Differential Replication of Two Chloroplast Genome Forms in Heteroplasmic *Chlamydomonas reinhardtii* Gametes Contributes to Alternative Inheritance Patterns

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Manuscript received April 28, 2010

Accepted for publication May 21, 2010

ABSTRACT

Two mechanisms for chloroplast DNA replication have been revealed through the study of an unusual heteroplasmic strain of the green alga *Chlamydomonas reinhardtii*. Heteroplasmy is a state in which more than one genome type occurs in a mitochondrion or chloroplast. The *Chlamydomonas* strain spa19 bears two distinct chloroplast genomes, termed PS+ and PS−. PS+ genomes predominate and are stably maintained in vegetative cells, despite their lack of known replication origins. In sexual crosses with spa19 as the mating type *plus* parent, however, PS+ genomes are transmitted in only ~25% of tetrads, whereas the PS− genomes are faithfully inherited in all progeny. In this research, we have explored the mechanism underlying this biased uniparental inheritance. We show that the relative reduction and dilution of PS+ *vs.* PS− genomes takes place during gametogenesis. Bromodeoxyuridine labeling, followed by immunoprecipitation and PCR, was used to compare replication activities of PS+ and PS− genomes. We found that the replication of PS+ genomes is specifically suppressed during gametogenesis and germination of zygospores, a phenomenon that also was observed when spa19 cells were treated with rifampicin, an inhibitor of the chloroplast RNA polymerase. Furthermore, when bromodeoxyuridine incorporation was compared at 11 sites within the chloroplast genome between vegetative cells, gametes, and rifampicin-treated cells by quantitative PCR, we found that incorporation was often reduced at the same sites in gametes that were also sensitive to rifampicin treatment. We conclude that a transcription-mediated form of DNA replication priming, which may be downregulated during gametogenesis, is indispensable for robust maintenance of PS+ genomes. These results highlight the potential for chloroplast genome copy number regulation through alternative replication strategies.

CHLOROPLAST genome replication mechanisms are not well understood; however, evidence exists for both priming at D-loop type origins (reviewed in HEINHORST and CANNON 1993) and priming via recombination (reviewed in BENDICH 2004). In addition, data from the green alga *Chlamydomonas reinhardtii* suggests that transcription initiated by RNA polymerase can prime replication, a mechanism that would be independent of DNA primase (CHANG and WU 2000). Evidence underlying each of these pathways has been recently reviewed in detail (DAY and MADEIRA 2007). Here we have focused on the *Chlamydomonas* strain spa19 (suppressor of *polyadenylation*), a persistently heteroplasmic strain that maintains two distinct chloroplast genomes (NISHIMURA *et al.* 2004). As we show below,

these two genome types are differentially inherited because they are replicated by different mechanisms.

Heteroplasmy is defined as a state in which more than one mitochondrial (mt) or chloroplast (cp) genotype occurs in an organism, and this state is widely found in plants, algae, fungi, and animals (BARR *et al.* 2005; KMEC *et al.* 2006; WHITE *et al.* 2008). Persistent cpDNA heteroplasmy has been reported in the green alga *Chlamydomonas reinhardtii* (BOLEN *et al.* 1980; SPREITZER and CHASTAIN 1987) and higher plants such as rice, Medicago, and *Senecio vulgaris* (FREY 1999; RAUBESON *et al.* 2007). How the heteroplasmic state is maintained and sexually transmitted to the next generation is still unclear.

The type of heteroplasmy found in the spa19 strain is illustrated in Figure 1A, with its two chloroplast genome types defined as PS+ (required for photosynthesis) and PS− (presence of this genome alone does not confer photosynthetic competence). Spa19 was isolated as a spontaneous photosynthetic revertant of the strain Δ26pAtE, which was created from wild-type (WT) *Chlamydomonas* through chloroplast transformation.

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.118265/DC1>.

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FIGURE 1.—Chloroplast genome organization in *spa19*. (A) Structures of the two chloroplast genomes of *spa19*, named PS+ and PS-. The PS+ genome deletion is shaded. The positions and orientations of *atpB*, *trnE*, *rpl16*, *OriA*, and *OriB* are indicated by circles and arrows. The inverted repeats (IR) are represented by solid boxes. (B) Detail of the PS- and PS+ genome *atpB* regions. In PS- genomes, the region downstream of *atpB* is engineered to encode a poly(A) sequence (pA), an RNase P site, and *trnE* (solid arrow) with its promoter (open triangle). In the PS+ genome, the sequence downstream of the *trnE* promoter is altered due to the PS+ genome deletion and is connected to the endogenous *trnE* promoter and *tufA* in inverted orientation, which is ~145 kb distant in WT cp genomes. The rearrangement breakpoint is delineated by a vertical dashed line. The rearrangement enables distinction between these two genomes either by DNA gel blot analysis (using *PstI*) or by PCR. Primer sets YK12/YK3+spatE and YK12/YK3+BM3'tE specifically amplify the PS+ and PS- genomes, respectively. (C) The PS- genome produces unstable polyadenylated *atpB* mRNA. Since *atpB* encodes the β -subunit of ATP synthetase, which is required for photosynthesis, the PS- genome alone cannot support photosynthesis (top). The PS+ genome generates antisense transcripts to the *atpB* mRNA 3' region, which suppress instability of the sense transcripts, thereby restoring photosynthesis (bottom).

In $\Delta 26pAtE$, which does not contain the PS+ genome, the *atpB* gene had been modified as depicted for the PS- genome in Figure 1B. This structure results in a constitutively polyadenylated *atpB* mRNA that is unstable in chloroplasts, and thus does not support synthesis of the *atpB* gene product, the ATPase β -subunit, which is essential for photoautotrophic growth (Figure 1C; KOMINE *et al.* 2002). Apart from the modified *atpB* gene, the PS- genome contains the same genetic information as WT cpDNA.

Photosynthetic reversion in *spa19* was found to be accompanied by the presence of the PS+ genome, which is a deletion derivative of the PS- genome, spanning the 58-kb white arc in Figure 1A. As shown in Figure 1B, PS+ genomes produce a novel *atpB* antisense transcript as a fortuitous consequence of the apposition of a promoter at the deletion junction. This antisense RNA blocks degradation of polyadenylated *atpB* mRNA and restores photosynthetic activity (NISHIMURA *et al.* 2004). This molecular basis of the *spa19* phenotype is outlined in Figure 1C.

The life cycle of *Chlamydomonas* is summarized in Figure 2, as its various stages are discussed in the context of this study. In *Chlamydomonas*, cp genomes are transmitted uniparentally from the mating type *plus* (mt+) parent (SAGER and LANE 1972; KUROIWA *et al.* 1982; NISHIMURA *et al.* 1999). However, while PS+ genomes occur in severalfold higher abundance than PS- genomes in *spa19* vegetative cells (NISHIMURA *et al.*

2004) and as shown below, the PS+ genome is very poorly transmitted from the mt+ parent following genetic crosses. Figure 3A illustrates that when *spa19* is crossed to the WT as a mt+ parent, two types of viable tetrads were obtained. In type I tetrads, comprising 20 out of 79, or ~25% of the progeny, chloroplasts of all four progeny were indistinguishable from that of *spa19* and the cells were photoautotrophic. But in the more frequent type II tetrad, comprising the other 75% of progeny, all clones were nonphotosynthetic. DNA gel blots (Figure 3B) revealed that whereas PS- genomes were transmitted to both type I and type II progeny, type II progeny universally lacked PS+ genomes. Tetrads that inherited only PS+ genomes were not observed, and we presume these progeny were inviable as they would lack many essential chloroplast genes (Figure 1A).

The presence of the PS- genome, rather than WT cpDNA, confirmed that type II progeny did not inherit cpDNA from the mating type *minus* (mt-) parent, CC-124. Instead, these genetic and molecular analyses indicate that PS+ genomes frequently fail to be transmitted, even if they are present and relatively abundant in mt+ chloroplasts during vegetative life cycle. In the current research, we examined when PS+ genomes were lost and found that this appears to primarily occur during gametogenesis. Our data further suggest that a major contribution to this phenomenon is differential replication of PS+ and PS- genomes, which is probably related to the type of replication origin each possesses.

FIGURE 2.—Sexual life cycle of *Chlamydomonas reinhardtii*.

MATERIALS AND METHODS

Strains, culture conditions, and mating reactions: Cells were grown on Tris–acetate–phosphate (TAP), Sager and Granick (SG) II, or minimal medium (MIN) agar plates at 22° under 14 hr of light followed by 10 hr of darkness to synchronize cell division, which occurs during the dark period (HARRIS 1989). The light intensity was approximately 6600 lux at the surface. The mating reaction was induced by transferring the cells onto SG II agar plates. After 2 days incubation, cells were suspended in nitrogen-free Tsubo mating buffer [1.2 mM HEPES (pH 6.8), 1 mM MgSO₄] at a concentration of approximately 5×10^6 cells/ml. After 5–8 hr under light, equal numbers of mt+ and mt– gametes were mixed and allowed to mate. Gametogenesis begins upon transfer to SG II agar plates, and the mating efficiency reached ~80% after the incubation in Tsubo mating buffer for 5–8 hr. To measure the cell number on SGII agar plates, the agar plates were flooded with 5 ml of Tsubo mating buffer at various time points and cells were suspended carefully with pipettes. The cells were fixed with 4% formaldehyde (final concentration) and the cell density was counted using a hemacytometer.

DNA gel blot analysis and qPCR: Total DNA was purified as previously described (ROCHAIX 1980), and digested DNA was resolved in 0.7% agarose gels, then transferred to GeneScreen nylon filters (NEN Life Science Research Products). Probes were labeled by random priming, and hybridizations were performed as previously described (SAMBROOK *et al.* 1989). Quantitative real-time PCR was performed using the FastStart SYBR Green Master (ROX, Roche, Basel, Switzerland) and the MX3000PTM System following the manufacturer's instructions, and calculations were performed using the associated software MxProTM (Stratagene, Agilent Technologies, Santa Clara, CA).

RNA analysis: Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH). Equal volumes of RNA were used to synthesize single-stranded cDNA in a total volume of 10–20 μ l, using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) with random hexamers as primers.

Single-cell capture and PCR analysis: Cells were suspended at a concentration of 10^{2-3} cells/ml and spread onto 3% agar plates. After drying the plates, 1-mm³ agar blocks containing single cells were excised under a binocular microscope using ultrafine tweezers (Type 5 Titanium, Vigor, Switzerland). The agar blocks were added directly to the PCR reaction mixture. Nested PCR was used to analyze the PS+ and PS– genomes separately. For the PS+ genome, the first PCR was performed with YK12 + spatE, and the second PCR was with YK3 + spatE. For the PS– genome, YK12 + BM3'tE was used for the first PCR and YK3 + BM3'tE for the second PCR. The locations of the primers are represented in Figure 1B, and sequences not previously published are given in Table 1. The PCR conditions were optimized to enhance DNA extraction from cells, by including an initial incubation at 94° for 5 min. Cycles of PCR (35 $\times$ 2) were performed [94° for 30 sec, 55° for 30 sec, and 72° for 30 sec], followed by incubation at 72° for 7 min. One microliter of product from the first reaction was used as a template for the nested reaction.

DNA extraction from zygospores: *Chlamydomonas* zygospores bear chemically and physically robust cell walls with a large component of hydroxyproline-rich glycoproteins (WOESSNER and GOODENOUGH 1989). When matured on agar plates, they adhere tightly to the surface and are also prone to contamination by unmated gametes. To overcome these problems, zygospores were matured in 1.5 ml liquid Tsubo mating buffer and kept in the dark for 1 week. When the mating efficiency was high, pellicles were formed and the supernatant became almost clear. The supernatant was removed carefully and the pellicles were extensively washed with sterile Tsubo mating buffer three times to eliminate unmated gametes, frozen in liquid nitrogen, homogenized with a plastic pestle, and used for DNA extraction.

Fluorescent microscopy and SYBR Green I staining: Vital staining of DNA was performed as previously described (NISHIMURA *et al.* 1998). SYBR Green I (Invitrogen) was used at a final dilution of 1:1,000. In all experiments, the mixture was kept in the same conditions as the original culture and either illuminated or placed in the dark. Stained cells were observed under blue excitation with a fluorescence microscope (Axioskop, Zeiss, Germany) connected to a CCD camera (ProgRes C14, Jenoptik, Jena, Germany).

Bromodeoxyuridine labeling and immunofluorescence microscopy: Log phase cells ($\sim 10^6$ cells/ml) were cultured for 16 hr in TAP containing 1 mM 5-bromo-2-deoxyuridine (BrdU) and 0.1 mM fluorodeoxyuridine (FdUrd). After this treatment, cells were fixed in 4% formaldehyde, and washed three times with phosphate-buffered saline (PBS). Since the anti-BrdU mouse monoclonal antibody cannot detect BrdU in intact DNA double helices, cells were treated with 3 M HCl for 30 min to depurinate and relax DNA structure. After HCl treatment, cells were washed three times in PBS, blocked with 1% bovine serum albumin in PBS for 1 hr at room temperature, and reacted with a 1:1000 dilution of a mouse anti-BrdU antibody (Invitrogen) overnight at room temperature. The cells were then washed three times and reacted with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, highly cross-adsorbed) at 37° for 1 hr. Cells were washed three times again and stained with 0.5% 4,6-diamidine-2-phenylindole dihydrochloride, and then observed under a fluorescent microscope (BHS-RFK, Olympus, Tokyo, Japan). Immunofluorescence microscopy was performed as previously described (MUNAUT *et al.* 1990).

Immunoprecipitation and analysis of BrdU-labeled DNA: BrdU-labeled DNA was extracted, suspended in 250 μ l of IP buffer [25 mM Tris–HCl (pH 7.2), 150 mM NaCl], and sonicated on ice for 30 sec using an ultrasonic homogenizer

FIGURE 3.—*spa19* inheritance patterns. (A) Scheme of the observed inheritance patterns. According to classic chloroplast genetics, all four tetrad progeny inherit cpDNA from the mt+ parent (left column). In this cross, however, both type I and type II tetrads were obtained, as described in the text. For the mt+ parents, WT cpDNA is represented as a solid circle, or in the case of *spa19* the PS+ and PS- genomes as open squares and open circles, respectively. The PS+ label is larger to represent its increased stoichiometry relative to the PS- genome. (B) Genotypes of the resulting progeny were verified by DNA gel blot. Total DNAs of WT, *spa19*, and tetrad progeny were digested with *Pst*I, and a gel blot was probed with the *atpB* coding region. The migrations of PS+ and PS- specific genome fragments are indicated at the right. The WT *atpB* band migrates slightly faster than that of the PS- genome.

(UX050, Mitsui-EC, Chiba, Japan) connected to a microtip (UMS-2), generating an average DNA fragment size of 500~800 bp. To prepare PCR products as internal controls, PCR was performed in the presence of 1 mM BrdUTP (Invitrogen). Samples were denatured by boiling for 5 min, chilled on ice, then reacted with the anti-BrdU antibody for 1 hr at room temperature on a rotary shaker. Then 25 μ l of Protein G magnetic beads (PureProteome Protein G magnetic beads, Millipore Corp., Billerica, MA) were added to each sample, and incubation was continued at room temperature for 1 hr. The magnetic beads were collected and washed 4 times with IP buffer and resuspended in 50 μ l of TE. Immunoprecipitated DNA samples were released by boiling the beads for 5 min. One microliter of the supernatant was used for qPCR analysis.

PS+ and PS- competitors: The primer positions and restriction sites of the PS+ and PS- competitors are shown in Figure 8C. To prepare these competitors, PS+ and PS- fragments were obtained by PCR (primers YK12+spatE for

PS+ and YK12+BM3'tE for PS-), purified by gel extraction, and used as templates for the next PCR. 5' and 3' fragments were amplified by using primers with modified restriction sites (*Xba*I $\rightarrow$ *Spe*I in PS+ and *Eco*RI $\rightarrow$ *Spe*I in PS-), gel extracted, and mixed. Using the mixture of 5' and 3' fragments as a template, PS+ and PS- specific PCR was performed, generating products identically sized to the original ones but containing the restriction site changes. These fragments were inserted into the pGemT-Easy vector (Promega, Madison, WI). To confirm the incorporation of BrdU where it was used for PCR, products were denatured by boiling, chilled on ice, spotted on a nylon membrane, and UV crosslinked. The membrane was blocked in 5% skim milk and reacted with anti-BrdU antibody for 1 hr at room temperature in maleic acid buffer [0.1 M Maleic acid, 0.15 M NaCl pH 7.5]. After washing with maleic acid buffer three times, the membrane was reacted with HRP-conjugated anti-mouse IgG antibody (GE Healthcare, Waukesha, WI) for 1 hr at room temperature. The

TABLE 1
Oligonucleotide primers

Designation	Sequence (5' to 3')	Location	Direction
T7	TGTAATACGACTCACTATAGGGCGA	pGem T easy	F
SP6	ATTTAGGTGACACTATAGAATACT	pGem T easy	R
YK11	CCTTACTGACCCTGCTCCTG	<i>atpB</i> coding	F
KY09	GGTGAACCTGTAAATACTTC	<i>atpB</i> coding	R
YK3	CAAGCATTCTACTTAGTAGG	<i>atpB</i> coding	F
YK12	GCTGAAACTATTGAA	<i>atpB</i> coding	F
spatE	GTAAGGGGATGCCGGAG	<i>trnE1</i> 5' end	F (R in spa19)
B-M3'tE	AGATCTGAATTGAGATTCTCTGACACTGTC	<i>trnE1</i> 3' end	R
CrRecA_F1	GGGAGCTGCACTGATTTAGC	<i>recA</i> coding	F
CrRecA_R1	CTGCCAGTAACAACCCACCT	<i>recA</i> coding	R
RbcL-Fq	GACTGGTGTTCATGCCAGGTGTT	<i>rbcL</i> coding (qPCR)	F
RbcL_Rq	ACTGAAGACATGCGTCATCACCGA	<i>rbcL</i> coding (qPCR)	R
TufA_Fq	AGGTGGTTCGTCCTCTGCTTTTCAT	<i>tufA</i> coding (qPCR)	F
TufA_Rq	ATACGGTCACCAGGCATAGCCATT	<i>tufA</i> coding (qPCR)	R
PsbB_Fq	TTCCGTGACCCACGTACAGGTAAA	<i>psbB</i> coding (qPCR)	F
PsbB_Rq	AGAAGGAGCTACTGGTTGAACGCT	<i>psbB</i> coding (qPCR)	R
PS+ _Fq	AGTAAAGCTGCTTCATTTAA	<i>atpB</i> 3' UTR in PS+ genome (qPCR)	F
PS+ _Rq	ACTATTATTTAGCAAGAATCCTCT	<i>atpB</i> 4' UTR in PS+ genome (qPCR)	R
PS- _Fq	GGTGAATTAGATGATTTACCAGAACAAGC	<i>atpB</i> 5' UTR in PS- genome (qPCR)	F
PS- _Rq	TTTCTCCGTGAAAGGGAGGTGTCCTA	<i>atpB</i> 6' UTR in PS- genome (qPCR)	R
clpP_Fq	TGGCGGTTCTGTAGGTAATGGT	<i>clpP</i> coding (qPCR)	F
clpP_Rq	GCAGATGCAGCTACACCTAATGCT	<i>clpP</i> coding (qPCR)	R
rp116_Fq	TCCGTAAACCACACCGTGGTCATT	<i>rp116</i> coding (qPCR)	F
rp116_Rq	CGTCCGGCTTCAATTTGACGTGAT	<i>rp116</i> coding (qPCR)	R
psbE_Fq	ATGGCTGGTAAACCAGTAGAGCGT	<i>psbE</i> coding (qPCR)	F
psbE_Rq	GGACGTGGAGTACCAAATACGTCA	<i>psbE</i> coding (qPCR)	R
psbD_Fq	CACATGATGGGTGTTGCTGGTGTT	<i>psbD</i> coding (qPCR)	F
psbD_Rq	AATGCACGGAATGTGTTAGCACCG	<i>psbD</i> coding (qPCR)	R
psbC_Fq	TCCAAGTGGTCCAGAAGCAAGTCA	<i>psbC</i> coding (qPCR)	F
psbC_Rq	ACGGAAGTCCCAGAAACGCATAGT	<i>psbC</i> coding (qPCR)	R
atpB_Fq3	GATGATTTACCAGAACAAGCATTC	<i>atpB</i> coding (qPCR)	F
atpB_Rq3	TTGGCTCTTTAAGAAGAAAACAAC	<i>atpB</i> coding (qPCR)	R
maa7_Fq2	GTTCCGGCGGCAAGTACGT	<i>maa7</i> coding (qPCR)	F
maa7_Rq2	CGCAATCGCCTCGTTGTAGT	<i>maa7</i> coding (qPCR)	R
PS+-Xba-Spe-R	CAACTAGTGGCCTAGACG	PS+ point mutagenesis	R
PS+-Xba-Spe-F	CGTCTAGGCCACTAGTTG	PS+ point mutagenesis	F
PS--Eco-Spe-R	GCGACTAGTGAATCCGCG	PS- point mutagenesis	R
PS--Eco-Spe-F	CGCGGATTCACTAGTCGC	PS- point mutagenesis	F
aadA_F	GGCAATGCGTACTCCAGAAGAACT	<i>aadA</i> coding	F
aadA_R	GCCAAGCGATCTTCTTCTTGTTCCA	<i>aadA</i> coding	R
aadA_Rq	GTCGGCAGCGACATCCTT	<i>aadA</i> coding (qPCR)	F
aadA_Fq	TTGTCCCGCATTTGGTACAG	<i>aadA</i> coding (qPCR)	R
SAG1_Fq1	CTGCCAATAGCGTGTGTTGTGGTGT	<i>sag1</i> (qPCR)	F
SAG1_Rq1	CTACTGCCGTTTGTGTTGTTGCCT	<i>sag1</i> (qPCR)	R
Nit2_Fq1	TGAGCAACAGCAACAACAGCAGCA	<i>nit2</i> (qPCR)	F
Nit2_Rq1	AGCTTGTTGTTTCATGGAAGCTGCG	<i>nit2</i> (qPCR)	R

Direction specifies the orientation of the primer with respect to the gene it amplifies. F indicates the same orientation as the gene; R indicates the antisense orientation.

membranes were washed and the signals were detected using ECL chemiluminescence (GE Healthcare).

RESULTS

Reduction of the PS+/PS- genome ratio during gametogenesis: To understand the basis for frequent elimination of the spa19 PS+ genome during sexual

crosses, we analyzed cpDNA at various stages of the sexual cycle, ultimately focusing on gametogenesis, which profoundly affected the PS+/PS- genome ratio. Figure 4A shows this change in ratio in a comparison of DNAs from vegetative and gametic cells, the latter of which continued dividing under gametogenesis conditions (Figure 4C). In this experiment, the upper band, representing the PS- genome, becomes dominant in

FIGURE 4.—Behavior of PS+ and PS- genomes. Total DNAs were isolated from *spa19* cells grown on (A) TAP (vegetative cells, V) or SGII (gametes, G); or (B) nitrogen-depleted TAP (TAP-N) under high light or in the dark for 24 or 48 hr. The DNAs were digested with *Pst*I and analyzed by filter hybridization using the *atpB* PCR product amplified by YK11 and KY09 as a probe. Duplicate samples were blotted on another membrane and probed with *RECA*, which is a nuclear gene. Note that the DNA samples of 24 hr and 48 hr were overloaded in the right side of A. Mating efficiencies (see MATERIALS AND METHODS) are shown under the gels. (C) Cell growth of WT (solid line) and *spa19* (dashed line) during gametogenesis was measured by counting the number of cells on SGII agar plates (see MATERIALS AND METHODS) and in Tsubo mating buffer. The arrow indicates the timing when cells were suspended in Tsubo mating buffer as the final step of gametogenesis. (D) Total RNAs were isolated from cells grown on nitrogen-depleted TAP (TAP-N) under the high light or in the dark as in B. cDNAs were prepared and the relative abundances of *SAG1* and *NIT2* mRNAs were analyzed by qPCR. The data were normalized to the transcript level for *MAA7*, and then the levels for *SAG1* and *NIT2* were set to 1 in vegetative cells for comparative purposes. Error bars represent standard deviations based on three independent experiments. DNAs and mating efficiency were analyzed as for A and B for (E) cells grown in TAP with the indicated concentrations of FdUrd for 48 hr and (F) in TAP modified to contain the indicated concentrations of phosphate.

gametes within 48 hr, irrespective of the *spa19* mating type used. Because induction of gametogenesis includes nitrogen starvation, we examined DNA ratios after *spa19* cells were incubated on nitrogen-depleted medium (TAP-N) under the light or in the dark (Figure 4B). In the dark, cells cannot differentiate into gametes. The differentiation state was corroborated by checking

agglutination with CC-124 mt- gametes (+ and - symbols below gels) and by analyzing the transcript abundance for *SAG1* (a mt+ gamete-specific gene that encodes an agglutinin (FERRIS *et al.* 2002)) and *NIT2* (a nitrate assimilation-specific regulatory gene) by quantitative reverse transcriptase-PCR (qRT-PCR; Figure 4D). Although similar expression levels were observed for

FIGURE 5.—The relative abundances of PS+ and PS− genomes were compared by qPCR between of *spa19* vegetative cells (V) and cells incubated for 24 hr under nitrogen-depleted and high light condition (gametes, G). The regions analyzed are shown below each graph. Positions of the genes are shown on the left. The data were normalized to the nuclear gene *MAA7*, and then the amount in vegetative cells was set to 1. Error bars represent standard deviations based on three independent experiments.

NIT2 irrespective of the light condition, a 20-fold upregulation of *SAG1* mRNA was observed only when the cells were cultured in the light, indicating that *spa19* cells cannot fully differentiate into gametes in the dark. Under these conditions, no change in the PS+/PS− ratio was observed, confirming the association of the phenomenon with gametogenesis rather than N starvation.

Vegetative cells were also incubated under conditions known to reduce the copy number of cpDNA generally, to see whether the two genome types were differentially influenced (Figure, E and F). These conditions were growth in the presence of fluorodeoxyuridine (FdUrd, which inhibits cpDNA synthesis; WURTZ *et al.* 1977), and phosphorus deprivation, which reduces cpDNA content through an unknown mechanism (YEHUDAI-RESHEFF *et al.* 2007). As expected, overall reduction of cpDNA copy number was observed under both conditions; however, there was no apparent bias for PS+ or PS− genomes.

The relative reduction of PS+ genomes in gametes was subsequently quantified using qPCR (Figure 5). Primer pairs were designed for eight positions on the chloroplast genome (Figure 5, left) and a nuclear control gene, *MAA7*. The chloroplast primer pairs targeted PS+ and PS− genome-specific forms of *atpB*, four genes specific to the PS− genome (*clpP*, *rpl16*, *psbB*, and *rbcL*), and two shared genes (*petA* and *tufA*). DNAs were analyzed from *spa19* vegetative cells, and from cells incubated under gametogenesis conditions for 24 hr. By using the *MAA7* coding region as an internal control, relative PS− and PS+ genome abundance could be calculated. This analysis (Figure 5, right) revealed that during the first 24 hr of gametogenesis, while the abundance of PS− genomes remained at 83–96% of the vegetative cell level, the amount of PS+ genomes was reduced to approximately 37%. This is in agreement with the DNA gel blot data in Figure 4A.

Unequal distribution of PS+ genomes during gametic cell divisions: Because the above experiments were conducted on populations of cells, it was not possible to study the distribution of PS+ genomes. For example, a reduced PS+ genome abundance in a population could reflect a similar effect on all cells, or

an “all or nothing” effect that averages out to the reduction observed. To address this issue, individual vegetative cells and gametes (defined as *spa19* cells incubated for 48 hr on SGII plates) were collected under a microscope using fine forceps and analyzed by nested PCR to detect the PS− or PS+ genomes, as shown in Figure 6. In vegetative cells, both PS+ and PS− genomes were nearly universally detected (PS−, 15/15; PS+, 13/15). In gametes, the PS− genome was invariably detected, as in vegetative cells. However, the PS+ genome was detected in only 4 out of 15 gametes. This suggested that most gametes had either lost the PS+ genome or had a sharply reduced copy number.

Preferential increase of PS− genomes in germinating zygospores: The above results show that gametogenesis acts as a trigger to alter the PS+/PS− genome ratio. However, it seemed likely that another developmental stage also affected the PS+/PS− genome ratio, given the high (75%) frequency of type II tetrads, where the PS+ genome had been clonally lost. Indeed, we found that zygospore germination appeared to play an important role. We first examined the behavior of chloroplast nucleoids, which are protein–DNA complexes easily visualized using the DNA-specific fluorochrome SYBR Green I (Figure 7). In young WT zygotes (CC-125 mt+ × CC-124 mt−) 1 day post-mating, the cpDNA aggregated into two to three nucleoids, which are easily distinguished from the larger zone of nuclear DNA fluorescence and are generally much brighter than mitochondrial nucleoids (Figure 7, a and e). After dark treatment for 1 week, young zygotes had matured into zygospores with thick cell walls (Figure 7b). At this stage, the cytoplasmic nucleoids were small and the average size of fluorescent spots was ~0.5 μm (Figure 7f). With their small size, distinction between cp and mt nucleoids was difficult. It was earlier proposed that cpDNA molecules might be consumed at this stage to sustain cellular metabolism (SEARS and VANWINKLE-SWIFT 1994), and perhaps the reduced brightness of apparent chloroplast nucleoids reflects this. After transfer to the light, a dramatic increase in the brightness of cytoplasmic nucleoids was observed before meiosis (Figure 7, c and g), suggesting rapid amplification of

FIGURE 6.—Analysis of PS+ genome loss. Single vegetative cells (V) and gametes (G) were isolated and used for PS+/PS— genome-specific nested PCR analysis, as described in MATERIALS AND METHODS. PC, positive control for PCR. Note that individual cells were analyzed in each case; in other words, the same cell was never analyzed for the presence of both PS+ and PS— genomes due to the method employed.

organellar DNAs in germinating zygozspores. Finally, distinct nucleoids were distributed equally to the tetrad progeny (Figure 7, d and h).

If we were correct in our inference of rapid cpDNA amplification after transfer of zygotes to the light, it was of interest to know whether both PS+ and PS— genomes were being amplified. Due to the technical limitations that limit the quality of DNA extracted from zygozspores, gel blot analysis could not be employed, and we were also unsuccessful in employing quantitative PCR. However, we were able to conduct a semiquantitative competitive PCR assay using total DNA extracted from zygozspores, with the caveat that small numbers of unmated cells could have been trapped in the zygozspore pellicle (see MATERIALS AND METHODS). As shown in Figure 8, the two samples were spa19 zygozspore DNA from either before or after germination (labeled α and β , respectively, in Figure 8). Figure 8A shows amplification of a nuclear gene (*RECA*), and PS+ and PS— genome-specific fragments with 25 PCR cycles, using increasing dilutions of DNA. This result for zygozspores before light exposure (α) roughly reflects the expected relative DNA abundance for PS+ and PS— genomes, since a visible product is obtained for the PS+ genome with a higher DNA dilution (lower concentration). After light-stimulated germination (β), however, the PS— genome is amplified nearly as easily as the PS+ genome, which is consistent with them having comparable copy numbers.

To obtain more quantitative information, the experiment was repeated with a slightly different approach, as shown in Figure 8B. In this case, a constant amount of input DNA was used (10 ng), but increasing amounts of unmated a competitor PCR template were added. These templates had the same length and primer binding sites as the genomic targets, but included a restriction enzyme digestion site so that following PCR, they could be distinguished from genomic DNA by digesting the total reaction products. The position of primers, restriction sites, and the sizes of DNA fragments are shown in Figure 8C.

In the case of the PS+ genome prior to germination (α), amplification of the competitor does not occur

FIGURE 7.—Phase contrast (a–d) and fluorescent images (e–h) of SYBR Green I-stained young zygotes (a, e), zygozspores (b, f), germinating zygozspores (c, g), and tetrads (d, h), from the cross CC-125 mt+ × CC-124 mt—. The bar in e is 5 μ m. The white arrows in g highlight increased fluorescent intensity of cp/mtDNA. After organellar DNA amplification, meiosis began and organellar DNAs were distributed equally to the progeny (d, h).

until 100 pg is added, whereas a small amount of the PS— competitor is amplified when only 10 pg is added. This is again consistent with a higher abundance of PS+ genomes in these cells. When DNA was analyzed after germination (β), however, only 10 pg (*vs.* 100 pg) of PS+ genome competitor was required for it to be amplified, suggesting that the PS+ genome concentration had decreased. In contrast, 1 ng of PS— competitor was required for it to be amplified instead of genomic DNA. Since more competitor was required in the case of PS— *vs.* PS+ to interfere with genomic DNA amplification, we conclude that the relative abundance of PS— DNA is higher in germinating zygotes. We therefore tentatively conclude that PS— genomes can be replicated more efficiently than PS+ genomes in germinating zygozspores.

The replication of PS+ genomes becomes less active in gametes: Altered abundance of PS+ genomes could have at its root reduced replication and/or decreased DNA stability relative to the PS— genome. Differential replication could be a consequence of size and/or sequence distinctions, whereas genome-specific instability seems a less plausible target for regulation. Replication activities of PS+ and PS— genomes were compared using BrdU, a thymidine analog that can be incorporated into replicating DNA. DNA with incorporated BrdU can be enriched by immunoprecipitation with an anti-BrdU antibody and used for subsequent molecular analysis (supporting information, Figure S1).

To compare PS+ and PS— genome replication, cells cultured on TAP or SGII agar plates for 2 days were resuspended in TAP liquid medium or Tsubo mating

FIGURE 8.—Preferential increase of PS[−] genomes in germinating zygotes. (A) PCR analysis to compare the amplification of PS⁺ and PS[−] genomes during zygote germination. Total DNAs from zygospores before (α) and after (β) light treatment were compared using 25 PCR cycles. The indicated amounts (10^{−1} to 10^{−6} ng) of total DNA were used as template, and the nuclear *RECA* signal was used as an indicator of DNA concentration. Primer sets are indicated at the left for each set of reactions. (B) Competitive PCR to compare the amplification of PS⁺ and PS[−] genomes in germinating zygospores. Ten nanograms of total DNA (α or β) was mixed with the indicated amount of competitor and used as templates for PS⁺ and PS[−] specific PCR. The PCR products were digested with *SpeI* to distinguish genomic DNA from the competitor. Primer sets and restriction enzymes used are shown at the left. (C) Top, schematic of competitors for PS⁺ and PS[−] genomes with altered restriction sites (PS⁺, *XbaI* → *SpeI*; PS[−], *EcoRI* → *SpeI*). These fragments were cloned, enabling their amplification with either T7/SP6 primers or with the PS⁺/PS[−] genome-specific primer pairs. Bottom, PCR fragments amplified from spa19 total DNA and competitors with the genome-specific primers, digested with indicated restriction enzymes.

FIGURE 9.—Analysis of DNA replication. (A) Chloroplast DNA replication activity was compared between PS+ and PS- genomes by BrdU labeling/immunoprecipitation analysis. *spa19* vegetative cells and gametes were incubated for the indicated number of hours in the presence of 1 mM BrdU with or without 0.1 mM FdUrd. Total DNA was extracted, 25 ng of BrdUTP-labeled PS+ (X/S) or PS- (E/S) fragments were added, the samples were sonicated and denatured, and immunoprecipitation was carried out with an anti-BrdU antibody. The precipitated DNA was analyzed by PS+ or PS- genome-specific PCR. To distinguish the target and control, PCR products were digested with *SpeI*. (B) Fluorescent images of a single WT cell treated with 1 mM BrdU and 0.1 mM FdUrd for 16 hr. The cell was simultaneously stained with DAPI (left) and an anti-BrdU antibody (center); the images are merged at right. In the merged image, the α -BrdU signal has been recolored in pink for clarity.

buffer, respectively. These vegetative cells and gametes, respectively, were cultured for 0, 1, 2, or 16 hr in the presence of 1 mM BrdU, which preliminary experiments had shown was sufficient to label cpDNA *in vivo* (data not shown). As it is known that BrdU incorporation into cpDNA can be enhanced by pre-incubation with the chloroplast thymidilate synthase inhibitor FdUrd, to reduce the chloroplast thymidine pool (WURTZ *et al.* 1977), two sets of samples with or without FdUrd were prepared. The -FdUrd sample was used to control for any mutagenic effects of FdUrd leading to DNA repair synthesis rather than DNA replication.

Total DNAs were extracted and sonicated, their concentrations were equalized, and 25 ng of BrdUTP-labeled PS+ and PS- control PCR fragments were added to each of the samples. Using the normalized samples, immunoprecipitation was performed with an anti-BrdU antibody. After extensive washes, PS+ and PS- genome-specific PCR was performed to compare the respective incorporation of BrdU (Figure 9A). In vegetative cells, PS+ and PS- signals were clearly detected from BrdU-treated cells, whereas in the negative controls (0 hr), the target sequence was not detected. The control PCR fragment demonstrated that the efficiency of immunoprecipitation was roughly equal for all samples.

When gametes were analyzed, a contrasting result was obtained. While the PS- signals appeared to be at most slightly weaker compared to those from vegetative cells, which may reflect the slight reduction of cpDNA copy number during gametogenesis (HARRIS 1989), the PS+ signals were reduced to the level of the negative control. These results indicate that gametic replication of PS+

genomes is far less efficient than that of PS- genomes under these experimental conditions.

We noted that in cases where genomic products were obtained, a shorter incubation with BrdU was required when FdUrd was used, but the results were qualitatively the same. This was expected, since FdUrd would lead to preferential incorporation of BrdU into cpDNA. This is illustrated in Figure 9B, where in the presence of FdUrd and BrdU, most of the anti-BrdU signal is localized to the chloroplast nucleoids.

Inhibition of chloroplast transcription results in reduced PS+ genome abundance: Prior studies in *Chlamydomonas* chloroplasts have developed evidence for both classical primase-driven DNA replication and RNA-primed DNA replication (NIE and WU 1999; CHANG and WU 2000). To assess whether RNA-primed DNA replication might be implicated in the genome accumulation phenomenon we had observed, we treated vegetative cells with rifampicin, which inhibits the sole type of RNA polymerase known to be present in *Chlamydomonas* chloroplasts (EBERHARD *et al.* 2002). Cells were grown in the presence of 0–3.5 mg/ml rifampicin for 16 hr, and the PS+/PS- genome ratio was initially observed using DNA gel blot analysis. As shown in Figure 10A, *spa19* cells grown in the higher concentrations of rifampicin became partially depleted for PS+ genomes, when equal amounts of total cellular DNA were analyzed. The abundances of PS+ and PS- genomes were then quantified by qPCR, using cells incubated for 16 hr in the presence or absence of 350 μ g/ml rifampicin, and with normalization to a single-copy nuclear gene, *MAA7*. As shown in Figure 10B, in the presence of rifampicin, PS+ genomes were reduced

FIGURE 10.—Effect of rifampicin on cpDNA abundance. (A) Gel blot analysis of *PstI*-digested total DNA. Rifampicin was added to the indicated concentrations to *spa19* vegetative cells at the beginning of a dark period, and cells were cultured 16 hr before DNA extraction. (B) The relative abundances of PS+ and PS- genomes in *spa19* cells treated or untreated with 350 µg/ml rifampicin for 16 hr was analyzed by qPCR using primer sets specific for the *atpB* region of the PS+ or PS- genome. The data were normalized to the nuclear gene *MAA7*, and then the amount in untreated cells was set to 1. Error bars represent standard deviations based on three independent experiments. (C) CC-125 vegetative cells, gametes, and cells treated with 350 µg/ml rifampicin for 3 or 6 hr were labeled with 1 mM BrdU +

0.1 mM FdUrd for 1 hr. BrdU-labeled total DNA was extracted. BrdUTP-labeled PCR product, 25 ng (*aadA*), was added as an internal control. The DNA samples were heat denatured, immunoprecipitated, and analyzed by qPCR using primers for the genes shown beneath each set of bars. The DNA level for each gene was set to 1 for untreated vegetative cells, as indicated by the shaded horizontal line across the graph. The locations of the genes are shown below as related to the entire chloroplast genome. Error bars represent standard deviations based on three PCR analyses.

by slightly more than 50%, whereas PS- genomes declined by slightly less than 10%, as compared to untreated cells. This confirms the result of the DNA gel blot in Figure 10A.

As an independent assessment of chloroplast genome region-specific differences in their dependence on replication-mediated replication, BrdU incorporation was compared at 11 dispersed sites in the WT chloroplast genome. The incorporation was compared between vegetative cells, gametes, and vegetative cells treated with rifampicin for 3 or 6 hr. BrdU incorporation was enhanced by the addition of 0.1 mM FdUrd, allowing the labeling time to be kept to 30 min. Total DNAs were extracted, sonicated, immunoprecipitated, and analyzed by qPCR for the genomic regions shown at the left of Figure 10C. As an internal control, 25 ng of a BrdUTP-labeled control PCR product (*aadA*) was added to each of the samples. Also, total DNA extracted prior to immunoprecipitation was measured by qPCR. This allowed us to normalize qPCR data to both total DNA (for efficiency of DNA extraction) and the internal control (for efficiency of immunoprecipitation). We then compared labeling of different regions of the cp genome in the different samples, setting the amount of

labeling for each region to a baseline (=1) for untreated vegetative cells. The results in Figure 10C show an overall similar trend in BrdU incorporation pattern between gametes and cells treated with rifampicin for 6 hr. Of particular note are sites such as *atpB*, where BrdU incorporation is markedly reduced in gametes and is also relatively rifampicin sensitive in vegetative cells. While the effect is greatest for *atpB*, several other sites with the same character are mainly found in regions shared by the PS+ and PS- genomes of *spa19*. Thus, we can tentatively extrapolate at least some of the replication characteristics of the two *spa19* genomes to regional replication differences in WT cpDNA.

DISCUSSION

The biased inheritance of PS+ and PS- genomes from the mt+ parent following sexual crosses of *spa19*, which ultimately results in PS+ genome loss in >75% of tetrads (Figure 3), was the starting point for this investigation. Considering the four- to fivefold excess of PS+ genomes in vegetative cells, and the presumed selective disadvantage of losing PS+ genomes in that they are required for photosynthetic competence, this bias was

perplexing. In this study, we have identified two developmental stages during which the PS+ /PS− genome ratio undergoes substantial changes, namely gametogenesis (Figure 4 and 5) and zygospore germination (Figure 8). Many *spa19* gametes appear to lose or become severely depleted for the PS+ genome, and this phenomenon appears to be amplified when zygospores germinate. If the PS+ genome survives these two stages, however, it returns to its vegetative cell ratio of approximately fourfold excess over the PS− genome (Figure 3).

One obvious possibility for a mechanism underlying the changes in the PS+ /PS− genome ratio was altered replication patterns of PS+ and PS− genomes in vegetative cells *vs.* gametes. The replication activities of PS+ and PS− genomes were compared following incorporation of BrdU, in the presence or absence of FdUrd. When BrdU-labeled DNA was enriched by immunoprecipitation and analyzed by PCR, we found that BrdU incorporation into PS+ genomes was virtually undetectable in gametes, whereas PS− genome incorporation did not differ between vegetative cells and gametes (Figure 9A). This result suggests that PS+ genome replication is preferentially depressed in gametes. Although we cannot exclude that the PS+ genome has a selective instability in gametes, how a particular genome type within a single, heteroplasmic mt+ chloroplast might be selectively identified for degradation is not immediately obvious.

FdUrd, a known mutagen, was used to enhance the incorporation of BrdU into cpDNA, raising the concern that BrdU incorporation was due in part to cpDNA repair, rather than replication. However, results in the absence of FdUrd showed no qualitative difference, perhaps because the 0.1 mM concentration/16-hr maximum treatment used was well below those traditionally used for cpDNA mutagenesis (MYERS *et al.* 1982). Furthermore, FdUrd induces deletions and point mutations by inhibiting chloroplastic thymidine synthase and thereby causing a thymidine shortage in chloroplasts (WURTZ *et al.* 1977). Therefore, enhanced BrdU incorporation should be the primary effect of FdUrd addition.

Given the premise that PS+ genome replication is depressed in gametes, its mechanistic basis becomes a key issue. One possibility is preferential methylation of PS− genomes, as it was previously proposed that methylated cpDNA is replicated more efficiently (UMEN and GOODENOUGH 2001). However, no role for cpDNA methylation has been observed during early events before or following mating (UMEN and GOODENOUGH 2001), the time at which PS+ genome abundance declines. A more likely explanation would derive from the massive deletion in the PS+ genome, in particular its lack of *OriA*, an origin initially identified by displacement loop mapping, along with the nearby *OriB* (WADDELL *et al.* 1984). Discounting the remote possibility that PS+ genomes are continuously generated from PS− genomes by recombination, it must be

concluded that PS+ genomes replicate independently of *OriA/B*. *OriA/B*-independent replication of the *spa19* PS+ genome is conceptually consistent with other examples. For example, deletion of *oriA* in tobacco chloroplasts had no obvious effect, but both copies of *oriB* could not be deleted (MUHLBAUER *et al.* 2002) and indeed, tobacco cpDNA appears to have multiple replication origins (SCHARFF and KOOP 2007). We also note that *OriA* could not by itself promote maintenance of an extrachromosomal element in *Chlamydomonas* chloroplasts (SUZUKI *et al.* 1997).

While biochemical details and the locations of all origins remain to be established, evidence exists for several nonexclusive mechanisms for *Chlamydomonas* replication priming. One is recombination (BENDICH 2004), another is RNA synthesis by DNA primase (NIE and WU 1999), as in bacteria, and the third is transcription by RNA polymerase, followed by endonucleolytic processing (CHANG and WU 2000), which is thought to be the major mechanism for the initiation of mtDNA replication in yeast and vertebrates (SHADEL and CLAYTON 1997). While all these mechanisms appear to operate at *OriA* in *Chlamydomonas*, transcription-mediated priming could certainly occur elsewhere, as has been suggested (CHANG and WU 2000). Thus, we hypothesize that PS+ genome replication is primed by RNA polymerase and RNA processing activity, rather than at a canonical origin(s).

While we do not know the location(s) of the active origin(s) in the PS+ genome, it is tempting to speculate that its efficient replication activity as compared to the PS− genome is associated with its unique junction formed through recombination (Figure 1B). It is possible that the novel antisense transcript promoted from *tufA* somehow leads to replication initiation. This transcript is known to form stable duplexes with the *atpB* coding strand (NISHIMURA *et al.* 2004); perhaps this is a substrate for an endonuclease that generates a replication primer. In animal mitochondria the analogous activity would be MRP (BENNETT and CLAYTON 1990), but there is no evidence that an analogous ribonucleoprotein exists in plastids. However, chloroplasts do contain numerous endonucleolytic cleavage activities (STERN *et al.* 2010), whose roles in DNA replication are still unknown.

The hypothesis that the PS+ genome is primed by RNA polymerase was tested by treating *spa19* vegetative cells with the transcriptional inhibitor rifampicin, which led to depletion of PS+ genomes at higher concentrations, as well as reduced incorporation of BrdU into the region shared by the PS+ and PS− genomes in a WT strain (Figure 10). We cannot exclude that replication of PS+ DNA was indirectly affected by rifampicin, through reduced expression of a chloroplast transcript and ultimately a gene product required for PS+ genome maintenance. This is very unlikely, however, since the chloroplast genome does not encode known DNA maintenance factors and since transcription inhibition has in

FIGURE 11.—Model for preferential replication of the PS- genomes in gametes. (A) In vegetative cells, WT cpDNA, represented here as the *spa19* PS- genome, can be replicated following either transcription-, recombination-, or primase-mediated priming. On the other hand, PS+ genomes are maintained solely by transcription-mediated priming (shaded circle), since they lack *OriA* and *OriB*. (B) In gametes, the transcription-dependent mechanism is depressed because of downregulated chloroplast transcription, slowing PS+ genome replication, whereas PS- genomes are replicated via *OriA* or *OriB*. Due to their inefficient replication, PS+ genomes are infrequently passed to daughter gametes. Thus, the remaining PS- genomes are not replicated and become diluted to produce type II tetrads, which lack PS+ genomes. (C) The accumulation of cpDNA at any given point is determined by the balance between input (cpDNA replication) and outputs (cpDNA turnover and dilution through cell divisions) of cpDNA. (D) In gametes, however, the accumulation level of

PS+ genomes is preferentially lowered due to the reduced replication of PS+ genomes. It is also possible that this process is facilitated by preferential degradation of PS+ genomes in gametes.

any case a very delayed effect on protein accumulation in *Chlamydomonas* chloroplasts (EBERHARD *et al.* 2002).

Figure 11 presents one model that could explain our results and is based on the existence of two different modes of replication, one *OriA/B* dependent and the other *OriA/B* independent. In the model, PS- (and by extension, WT) genomes possess *OriA/B* and are maintained by both mechanisms. However, PS+ genomes lack *OriA/B* and their replication is dependent on transcription initiation at one or more promoters. The model presumes that both primase and transcription-dependent replication are active in vegetative cells, leading to maintenance of both genome types. We hypothesize that replication of PS+ genomes is more efficient than PS- genomes due to their smaller size or increased replication activity, leading to their increased abundance in vegetative cells. An alternative possibility to explain PS+ genome predominance is unequal mitotic

segregation of PS+ genomes, combined with a selective growth advantage for cells that receive more copies of the PS+ genome. We also have not determined any contribution of DNA degradation, either of both genome types or selectively of the PS+ genome; however, these are raised as possibilities at the bottom of Figure 11.

The differentiation of vegetative cells into gametes triggers a process leading to the dormant zygospore stage in *Chlamydomonas*. During dormancy, photosynthesis is inactivated (BULTÉ and WOLLMAN 1992), thylakoid structure disintegrates, and chloroplast pigments are degraded (MARTIN and GOODENOUGH 1975). Importantly, reduced cpRNA accumulation has been observed during gametogenesis (CHANG and WU 2000), consistent with the hypothesis that replication becomes *OriA/B* dependent during this period. While *OriA/B* dependence might marginally affect replication of PS- or WT genomes, replication of PS+ genomes would

essentially be switched off. This would lead, as observed, to the reduction of the PS+/PS− genome ratio both in gametes (Figure 4, 5, and 6) and germinating zygotes (Figure 7 and 8), unequal distribution of PS+ genomes to daughter cells during gamete division (Figure 6), and the production of type I and II tetrads (Figures 3). Even if one or a few copies of PS+ DNA were initially present in “type II” zygospores, their inefficient replication or degradation would lead to dilution in germinating zygospores (Figure 8). Eventually, the transmission of PS+ genomes would be restricted despite the fact that they were present at high copy number in mt+ chloroplasts.

Why chloroplasts would utilize multiple DNA replication strategies is an as-yet unanswered question. It could be argued that redundancy is a strategy driven by the essential nature of genome replication. Having genome copy number partly linked to gene expression could also be a regulatory strategy, and indeed chloroplast genome copy number changes dynamically depending on developmental stage (ROWAN *et al.* 2007), tissue (OLDENBURG and BENDICH 2004), and environmental factors (YEHUDAI-RESHEFF *et al.* 2007), perturbations that are also associated with chloroplast gene expression modulation.

Heteroplasmy has received considerable attention in recent years, partly because of reports showing the paternal leakage of organellar DNA inheritance in plants (AZHAGIRI and MALIGA 2007; SVAB and MALIGA 2007), insects (SHERENGUL *et al.* 2006; FONTAINE *et al.* 2007), and animals including humans (SCHWARTZ and VISSING 2002); paternal leakage is thought to be the major source for heteroplasmy. While *spa19* is an unusual heteroplasmic mutant given its stable heteroplasmy and inheritance pattern, it nonetheless offers insight into the possible impact in WT cells of switching on and off specific replication modes.

We gratefully acknowledge Toshiharu Shikanai, Hirofumi Uchi-miya, Tsuneyoshi Kuroiwa, Haruko Kuroiwa, and Misumi Osami for technical advice, Shinya Murakami for thoughtful insights, and Hitomi Tanaka for technical assistance. This work was supported by PRESTO (Precursory Research for Embryonic Science and Technology), Japan Society of the Promotion of Science Grant-in-Aid for Young Scientists (B 19770050), and Kyoto University Step-Up Grant-in Aid for Young Scientists to Y.N. and awards from United States–Israel Binational Agricultural Research and Development Fund (IS-3605-04) and National Science Foundation (MCB 0091020) to D.B.S.

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Communicating editor: S. DUTCHER

GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.110.118265/DC1>

**Differential Replication of Two Chloroplast Genome Forms in
Heteroplasmic *Chlamydomonas reinhardtii* Gametes Contributes
to Alternative Inheritance Patterns**

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DOI: 10.1534/genetics.110.118265

FIGURE S1.—BrdU labeling of DNA. (A) Schematic of BrdU labeling, Immunoprecipitation, and PCR analysis. (B) PCR was performed in the presence of the indicated concentrations of BrdUTP. To confirm the incorporation of BrdUTP, PCR products were denatured by boiling and spotted on a Hybond N+ membrane. The membrane was reacted with an anti-BrdU monoclonal antibody and an HRP-conjugated secondary antibody, then developed using immunohistochemistry. (C) The indicated quantities of PCR product labeled in 1 mM BrdUTP (PS+ fragment amplified from the clone shown in Figure 7B, with the T7 and SP6 primers) were immunoprecipitated using Protein G beads, and analyzed by nested PCR using the PS+ specific primers YK12 and spatE.