events at the GAAA-repeat region. The results of Table 4-3 show that spontaneous replication slippage conferring photosynthetic competence even in a recombination-deficient background occurred at a range of 52-54 per 10^6 viable cells plated. These rates do not differ from those in a recombination-proficient background (1-70 per 10^6 viable cells plated) of *C. reinhardtii.*

Discussion

Replication slippage has contributed significantly to genome evolution in organisms as diverse as eubacteria, humans and plants (Hancock 1996). Replication slippage affcets the size of genomes in both ORFs and non-coding regions in bacteria (Gur-Arie et al. 2000) and in eukaryotes (Metzgar et al. 2000). Polymorphisms of microsatellite regions have made them useful as markers in genetic studies especially in forensics and genome mapping (Udupa et al. 1999). In plants microsatellites have been reported from the nuclear DNA of a number of species; examples include *Arabidopsis*, soybean, corn, tomato, barley, and chickpea (Broun and Tanksley 1996; Udupa and Baum 2001). Chloroplast genomes have also been shown to contain large numbers of microsatellites or SSRs, with their presence documented in algae such as *C. reinhardtii* (Maul et al. 2002), in bryophytes such as *Marchantia polymorpha* (Powell 1995a), and also in seed plants such as the gymnosperm *Pinus* and in angiosperms such as *Glycine*

Colony #	# of viable cells plated (X 10^6)	# of cells that showed photosynthetic competence	Rate of mutation to photosynthetic competence (per 10^6 viable cells plated)
1.	65	3360	**51.7**
2.	104.2	5640	**54.1**

Table 4-3. Rates of spontaneous replication slippage events in the chloroplast DNA of *Chlamydomonas reinhardtii* containing the OF construct in a recombination-deficient background.

max, *Zea mays and Oryza sativa* (Doebley et al. 1987; Hiratsuka et al. 1989; Powell et al. 1995a; Powell et al. 1995b; Provan et al. 1996).

Although the existence of microsatellites and their variability has been well documented in the chloroplast genomes of many plant species, few studies have been done to observe their rates of change. Phylogenetic comparisons have determined the relative rates of insertion/deletion events at these cpDNA microsatellites in an evolutionary context, and results have indicated that in comparison to nuclear DNA, the chloroplast DNA has a low mutation rate for microsatellites (Provan et al. 1999; Udupa and Baum 2001). The evolutionary studies have also indicated that insertion/deletion events at the cpDNA microsatellites occur at a much higher rate than do base substitutions (Clegg et al 1994; Provan et al. 1999; Sears et al. 1994). However it should be noted that the assessment of mutation rates at microsatellites is more difficult to assay than base substitutions, as one change often obscures the next. In addition, evolutionary data is often unable to assess the directionality of repeat length changes at the microsatellites. Our goal was to establish a reporter assay that would allow us to determine the rates of replication slippage at the cpDNA microsatellites and also ascertain the exact nature of the changes at the repeat regions (insertions/deletions).

In order to experimentally assess the rates of replication slippage in the cpDNA, a model system for chloroplast studies was used: *C. reinhardtii*, the unicellular green alga, was ideal for such studies as its cpDNA can easily be manipulated and also large numbers of cells can be observed, utilizing very little space. Since the naturally occurring

microsatellites of the cpDNA are mostly present in the non-coding regions of the plastid genome, a reporter construct was created to monitor replication slippage events *in vivo* using a native cpDNA gene, *rbcL,* in order to provide a phenotypic selection for the slippage event. The *rbcL* gene product is essential for the fixation of carbon from CO_2, and an out-of-frame insertion in the gene would therefore disrupt its reading frame and would result in the death of *C. reinhardtii* cells on media without an organic carbon source (acetate). Therefore if a stretch of microsatellites was inserted into the *rbcL* gene, disrupting its reading frame, the *C. reinhardtii* cells with this construct would not be able to grow on media lacking acetate. Slippage in the cpDNA could then be phenotypically observed by the restoration of photosynthetic competence in these cells: a deletion of one or more repeat units or an insertion event due to replication slippage would result in the above phenotype. However, since this assay required that the *rbcL* gene product would be functional with the addition of extra codons, initial experiments were done to prove that an in-frame insertion of additional codons in the *rbcL* gene of *C. reinhardtii* would allow the cells to remain photosynthetically competent. Results showed that an in-frame insertion (IF) of 24-bp composed of five GAAA repeat in the chloroplast encoded *rbcL* gene of *C. reinhardtii* did not affect the photosynthetic competence of the cells (Table 4-1 and Figure 4-5). On the other hand, an out-of-frame insertion (OF) of 28-bp composed of six of the same repeats resulted in cells that were unable to live on media without acetate. Therefore, the phenotypic assay for monitoring replication slippage *in vivo* in the cpDNA could be used for the OF cells.

Rates of replication slippage in the OF cells could then be calculated by comparing the numbers of cells with restored photosynthetic ability with the original numbers of cells plated. In three experiments, the rates of insertion/deletion events in the cpDNA at the GAAA-repeat site occurred from 1 – 70 per 10^6 viable cells plated. There is however a limitation in this experimental approach: the rates obtained in our study represent only the slippage events that have restored the *rbcL* gene function and thus, changes that would have occurred at the repeats without restoring the reading frame do not contribute to the rates. However, slippage events involving a single repeat have been reported to be the most frequent in bacteria (Levinson and Gutman 1987) and plants (Provan et al. 1996). In comparison, my results have shown a nearly equal frequency of deletions of one and four-repeat units, with one repeat deletion being slightly more frequent.

Several factors affect the rates of replication slippage, these include: the size of the repeat units, length of the microsatellite stretch, sequence context of the repeats themselves and finally the position of the repeat units in the genome (Blasko et al. 1988; Clegg et al. 1994; Gragg et al. 2002; Harfe and Jinks-Robertson 2000; Yamada et al. 2002). To enable experiments to proceed that will determine whether different substrates in the same position of the *rbcL* gene would slip at similar rates, I have created a *C. reinhardtii* strain that has a stretch of adenines inserted at the same site. In addition, a new reporter gene containing the same GAAA repeats is being constructed that can be placed at different regions of the cpDNA to assess the effects of genome context in replication slippage rates.

Features of replication that are intrinsic to chloroplast genomes can explain the seventy-fold difference in the rates for replication slippage in the cpDNA among the three different trials. Organelle DNA replication occurs in a relaxed rather than stringent fashion: DNA is replicated randomly at every round. In such a case one type of cpDNA molecule may be replicated more often than the others by chance alone (Birky and Walsh 1992; Birky 1994). In addition, the partitioning of the cpDNA molecules to daughter cells is also under relaxed control, which means that during chloroplast division, the cpDNA molecules are apportioned randomly between the daughter cells. In my experiments, a higher frequency of replication slippage may have been observed if the initial slippage event occurred early, while fewer slippage events may have been observed if an event occurred late during the growth of the culture. In order to avoid a founder's effect, precautions were taken to exclude any pre-existing slippage present in the starter cells of the assay: these included plating of the early pre-culture cells on media without acetate to look for early revertants, and checking the sizes of their *rbcL* gene with PCR amplification. Furthermore, the three-week delay in growth of the revertant photosynthetic colonies on the selective plates indicates that pre-existing revertants were absent in these trials.

Results from all three experiments share a common feature: with only one exception, all the slipped colonies had experienced deletion of one or four repeat units (Table 4-2 and Figures 4-6 A and 4-6 B). Although deletion of one repeat unit was the most common slippage event (58 out of 106), deletion of four repeats was also frequent (48 out of 106). Since the addition of two repeat units could have also restored the

photosynthetic competence, we conclude that the chloroplast DNA of *Chlamydomonas* has a bias towards deletions rather than insertions by replication slippage. We know that addition of two repeat units can occur spontaneously and can confer photosynthetic competence because we have observed a 2-repeat addition twice in OF lines under long-term maintenance even though none were seen in the experiments described here. These observations give us confidence in concluding that the template strand is more prone to slippage than is the daughter strand in the *Chlamydomonas* cpDNA (Figure 4-1). 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 (Aldrich et al. 1988), mitochondrial microsatellites of yeast (Sia et al. 2000) and nuclear genomes of animals such as snails (Weetman et al. 2002). The deletion bias observed in this report was surprising because short dispersed repeats in the *Chlamydomonas* chloroplast genome have proliferated to a great abundance (Maul et al. 2002). Since these repeats occur mostly in the intergenic regions, and hence it is not known whether the deletion bias seen in our experiments may reflect constraints related to function. Studies in *E. coli* and yeast have shown that general differences in the types of repeats and their expansion exist between coding and noncoding regions of the genome (Tautz et al. 1986; Gur-Arie et al. 2000; Metzgar et al. 2000). It has been hypothesized that this limitation of microsatellite expansion in coding regions is due to selection against frameshift mutations as well as due to specific constraints such as maintenance of the correct distance between regulatory elements or prevention of formation of nucleic acid secondary structures that would affect gene expression (Metzgar et al. 2000).

In order to determine whether the frequency of duplication/deletion of short direct repeats is affected by recombination in the OF lines, the rates of change were determined in recombination-deficient backgrounds. My results have shown that spontaneous insertion/deletion rates in these lines were similar to those in the recombination-proficient background (Tables 4-2 and 4-3), indicating that the photosynthetically competent cells arose mainly due to replication slippage and not recombination. Furthermore, screening the photosynthetic revertants obtained from all three experiments using PCR amplification enabled me to distinguish between recombination and slippage events as the cause of restoration of photosynthetic competence. Among all the colonies screened, only one colony showed apparent recombination products (Figure 4-7, line 3) where one of the two bands was around 4-bp larger than the OF band and the lower band represented the reciprocal recombinant molecule with a 4-bp deletion. In contrast, PCR amplification of all the other photosynthetic revertants showed either a single smaller sized band than OF, representing the slippage event in homoplasmic condition or in some cases a heteroplasmic condition was observed in which the original size of the microsatellite tract and a deleted product was seen (for example, Figure 4-11 lane 3). Collectively, these observations have led me to conclude that the insertion/deletion events at the microsatellites that resulted in the restoration of a photosynthetic phenotype were almost solely due to replication slippage and not recombination.

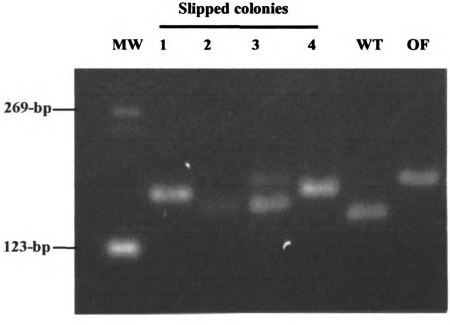


Figure 4-11. Agarose gel showing the heteroplasmic condition of the slipped cell line # 3 containing cpDNAs with the original OF-size insert as well as a 4-repeat deleted cpDNA.
MW refers to the wild-type product and theOF-lane contains the PCR product from the OF line. The slipped colonies are numbered from 1 through 4.

An experimental assay in chickpea has indicated that replication slippage in the nuclear DNA occurs at rates ranging from 1×10^{-2} to 3.9×10^{-3} per locus per generation. In contrast to this, the rates of slippage in the cpDNA from our experimental study with *Chlamydomonas* are at a much lower frequency. Comparing these observations could lead one to hypothesize that the chloroplast may possess more efficient replication or repair machinery than the nucleus resulting in a lower slippage rate. However, it needs to be stated that these studies include substrates that have different sequence contexts, and therefore, a true comparison cannot be made between the mutation rates of these two genomes.

In Chapter 3 of the thesis, rates of base substitutions at a particular target site in the cpDNA were assayed experimentally and the results showed a frequency of 3×10^{-11} to 2×10^{-8}. Those values are 100-10,000 times lower than the frequency of cpDNA replication slippage. A similar trend was also observed in phylogenetic comparisons of cpDNA (Provan et al. 1999; Wolfe et al. 1987). It should be mentioned that the rates of mutations in our studies were observed for two different regions of the chloroplast genome: the base substitution rates were observed for the *16S rRNA* gene that is within the inverted repeat region (IR) while the slippage events occurred in the single copy regions. Because the IRs contain identical repeat units, copy correction can occur and act to either eliminate or fix new mutations occurring in the IR region. If copy correction is likely to occur one way or the other, the mutation rates of genes in the IRs may be half of that or two-fold higher than genes in the single copy regions. Under any circumstance, my results show that base substitutions occurred at 100-10,000 times less often than did

replication slippage of the cpDNA. Hence, I believe that the mismatch repair machinery of the chloroplasts may be able to handle base mismatches more efficiently than the mismatches generated due to slippage. It could also be true that the replication machinery of the cpDNA may have intrinsically low rates of processivity (provided by factors such as SSB, Clamp loader, β-sliding clamp, etc.) that would stall the replicating polymerase and generate a high slippage rate.

In conclusion, this study provides the first experimental assessment of the rates of replication slippage in the cpDNA. Deletion events were by far the most frequent slippage event observed in the OF cells containing the $(GAAA)_n$ microsatellites, and slippage occurred at a range of 1 to 70 X 10^{-6}. Only one recombination event was observed. These observations lead me to believe that primarily the template strand is susceptible to slippage during replication of the cpDNA in *Chlamydomonas reinhardtii*. The reporter construct can now be used to monitor replication slippage events using several different types of repeats to observe whether sequence context plays a role in replication slippage rates in the cpDNA. These rates also provide a background rate for insertion/deletion events at the cpDNA of *C. reinhardtii*, which is an essential piece of information for isolation of mutator strains that show elevated levels of slippage in the cpDNA. Isolation of the defective genes in these mutator strains will provide us with an insight into genes that control cpDNA replication slippage rates in *C. reinhardtii*.

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CHAPTER 5

Isolation of chloroplast mutators in *Chlamydomonas reinhardtii*

Abstract

The isolation and characterization of mutator strains in prokaryotes and eukaryotes have helped to identify essential genes that are involved in mutation avoidance pathways. Since the genes involved in the maintenance of replication slippage stability have not been identified in plants, this study was undertaken to isolate mutator strains that would show an elevated frequency of replication slippage in the cpDNA of *Chlamydomonas reinhardtii.* A plasmid based mutagenesis system was used to isolate these mutators. To date, we have been able to identify one such mutator strain that will now be analyzed to identify the gene disruption that has resulted in the mutator phenotype. Utilizing this assay we could thus isolate a library of mutators, which would help us identify components of the cpDNA mutation avoidance pathways.

Introduction

Mutator cells are those which display a higher incidence of spontaneous mutations than a normal or a wild-type cell. Analysis of mutators has contributed to the study of mutation avoidance and repair pathways, and they have often helped define new pathways of mutagenesis (Miller 1998). Since mutator strains have increased levels of mutation frequencies, it is expected that the mutator phenotypes arise due to defects in genes whose products act to minimize genetic instability (Kunz et al. 1999). In bacteria and yeasts, most of the knowledge about DNA repair has been obtained by the isolation and analyses of the mutator strains. Mutator loci encode components of the mismatch

repair system, exonucleolytic proof reading domains of DNA polymerases, components of oxidative repair pathways, etc (Schaaper and Dunn 1987; Miller 1998; Yang et al. 1999).

Chloroplast DNA replication has been well documented in several plant species using microscopy, genetic analyses and in vitro experiments (reviewed by Heinhorst and Cannon 1993). In addition DNA polymerases and their associated 3'to 5' exonucleases have also been purified from plants such as soybean and pea (Heinhorst et al. 1990; Gaikwad et al. 2002). However to date, none of the genes encoding for the polymerases or other enzymes required during chloroplast DNA (cpDNA) replication has been found. Although genes coding for the repair of the cpDNA has also not been reported in plants, a few cpDNA repair enzymes have been isolated from plants: these include a bacterial homolog of the RECA protein, which is essential in prokaryotes for both homologous recombination and recombinational repair (Cerrutti et al. 1992) and certain DNA photolyases that are important for repair of UV-induced damages in the cpDNA (Small and Greimann 1987; Petersen and Small 2001). Furthermore, the evolutionary comparisons of different plant species have reported that the cpDNA has a lower rate of mutation than the nuclear and mitochondrial genomes (Wolfe et al. 1987), and this suggests the presence of mutation avoidance pathways in the chloroplast.

The complete sequence data from the chloroplast genomes of several plants have all demonstrated the lack of any of the genes involved in cpDNA replication or repair in the chloroplast genome. Therefore the mutator genes that would induce higher rates of

mutations for the chloroplast DNA would be expected to be nuclear-encoded. Chloroplast DNA mutators have been isolated from plants such as barley (*albostrians* and *chloroplast mutator* or *cpm*), maize (*iojap*) and *Oenothera* (*plastome mutator*). The *plastome mutator* of *Oenothera* has been the best characterized among all these mutators: when homozygous it causes a 200-1000 fold increase in the spontaneous mutation frequencies of the cpDNA (Epp 1973; Sears and Sokalski 1991). *The plastome mutator* affected both base substitutions and insertion/deletion events rates, although the latter type of mutations was observed predominantly in the *pm* lines (Chiu et al. 1990; Chang et al. 1996). Further analyses showed that these insertions/duplications occurred primarily at regions of short direct repeats, and hence elevated levels of replication slippage were assumed to be the mechanism by which these changes occurred in the cpDNA substrates targeted upon by the *plastome mutator*. This identity of the *pm*-encoded gene product is till unknown, but based on its mutational spectrum it can be hypothesized that it may code for a gene involved in mismatch repair or factors that affect the processivity of the cpDNA polymerase.

Our objective was to utilize the mutator approach to isolate genes that are involved in the repair and replication of chloroplast DNA. Initial mutator screens would be done to look for strains that would show an increase in the frequency of replication slippage of the cpDNA, and one of these could include the gene homologous to the *plastome mutator* in *Oenothera*. For this purpose, microsatellites were chosen as the substrates for the assay that monitor for a mutator activity; however, the naturally occurring microsatellites could not be used for the assay since a change in repeat length

at the site would not result in a detectable phenotype. Therefore, the replication slippage substrate was created in a reporter construct that would allow the repeat length changes to be observed at phenotypic and molecular levels (See Chapter 4 for details). *Chlamydomonas reinhardtii* was chosen as the experimental organism as it would be possible to easily create a library of mutators that could define several loci involved in the control of replication slippage frequencies in the cpDNA.

Rates of slippage in the cpDNA of wild–type *C. reinhardtii* strains occurred at a range of 1 - 70 per 10^6 viable cells (Chapter 4). Experiments were conducted to create nuclear disruptions by random integration of the transforming plasmid pSP124S (Figure 5-1) which contains a chimeric gene composed of the coding sequence of the *ble* gene from *Streptoalloteichus hindustanus* fused to the 5' and 3' untranslated regions of the *C. reinhardtii* nuclear gene *RBCS2* (Stevens et al. 1996). The *ble* gene confers resistance to antibiotics such as phleomycin or related antibiotics such as zeocin, and transformants were screened by growing the cells on zeocin. Successful transformants were then screened to identify lines that would show higher frequencies of replication slippage over the wild-type. Since a true mutator will be defined by its ability to induce similar elevated levels of slippage mutations at newly introduced cpDNA microsatellites, the "mutator" phenotype of the putative strains were rechecked by introducing a fresh cpDNA slippage substrate into their backgrounds. Once true mutators are identified, further

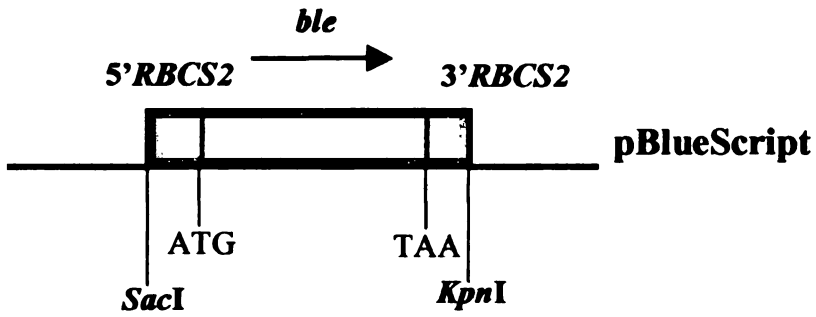


Figure 5-1. Diagrammatic representation of the pSP124S plasmid used for the nuclear transformation of the *Chlamydomonas* cw OF cells. The pSP124S plasmid contains the bacterial gene, "*ble*", which confers resistance to cells against the antibiotics zeocin or phleomycin. The transforming plasmid also has the regulatory regions (5' and 3' *RBCS2*) from a nuclear gene that enables the expression of the bacterial *ble* gene in *Chlamydomonas* cells. The whole size of the plasmid is ~ 4.3 kb (insert is 1.3 kb and pBluescript is 3kb).

characterizations of the disrupted nuclear gene that led to the mutator phenotype would allow us to identify nuclear genes involved in the maintenance of stability at the repeated sequences of the chloroplast DNA in *Chlamydomonas*.

Materials and Methods

Construction of Chlamydomonas strains, growth and crosses

A *Chlamydomonas* cell line (OF, mt+) containing a reporter construct with slippage substrate (used in assays for spontaneous replication slippage rates in Chapter 4) was crossed with cc849, a cell wall-less strain, mt- to produce a strain that would contain the cpDNA mutator target in a cell wall less background. The mt- parent, cc849 was obtained from the *Chlamydomonas* culture collection at Duke University. The OF strain and cc849 were maintained on media as described in Chapters 3 and 4. The nuclear transformants were selected on TAP media supplemented with agar and supplemented with 10μg/ml Zeocin (Invitrogen, USA) and kept under low light conditions. The Chlamydomonas strains were crossed using the protocol described in Chapter 4. The progeny from the cross OF X cc849 were grown under low lights on TAP media.

Selection for cell wall less phenotype in the meiotic progeny

The meiotic progeny that had the slippage substrate in the cpDNA and a cell wall less phenotype was selected as the recipient strain for nuclear transformations. To select for the cell wall less phenotype a modified protocol adapted from the *Chlamydomonas*

sourcebook (Harris 1984) was followed. The cells were scraped off from the plate, dissolved in one ml of TAP in an eppendorf tube, and then centrifuged at 13K rpm for 5 mins. The supernatant was discarded and cells resuspended in an appropriate volume of TAP (depending on the size of the pellet) to obtain a concentrated cell suspension. For each sample, equal volumes of the cell suspensions were added to two eppendorf tubes: one tube contained one ml of sterile water, while the other tube contained a ml of Triton/EDTA solution (Harris 1988). The cells were mixed well, and then cell counts done using the hemocytometer as described in the *Chlamydomonas* sourcebook (Harris 1988). The efficiency of cell lysis was expressed as:

$$\frac{\text{(average cell count in water – average cell count in Triton/EDTA)}}{\text{average cell count in water}}$$

Nuclear transformation of C. reinhardtii

The cwOF strain constructed was used as the recipient strain and 1 μg of digested or undigested pSP124S plasmids were used for transformation. The nuclear transformation was carried out using the glass bead method as described in Kindle (1990) and Stevens et al. (1996), with a few modifications. Sheared salmon sperm DNA (10μg/ml) was used as carrier DNA and the transformants were plated with corn starch for selection, on TAP plates supplemented with Zeocin (Invitrogen, USA).

PCR amplification

PCR amplification was done using the primers rbcL for2 and rbcL rev using the same conditions as described in Chapter 4.

Assay for isolating putative mutators among the Zeocin-resistant nuclear transformants

The transformants were replica plated on TAP media supplemented with Zeocin and kept under low lights to check their viability and growth patterns on non-selective media; and also on selective media containing HS (no acetate) and kept under high lights to select for lines that grow many colonies (compared to the original OF strain that has no nuclear disruptions and grown under similar conditions).

Restesting of the putative mutators to confirm their mutator phenotypes

Crosses were done with the putative mutator strains (photosynthetically competent) with the original OF strain (photosynthetically incompetent) to reintroduce a new slippage substrate into the nuclear disrupted background of the putative mutators. Crosses were done according to the method described in Chapter 4. The meiotic progeny were dissected and then each grown on three different growth conditions: TAP plates kept under low lights, representing the non-selective condition: TAP plates supplemented with Zeocin kept under low lights to identify the progeny that contains the nuclear *ble* gene; HS plates kept under high lights to identify the progeny in which replication slippage has occurred in high frequencies which could be phenotypically observed as green patches (Figure 5-6).

Results

Construction of strains for nuclear transformation

The nuclear transformations were done using the glass bead method of transformation as described by (Kindle 1990; Stevens et al. 1996) and for this purpose, an appropriate cell wall-less recipient strain containing the slippage substrate in its cpDNA was constructed by crosses (see Materials and Methods for details). The recipient strain, cw OF, was selected by checking the meiotic progeny from the crosses using PCR amplification with primers flanking the region of the *rbcL* gene containing the microsatellites. Figure 5-2 shows that the cw OF cells contains the 28-bp insertion of GAAA-repeats in its chloroplast encoded *rbcL* gene. The cell wall less phenotype was also checked for in the same progeny cw OF, using the protocol described in Materials and Methods. Nuclear transformations were then performed with this strain, using both digested and undigested DNAs of pSP124S (Figure 5-3) and also in the presence and absence of a carrier DNA (sheared salmon sperm DNA) to establish optimal transformation conditions.

Conditions for nuclear transformation

cw OFwas used for nuclear transformation using the glass bead method (Kindle 1990) with pSP124S as the transforming plasmid. The transformation efficiencies compared among the different transforming parameters used. The glass bead method for nuclear transformation is explained in details in the Materials and Methods section. Successful nuclear transformants were selected by plating them on TAP media containing Zeocin (10μg/ml). Transformants were obtained from each type of treatment and the

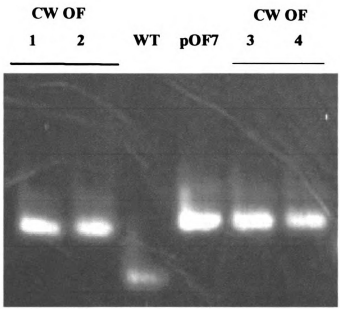


Figure 5-2. Agarose gel picture showing the PCR amplified products from the wild type (WT) , plasmid-pOF7, and the cell wall-less out-of-frame cells (CW OF). The PCR amplified products in lanes numbered 1 and 2 were from the cw OF cells that were used as the starter culture. The PCR products in lanes 3 and 4 were from the DNAs that were obtained from the final culture of cw OF cells used in the transformation experiment.

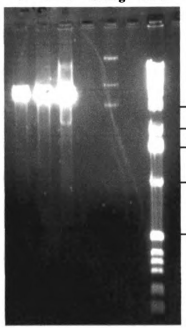


Figure 5-3. Agarose gel showing the pSP124S DNAs used for nuclear transformation of the cw OF cells. The *Kpn*I digested DNAs are labelled from 1 thorugh 3, and the undigested DNA lane is labeled as Undig. MW denotes a lane with the molecular weight standards (1 Kb DNA ladder from Gibco BRL).

results graphed in Figure 5-4. Results showed that both undigested and digested plasmid DNA were able to integrate into the nuclear genome of the cw OF cells, but two of the digested DNA samples (Dig-1 and Dig-2, Figure 5-4), produced Zeocin-resistant transformants at higher frequencies than did the undigested DNAs. The presence of the carrier DNA did not affect transformation frequencies when undigested DNAs were used, but did produce higher efficiency when the KpnI digested psP124S was used as the transforming plasmid (Figure 5-4).

Assay for isolating chloroplast DNA mutators from the Zeocin-resistant transformants

The Zeocin-resistant transformants had the randomly integrated pSP124S plasmid in their respective nuclear genomes as well as the slippage substrate in their cpDNA. The presence of the 28-bp insertion in the *rbcL* gene of the transformants caused them to be non-photosynthetic, as a result of which they were unable to live on media without acetate (HS). As outlined in the overview of the experimental plan in Figure 5-5, our next step was to identify the mutator lines that show increased numbers of cells with restored photosynthetic competence. For this an assay was performed to check for rates of restoration to photosynthetic competence in the different Zeocin-resistant transformants. Out of the 1534 original transformants, only 617 were recovered when they were replated on fresh TAP media supplemented with Zeocin, for maintenance. As shown in Table 5-1, 44 among them displayed a phenotype in which the rates of photosynethetic competence were higher than the original cwOF strain without any nuclear disruption.

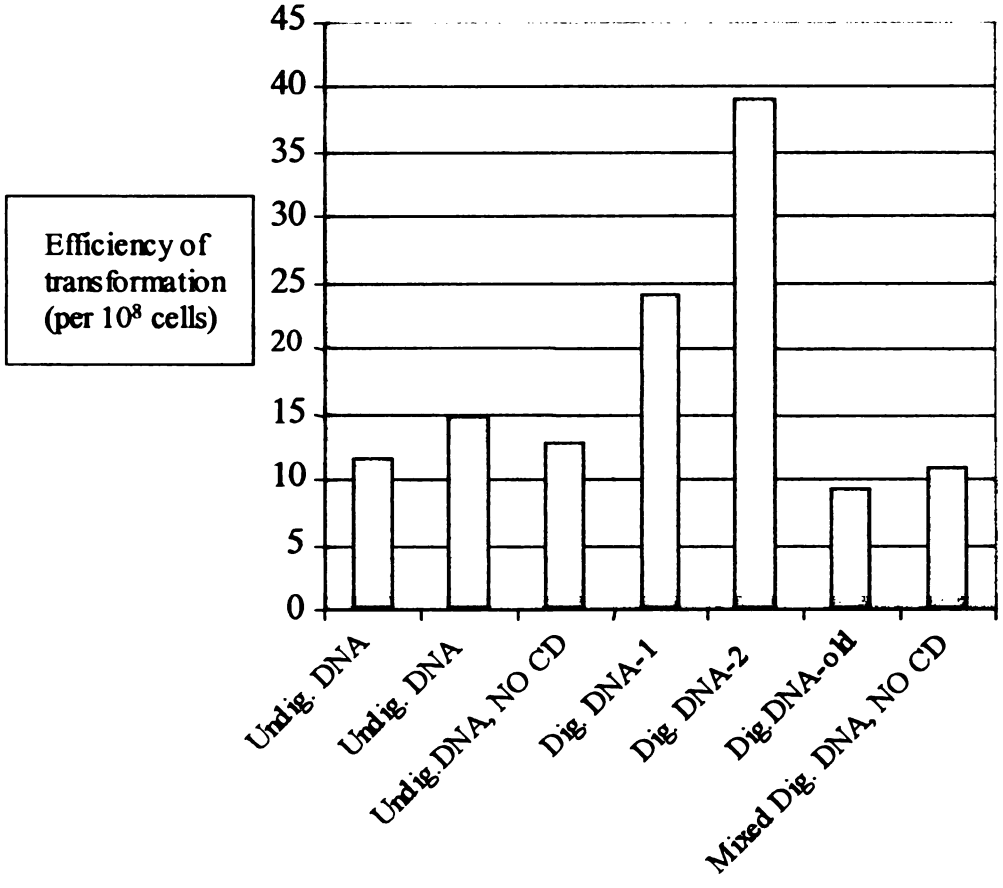


Figure 5-4. Graphical representation of the comparisons of efficiency of nuclear transformation in cw OF cells under different conditions.
Dig. DNA refers to digested DNA with the numbers representing two different tubes from which the respective DNAs were used; undigested DNA is indicated by Undig. DNA on the graph, and CD refers to carrier DNA (sheared salmon sperm DNA). Mixed Dig. DNA refers to the condition when the digested DNAs from the tubes were combined together.

# of initial Zeocin-resistant transformants	# of transformants that were replica plated	# of "putative mutator" lines obtained	# of putative mutator lines tested by crosses	# showing a "true mutator" phenotype
1534	617	44	9	1

Table 5-1. Number of "true mutators" obtained from screening the initial Zeocin-resistant nuclear transformants. Only a subset of the original transformants (non-photosynthetic) was used for replica plating on the non-selective and selective media (for photosynthetic competence). Among the 617 colonies tested, 44 showed an elevated reversion frequency to photosynthetic competence. Crosses were done to introduce a fresh cpDNA slippage substrate into the nuclear background of nine of the putative mutators. One of the re-tested putative mutators showed a "true" mutator phenotype.

Selection of true mutator lines

These 44 putative mutator strains may show a higher level of replication slippage because of an earlier random event, or due to a nuclear disruption in an essential gene that plays a role in the maintenance of the repeat tract stability of the cpDNA. In order to test whether the nuclear gene disruption has caused the elevated level of replication slippage in the cpDNA of a putative mutator line, a new cpDNA microsatellite substrate was crossed into the nuclear backdrop of each of the putative mutators. To date, only nine out of the 44 putative mutator lines have been crossed (Table 5-1).

In a sexual cross of *Chlamydomonas*, the meiotic progeny all contain the cpDNA of the mating type + parent (Boynton et al. 1987) while the nuclear markers are inherited in a Mendelian ratio. Since the line in which the putative mutators were isolated is a mating type - parent, the new slippage substrate in the cpDNA is contributed by the mating type + parent. Hence, almost all the progeny from the cross would be photosynthetically incompetent and their slippage rates similar to the original OF strain. In contrast, the *ble* gene is a nuclear marker and would be present in only half of the meiotic progeny. If the putative mutator lines were true mutators due to a disruption caused by the pSP124S plasmid, then the presence of the Zeocin marker should increase the slippage frequency, resulting in more cells that are photosynthetic. This condition is illustrated in Figure 5-6, which shows one set of the four meiotic progenies labeled as 11-2 through 11-4 from a zygote. As summarized in Table 5-2, the progeny numbers 11-1 and 11-2 were resistant to Zeocin, and also were the only two which showed higher

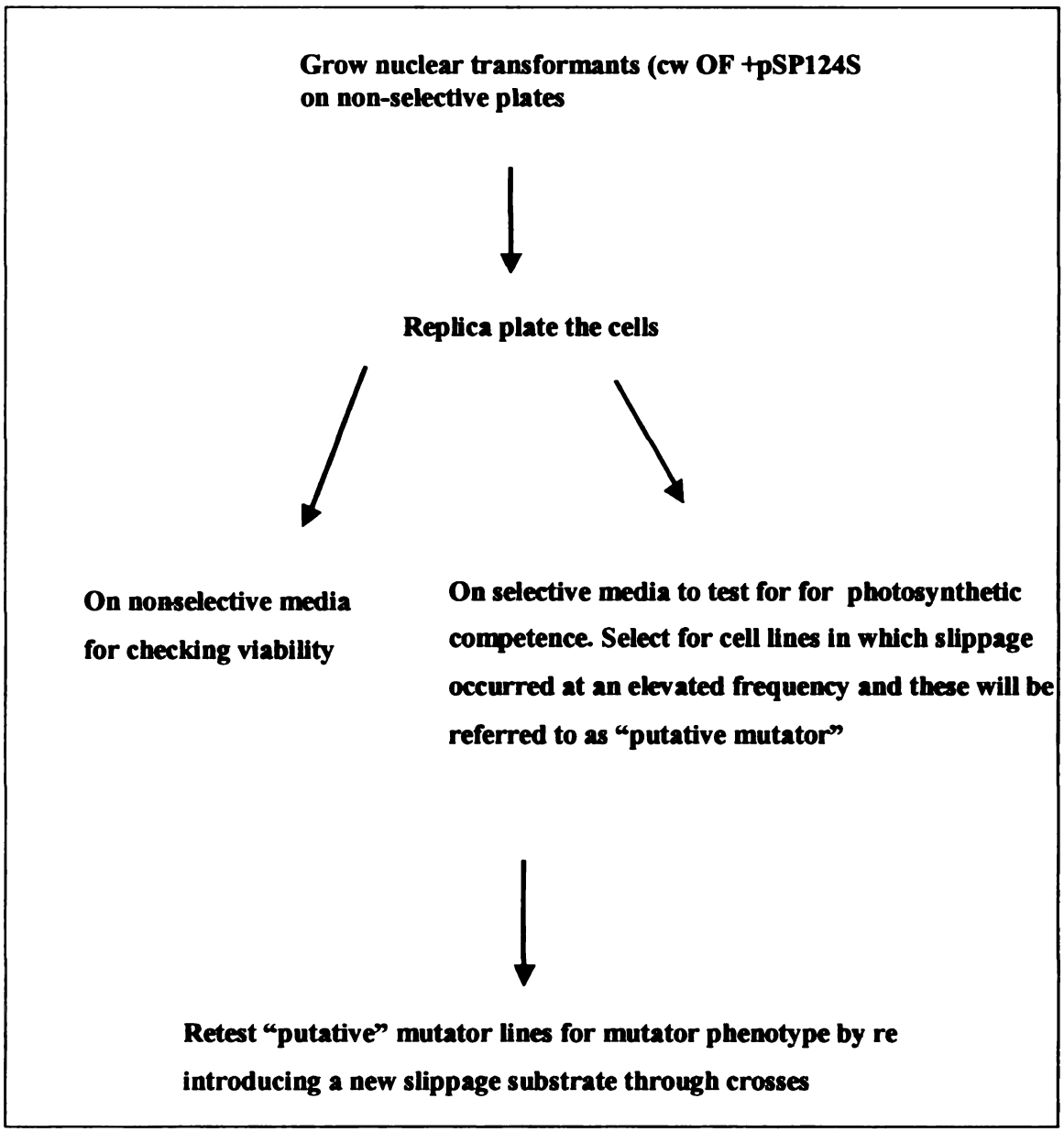
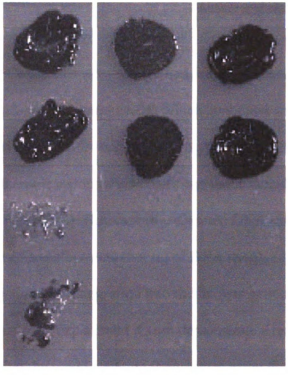


Figure 5-5. Assay for isolating the cpDNA mutator strain

Tetrad progeny	TAP +Low Light (non-selective)	TAP + Zeocin + Low Light (selective for the nuclear marker, *ble*-gene)	HS + High Light (selective for photosynthetic competence)	Mating Type (nuclear marker)
11-1	Y	Y	Y	+
11-2	Y	Y	Y	+
11-3	Y	N	N	-
11-4	Y	N	N	-
14-1	Y	Y	Y	-
14-2	Y	N	N	+
14-3	Y	Y	Y	-

Table 5-2. Phenotypes of two tetrad progenies from a cross between *pm1* (with the putative mutator phenotype, mt-) and an OF line (with the cpDNA slippage substrate, mt+). The chloroplast DNA with the slippage substrate from the mt+ parent (OF) is transmitted to all the tetrad progeny from the cross, whereas the nuclear markers are transmitted in the regular Mendelian ratio (2:2). Phenotypes of two sets of tetrad progenies from the cross are shown here. Y refers to growth on the medium, whereas N indicates that the cells died on this medium.

Non-selective	Zeocin Resistant?	Photo-synthetic?
Y	Y	Y
Y	Y	Y
Y	N	N
Y	N	N



11-1

11-2

11-3

11-4

A B C

Figure 5-6. Growth of tetrad progenies 11-1 to 11-4 obtained from the cross PM1 X OF, under different growing conditions. Panel A shows the growth of the progenies on TAP (+ low light), a non-selective condition; panel B shows the same colonies plated on TAP + Zeocin (+low light), that will only allow the growth of *Chlamydomonas* cells with the *ble* gene, and panel C shows the colonies on HS media (kept under high light), a condition to select for photosynthetic competence.

numbers of cells with restored photosynthetic ability, resulting in a green patch of growth.

Discussion

Conditions were established for the successful transformation of the *Chlamydomonas strain*, cwOF, which contained an out-of-frame slippage substrate in its *rbcL* gene and a cell wall-less phenotype. Nuclear transformants were obtained from all the different transformation conditions used, including both digested and undigested DNA (Figure 5-4). However, the digested DNA samples consistently produced a higher number of transformants (per μg of DNA used), and therefore it seems that it may be more optimal for integration into the nuclear genome. In contrast, results from the nuclear transformation experiment do not demonstrate a requirement for the carrier DNA in increasing the transformation frequencies using the glass bead method when undigested DNA was used as the transforming plasmid. Form this experiment, it is seen that the most optimal condition for producing the highest frequencies of nuclear transformation is when the single digested plasmid DNA is used for transformation along with the carrier DNA. Future experiments need to be done in order to firmly establish the optimal conditions for glass bead transformantion for our strain, as our conclusions are based on a single experiment.

Out of the 600 Zeocin-resistant transformants that were selected from the above glass bead experiment, 44 had shown a "putative" mutator phenotype (Table 5-1).

However, when these were retested for verification of their mutator, only one *(pm1)* out of the nine tested thus far, had a true mutator phenotype. During the growing of the cwOF cells for nuclear transformation, few cells may have undergone slippage with a reversion to photosynthetic competence. These could be mistaken as putative mutator lines if they also were also among the successful nuclear transformants, and this could be a reason why our assay shows a high number of false positives (8 out of nine putative mutators retested). The true mutator nature was established in the *pm1* strain by re-introducing a new cpDNA slippage substrate against the nuclear background of the putative mutator, which would correlate the gene disruption in the putative mutator strain with the increased slippage frequency phenotype of the cpDNA. This true mutator line (*pm1*) is now being analyzed to isolate and identify the disrupted nuclear gene using the plasmid rescue method. This would hopefully lead to the identification of the first gene involved in the maintenance of stability of cpDNA microsatellites.

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CHAPTER 6

Conclusions

Base substitutions and insertion/ deletion events are minimized in organisms through error-free DNA replication, successful inactivation of DNA damaging agents, as well as by the recognition and repair of the DNA damage after it has occurred. Mutation rates of organisms are usually considered as indicators about whether a high degree of fidelity exists during replication, and also as a testament to the efficiency of the DNA repair machinery. Rates of base substitutions and insertion/deletion events have been measured in organisms through experimental investigations as well as by evolutionary studies. Spontaneous mutation rates that have been obtained through experimental evaluations provide a baseline for selecting mutator strains. The isolation and characterization of mutator strains in prokaryotes and eukaryotes have helped to identify essential genes that are involved in their mutation avoidance pathways.

For the chloroplast DNA, investigations to examine the rates of base substitutions and replication slippage through experimental analyses are lacking. Current estimates of cpDNA mutation rates are based solely on phylogenetic comparisons: these studies have indicated that the cpDNA mutation rates are slower than their nuclear counterpart, with synonymous base substitutions occurring at a range of 1.1 to 3 X 10^{-9} synonymous base substitutions per site per year (Wolfe et al. 1987). This low rate of mutation has been hypothesized to be due to efficient replication and repair systems of the cpDNA. In order to have confidence in the existence of a slow molecular clock for the cpDNA sequences, the true rates of mutations occurring *in vivo* need to be examined experimentally.

Our investigation provided the first experimental assessment of base substitution mutations occurring at a target site in the chloroplast DNA of the green alga, *Chlamydomonas reinhardtii*. Our data showed that these mutations occurred in the chloroplast DNA at a range of 3 in 10^{11} to 1 in 10^{8} viable cells plated. Analyses of the different types of base changes allowed us to conclude that transversions occurred about four times more frequently in the cpDNA than transitions. In addition, the spectrum of mutations that occur spontaneously in the cpDNA provided information regarding the existence of different repair pathways that act to minimize their formation or are involved in their repair.

Rates of insertion/ deletion events at microsatellites were also assayed for a particular region in the cpDNA of *C. reinhardtii*. Replication slippage has been identified as the mechanism by which such additions or deletions occur at the repeated sequences. Compared to the base substitution events, replication slippage at a stretch of GAAA repeats occurred 100 –10,000 times more frequently, at a rate of 1 to 70 per 10^{6} cells plated. Furthermore, the cpDNA showed a strong bias towards deletion events, indicating that the template strand is prone to slip more during cpDNA replication, at least at this particular site. In contrast, similar substrates carried on plasmids in *E. coli* showed a higher frequency of slippage, and insertions of repeat units occurred predominantly (Appendix A). This result is particularly interesting as the cpDNA is known to have a cyanobacterial ancestry and hence it was expected that its replication pattern would mirror that of prokaryotes. Experiments are now being conducted in our laboratory to determine the frequency and type of replication slippage in the cyanobacterium

Synechocystis. If the cpDNA slippage event resembles that of the cyanobacteria, it would suggest that the mutational machinery that maintains stability at the cpDNA repeats have remnants of its prokaryotic ancestry.

Mutation rates are dependent on the types of sequences assayed for, as well as on their respective positions in the genome and may also vary depending on the flanking sequences. Future experiments need to be conducted to introduce different substrates for both slippage and nucleotide substitutions in order to get a true assessment of the range of mutations in the cpDNA. In addition, the presence of copy correction in the cpDNA, especially between sequences in the inverted repeat region, should be considered while comparing the rates between different target loci assayed for mutations.

Since the genes involved in the maintenance of replication slippage stability or those that repair the mismatches due to slippage have not been identified in plants, we decided to isolate mutator strains that would show an elevated frequency of replication slippage in the cpDNA of *Chlamydomonas reinhardtii*. The rationale for this approach was that such a mutator phenotype would be caused due to deletion in an essential gene that is otherwise involved in the maintenance of the repeat lengths in wild-type cells. A plasmid-based mutagenesis system was used for gene disruptions. To date, we have been able to identify one putative mutator strain in which a nuclear gene disruption has occurred that appears to control the rates of slippage in the cpDNA containing the microsatellites. The mutator strain is currently being analyzed to identify the gene disruption that has resulted in the mutator phenotype. Utilizing this assay it should be

possible to isolate a library of mutators, which would help us identify components involved in the maintenance of stability of cpDNA microsatellites. In this process we could unravel the different components of the replication machinery that act to prevent slippage at repeats, or identify genes involved in the mismatch repair system of the cpDNA of plants. Since none of these genes have yet been identified or isolated in plants, this library should present us with a unique opportunity for gene discovery.

APPENDIX

Examining the patterns and rates of replication slippage in plasmids of *Escherichia coli*

Introduction

Insertion/ deletion events that occur mostly in regions of DNA containing short direct repeats (microsatellites) are major sources of mutations. Microsatellites are distributed throughout the genomes of both prokaryotes and eukaryotes (Metzgar et al. 2000; Gur-Arie et al. 2000). Eukaryotic microsatellites have been shown to have a high rate of mutation, as a result of which they have become a primary source of polymorphic markers in studies of population genetics (Metzgar et al. 2000). The addition or deletion of repeat units occurs mostly by a mechanism known as replication slippage (Levinson and Gutman, 1987). In bacteria such as *Escherichia coli*, the study of repetitive DNA sequence instability has provided insights into the molecular mechanisms by which cells avoid these insertion/deletion events at microsatellites.

My experimental assay of replication slippage of a cpDNA microsatellite region (Chapter 4 of thesis) has shown that the cpDNA microsatellites undergo mutations much more frequently than do base substitutions (100-10,000 times higher). In addition, the cpDNA microsatellites have a deletion bias, as almost all the "slipped" colonies contained a deletion of one or more repeat units. Therefore, since the same replication slippage substrate used in the cpDNA studies was also available to us on a plasmid contained in *E. coli* (GM2163), we decided to test for the patterns of slippage events occurring in the bacterial cells. Since chloroplasts have been proposed to be descendants of cyanobacterial endosymbionts (Gray 1992), it could be possible that the rates and patterns seen in our study with the cpDNA (Chapter 4) would be similar to those in *E.*

coli. However, since this gene has no phenotypic selection in bacteria, estimation of slippage rates would be difficult.

In order to assess rate of slippage in bacteria, the first goal was to create a reporter construct to monitor the rates and patterns of replication slippage in bacteria. The *aadA* gene is a eubacterial gene that confers resistance to the antibiotic, spectinomycin, in bacterial cells when present (Shaw et al. 1993). The *aadA* gene was chosen and a stretch of GAAA repeats was inserted to create an out-of-frame insertion. Cells containing this out-of-frame construct would thus be sensitive to spectinomycin, and therefore die on LB media containing the antibiotic. Restoration of resistance to the antibiotic will be a result of changes at the repeats, and by counting the number of cells with restored resistance, the rate of slippage in bacteria can be assessed. PCR amplification of the slipped colonies will then allow us to observe the different types of slippage events that would occur in bacteria. Since the same slippage substrate will be put in two different genes on the plasmid (*aadA* and *rbcL*), it could be deduced if genome position affects the rates and patterns of slippage events. Furthermore, a different type of slippage substrate (a 14-bp duplication) was introduced into the *aadA* gene at a different position to determine whether gene context and slippage substrate play a role in the replication slippage pattern and rates.

Materials and Methods

Construction of plasmid strains for the replication slippage assay

One of the plasmids pOF7 used for the assay had been used previously, and details are outlined in Chapter 4. The aadA-OF plasmid was made by creating appropriate primers that would contain the microsatellite of interest along with anchor sequences on either direction that would anneal to the wild type *aadA* sequences carried in a pUC 8 expression vector (p699, Sears lab). The out of frame forward primer used for PCR amplification had the following sequence: 5' AGGTATGCGTCCATGGAAAGAAAGAAAGAAAGAAAGAAAG AAAGCTCGTGAAGCGGT 3'. The reverse primer used for PCR amplification was *aadA* R, which had the following sequence: 5' AAT CTC GCT CTC TCC AGG 3. The PCR product had *Bcl* I and *Nco* I endonuclease cut sites on either side to facilitate directional cloning into the vector.

To create a different type of replication slippage substrate, a 14-bp duplication was generated in the *aadA* gene at the *Bcl* I cut site using restriction digestion and cloning techniques. The plasmid p699 containing the wild type *rbcL* gene and the *aadA* gene was used to create the new *aadA*-Dup plasmid using the oligonucleotides *Bcl* I F and *Bcl* I R to create a 14-bp duplication at the *Bcl* I cut site of the *aadA* gene that resulted in a disruption of its reading frame.

Bcl I F: GAT CAA CGA CCT TT

*Bcl*I R: GAT CAA AGG TCG TT

Replication slippage assays

The pOF7 colonies were grown under no selection in liquid media and then plated on non-selective media (LB-agar with ampicillin). Several assays were undertaken to establish the rates of replication slippage in the *aadA*-OF cells. For one assay, the *aadA*-OF cells were grown in LB media overnight and then plated on media containing spectinomycin to select for revertants with resistance to the antibiotic. For viable cell counts, cells were diluted and plated on media without selection (LB agar with ampicillin). The replication slippage rates were determined by comparing the numbers of colonies that grew on spectinomycin with the numbers of colonies on LB and ampicillin. A second type of assay was done which required plating the *aadA*-OF cells on a non-selective media at several dilutions as well as time points in order to quantify colony forming units. For this purpose, the *aadA*-OF cells were grown on LB media to a certain cell density such that the viable cells could be counted at dilution of 10^{-4}, 10^{-5} and 10^{-6}. Aliquots of the culture were plated at the above three dilutions on multiple plates of non-selective media, and the plates were placed in a 37 C incubator. A subset of the plates representing each dilution was removed at 1-hour intervals, and then a layer of top agar containing spectinomycin or ampicillin was poured over the non-selective media. The number of colonies that grew on the LB+ ampicillin media represented the numbers of viable cells plated at that particular time point, and the number of colonies that grew on the LB + spectinomycin plates represented the numbers of colonies that underwent slippage to restore a spectinomcyin-resistance phenotype.

PCR amplification

The slipped colonies using the pOF7 plasmid were checked by PCR amplification using the primers rbcL for2 and rbcL rev and the PCR program described in Chapter 4. The slipped colonies in the aadA-OF cell line were checked using *psbA* F (5'-AATGTGCTAGGTAACTAA-3') and a*adA* R (5'-AATCTCGCTCTCTCCAGG-3') using the same PCR program as in Chapter 4.

Results

Replication slippage in the GM 2163 cells containing the pOF7 plasmid

The pOF7 plasmid contains the GAAA stretch of repeats in the *rbcL* gene of *Chlamydomonas reinhardtii*, which has no phenotype in bacterial cells. In order to evaluate replication slippage events at this site, the bacteria were grown and plated under non-selective conditions, allowing for mutations to accumulate in the process. Single colonies were then randomly selected (see Materials and Methods for details) and checked using PCR amplification to assess the changes that had occurred at the GAAA-stretch within the *rbcL* gene carried on a plasmid (pUC8 derivative). Figure A-1, shows an agarose gel picture with the PCR amplified products containing the microsatellite region of the *rbcL* gene from the randomly selected colonies. Among the 22 colonies from which DNA was amplified, 10 colonies showed higher sized products than that were present in the original strain. Thus, it appears that replication slippage occurs frequently in bacteria, and that the repeat units show a

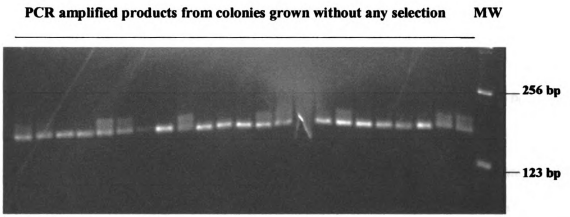

Figure A-1. Insertion events seen in a plasmid containing a stretch of GAAA repeats contained in *E. coli.* Agarose gel showing the PCR amplified products from random colonies of bacterial cells containing the pOF7 plasmid. MW denotes the lane that contains the molecular weight standard (123-bpDNA ladder, Gibco BRL).

bias towards amplification. Estimation of direct rates at this microsatellite region in pOF7 was not possible due to the lack of any phenotypic selection.

Selection of a site within the aadA gene to insert a slippage substrate that could be assessed for frequencies in bacterial cells

In order to assess the rates of replication slippage in bacterial cells, the *aadA* gene that confers resistance to spectinomycin was chosen for the insertion of slippage substrates. The principle was to insert an out-of-frame slippage substrate into the *aadA* gene, which will result in cells that are spectinomycin-sensitive, and a slippage event would subsequently restore the reading frame resulting in the restoration of spectinomycin resistance. At first the *Bcl I* cut site at the 66^{th} codon position of the *aadA* gene was chosen for inserting two different types of slippage substrates, a GAAA stretch of repeats and a 14-bp duplication, to assess for replication slippage frequencies in *E. coli*. My results showed that this region of the *aadA* gene is not amenable for accepting additional codons and hence both these substrates could not be inserted at that position of the *aadA* gene for assessing their slippage frequencies in *E coli*. A different region (the second codon position) of the *aadA* gene was then chosen for the insertion of the GAAA stretch of repeats to assess the rates of replication slippage events. This region as shown in the results below, proved to be flexible in accepting additional codons generated by the insertion of an in-frame slippage substrate, which still retained the spectinomycin resistance.

Estimation of rates and patterns in the aadA gene containing the GAAA microsatellites

The *aadA*-OF cells containing the out-of-frame insertion were assayed for rates and patterns of replication slippage that would occur in the microsatellites (for details, see Materials and Methods section). Figure A-2 shows PCR products from a subset of the slipped colonies, which had a spectinomycin-resistant phenotype. All of the slipped colonies (12 were analyzed) were heteroplasmic, containing the original sized repeats as well as a larger band representing an insertion event. Therefore, the GAAA repeats in the *aadA* gene also preferentially undergo expansions as none of the randomly chosen colonies showed any smaller sized product.

Results from the assays to estimate the rates of replication slippage by comparing the numbers of viable cells plated to the numbers of cells with restoration of the antibiotic-resistance phenotype obtained from several trials failed to establish a consistent rate for the replication slippage events. From the first type of assay for rates, 1 in 100 cells appeared to have undergone slippage (trial 1), whereas in the second trial, 1 in 10 cells showed slippage (data not shown). Form the overlay experiments to assay for rates of slippage; nearly every colony produced mutant cells showing spectinomycin resistance. This could be due to the very high mutation rates, along with the high copy number of plasmids and their short generation time within a bacterial cell as a result of which, the exact replication slippage rates could not be assessed. However, these data leave no doubt that replication slippage in the bacterial plasmids show a very high frequency of replication slippage.

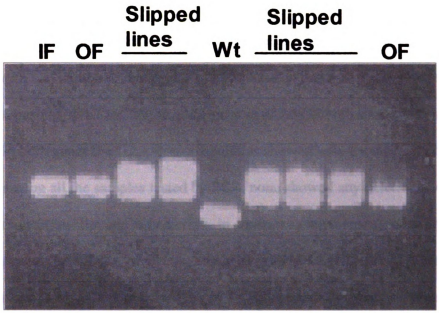

Figure A-2. Replication slippage at GAAA microsatellites in the 2nd codon position of the *aadA* gene. Agarose gel showing the PCR amplified products from the slipped lines that are resistant to the antibiotic; IF and OF are the original strains containing an in-frame or an out-of-frame insertion of GAAA repeats, respectively; along with the wild-type strain without any GAAA repeats (Wt).

Discussion

I observed that replication slippage occurred in plasmids being replicated within bacteria, with and without the presence of any selection pressure: GAAA repeats present in the *rbcL* gene in pOF7 and in the *aadA* gene in *aadA*-OF both showed replication slippage. In both genes the GAAA microsatellites showed only expansions of the repeat units and among all the samples tested by PCR, none showed any deletion events. This aspect of replication slippage in bacteria is in contrast to the scenario seen for the same type of repeat in the cpDNA of *Chlamydomonas reinhardtii* (Chapter 4), which only showed deletions of the repeat units. Therefore, it appears that according to the model proposed by Levinson and Gutman (1987), in *E. coli* plasmids, during replication at a GAAA repeat region, the daughter strand preferentially undergoes slippage, as a result of which duplications of the repeat units occur. In contrast in chloroplasts, the template strand of the replicating cpDNA molecule must be more prone to slippage. Experiments are now being conducted in our laboratory to introduce similar slippage substrates into a cyanobacterial genome in order to observe the types of slippage events for comparisons with the cpDNA.

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