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Inhibition of Chloroplast DNA Recombination and Repair by Dominant Negative Mutants of *Escherichia coli* RecA

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The occurrence of homologous DNA recombination in chloroplasts is well documented, but little is known about the molecular mechanisms involved or their biological significance. The endosymbiotic origin of plastids and the recent finding of an *Arabidopsis* nuclear gene, encoding a chloroplast-localized protein homologous to *Escherichia coli* RecA, suggest that the plastid recombination system is related to its eubacterial counterpart. Therefore, we examined whether dominant negative mutants of the *E. coli* RecA protein can interfere with the activity of their putative homolog in the chloroplast of the unicellular green alga *Chlamydomonas reinhardtii*. Transformants expressing these mutant RecA proteins showed reduced survival rates when exposed to DNA-damaging agents, deficient repair of chloroplast DNA, and diminished plastid DNA recombination. These results strongly support the existence of a RecA-mediated recombination system in chloroplasts. We also found that the wild-type *E. coli* RecA protein enhances the frequency of plastid DNA recombination over 15-fold, although it has no effect on DNA repair or cell survival. Thus, chloroplast DNA recombination appears to be limited by the availability of enzymes involved in strand exchange rather than by the level of initiating DNA substrates. Our observations suggest that a primary biological role of the recombination system in plastids is in the repair of their DNA, most likely needed to cope with damage due to photooxidation and other environmental stresses. This hypothesis could explain the evolutionary conservation of DNA recombination in chloroplasts despite the predominantly uniparental inheritance of their genomes.

Homologous genetic recombination is widespread in living organisms. In green algae and land plants it occurs in the nuclear, mitochondrial, and chloroplast genomes (1, 9, 10, 49, 52). However, the role of organelle DNA recombination in the survival and evolution of eukaryotic organisms is currently unknown. In chloroplasts, exchange of genetic information between genomes from different individuals rarely takes place because plastids are uniparentally inherited or, when biparentally inherited, chloroplast fusion is very infrequent (23, 38). Moreover, when chloroplasts fuse, as in zygotes of the unicellular green alga *Chlamydomonas reinhardtii*, one of the parental genomes is usually selectively degraded (23, 38). In addition, chloroplast DNA recombination is very active in vegetative cells of *C. reinhardtii*, as indicated by the dispersive labelling of plastid DNA in density transfer experiments (62). This suggests that the immediate selective value of chloroplast DNA recombination does not reside in the generation of genetic diversity by reshuffling genes in natural populations.

Instead, we hypothesize that a primary function of homologous recombination systems in chloroplasts is in the repair and, possibly, replication of DNA under genotoxic stress. The importance of DNA recombination to cell survival and growth in eubacteria and in the nuclear genome of several eukaryotes is well documented (6, 7, 16, 18, 58). In *Escherichia coli*, the multifunctional RecA protein is essential for homologous recombination and a variety of cellular responses to DNA damage (SOS response), including regulation of gene expression, recombinational repair, SOS mutagenesis, and DNA replication (2, 3, 16, 18, 33, 58, 64). The RecA protein of *E. coli* has also been implicated in the proper partitioning of newly replicated chromosomes, since many *recA* mutant cells have an abnormal number of chromosomes following the completion of ongoing rounds of DNA replication (16, 41, 56). However, recent studies have provided strong evidence that the role of RecA may be more indirect, presumably a protective function, than initially suggested (56). In the absence of functional RecA protein, individual chromosomes appear to be selectively and completely degraded by the RecBCD enzyme (56). In *Saccharomyces cerevisiae*, the lack of resolution of Holliday junctions has been shown to affect the partitioning of the mitochondrial genome (51, 67). Thus, chloroplast DNA recombination may also be involved in the proper segregation of the plastid genomes. In addition, it may play a role in generating intragenomic variation via adaptive mutation, as suggested for the RecA system in *E. coli* (20, 28).

As predicted from the endosymbiont theory (49), the chloroplast recombination system seems to be related to its eubacterial counterpart. A nuclear gene encoding a plastid-localized protein 53% identical to *E. coli* RecA has been cloned from *Arabidopsis thaliana* (15, 36). In addition, two *Arabidopsis* cDNAs, apparently encoding chloroplast-targeted proteins, have been isolated by complementation of a *ruvC recG* double mutant of *E. coli* deficient in the resolution of Holliday junctions (50). Moreover, a protein immunologically related to *E. coli* RecA and induced by DNA-damaging agents was detected in chloroplasts of pea and *C. reinhardtii* (12, 13). A RecA-like strand-transfer activity is also present in stromal extracts from pea (14, 36). The active form of the *E. coli* RecA protein in vitro is a nucleoprotein filament formed on single-stranded or gapped DNA in the presence of a nucleotide cofactor (16, 18, 36, 39, 60). By comparing the primary sequences of eubacterial and eukaryotic RecA homologs with the crystal structure of the *E. coli* RecA protein, the invariant residues were found to

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correspond to four functional or structural domains, including the hydrophobic interfaces of the subunits in the filament (59, 60). These residues are completely conserved in the chloroplast RecA homolog of *A. thaliana* (15), suggesting that it might form a similar (if not identical) filament.

In *E. coli*, certain missense or truncated RecA proteins inhibit the activity of the wild-type protein (31, 55). This type of negative complementation is characteristic of proteins that function as multimers and suggests that the mutant RecA proteins either form defective heteromultimers with the wild-type protein (39) or prevent the formation of an active filament. A truncated *E. coli* RecA protein has also been shown to decrease resistance to UV irradiation in other species of eubacteria (55), and direct interaction between RecA proteins from different enterobacteria has been demonstrated by affinity chromatography (22). Thus, we decided to examine whether expression of dominant negative mutants of *E. coli* RecA in the chloroplast of *C. reinhardtii* can interfere with the activity of the endogenous RecA homolog. This approach was designed to (i) verify the existence of a RecA-mediated recombination system in chloroplasts and (ii) test its role in DNA repair and cell survival under genotoxic stress.

MATERIALS AND METHODS

Construction of chimeric *E. coli recA* genes and their transformation into the *C. reinhardtii* chloroplast genome. The coding sequence of wild-type *E. coli recA* was obtained by PCR amplification (24) of the cloned gene with the primers recA5ter (gtgccatgGACGAAAACAAACAGAAAGC, adding an *Nco*I site [underlined] and homologous to *recA* from positions 58 to 77 [30, 54]) and recA3end (ctggcatgcTTAAAAATCTTCGTTAGTTTC, adding an *Sph*I site [underlined] and homologous to *recA* from positions 1110 to 1090 [30, 54]). After partial digestion with *Nco*I and complete digestion with *Sph*I, the PCR fragment was cloned into the corresponding sites of a pUC8 vector containing the 5′-*atpA*::*aadA*::*rbcL*-3′ selectable marker (24), replacing the coding sequence of *aadA*. In this construct (W*recA*) the first three amino acids of the RecA protein are replaced by Met-Ser-Met. The correct sequence of the cloned PCR fragment was verified by DNA sequencing (15). The ΔN33*recA* construct was obtained by deleting the amino-terminal end of W*recA* at an internal *Nco*I site (positions 152 to 157 [30, 54]) and the introduced site at the end of the gene. Similarly, two internal *Bst*EII sites were used to delete 72 nucleotides (from positions 589 to 661 [30, 54]), generating the ΔInt*recA* construct. The coding sequence of ΔN42*recA* was prepared by PCR amplification with the primers recAΔN1 (gtgccatgGGTTCGCTTTCACTGGATAT, adding an *Nco*I site [underlined] and homologous to *recA* from positions 178 to 197 [30, 54]) and recA3end. This PCR fragment was cloned into the pUC8 vector (24) as described above. The different chimeric *atpA*::*recA*::*rbcL* genes were excised by standard techniques (53) and inserted into the second *Kpn*I site, downstream from *atpB*, in the cloned chloroplast fragment *Bam*10 (9, 27). The direction of transcription of the different *recA* alleles is opposite to that of the *atpB* gene. Chloroplast transformants for each construct were obtained by microprojectile bombardment of *C. reinhardtii* cells containing a partially deleted *atpB* gene (CC-373) and subsequent selection for photosynthetic competence on minimal medium (9).

DNA, RNA, and protein analysis of *C. reinhardtii recA* transformants. Total-cell DNA was isolated (40), fractionated by agarose gel electrophoresis, and hybridized as previously described (15, 53). Standard techniques were used for RNA purification, formaldehyde gel electrophoresis, blotting, and hybridization (15, 53). Isolated proteins from whole cells (40) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to nitrocellulose filters, and detected by chemiluminescence according to published procedures (13).

Growth conditions and determination of growth rate. Unless noted otherwise, *C. reinhardtii* cells were grown photoautotrophically in minimal HS medium (27) under continuous light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation [PAR]) at 25°C, as previously described (40). To determine the growth rate, cells were cultured in 24-well plates (Falcon 3047; 1 ml of medium per well), mounted on a rotary shaker, under the same light conditions described above, without CO_2 bubbling. The optical density at 740 nm was measured every 2 h with a microplate reader (Cambridge Technology model 7520), as described in more detail elsewhere (29).

Determination of cell survival during exposure to DNA-damaging agents. *C. reinhardtii* cells were grown photoautotrophically to logarithmic phase (optical density at 740 nm = 0.1 to 0.2) and spread on minimal medium plates (HS [27]). For treatments with UV-C light, cells were irradiated on the plates with a germicidal lamp (GE G8T5) set 42 cm above the surface of the agar (energy fluence rate = 1.6 $J \cdot m^{-2} \cdot s^{-1}$). After 24 h in the dark to prevent photoreactivation, the plates were incubated under moderate light (110 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) at 25°C for 10 days before the surviving colonies were counted. For treatments with methyl methanesulfonate (MMS; Sigma) or 5-fluoro-2′-deoxyuridine (FdUrd; Sigma), cells were spread on HS plates (27) containing the appropriate concentrations of each genotoxic agent and incubated as described above.

Analysis of chloroplast DNA repair in cells treated with MMS. *C. reinhardtii* cells grown photoheterotrophically (TAP medium [27]) were collected in logarithmic phase and resuspended in the same medium to an optical density at 740 nm of 0.5. MMS was added to a final concentration of 25 mM, and the cells were incubated for 30 min in the dark at 25°C. Untreated control cells were incubated in TAP medium without MMS. Immediately afterwards, aliquots of control and MMS-treated cells were frozen for isolation of DNA corresponding to the zero time points. After three washes with TAP medium to remove unreacted MMS, the remaining cells were incubated in the dark, with moderate shaking, for 6 or 12 h, and aliquots were frozen as before. Total-cell DNA was isolated (40), separated by denaturing gel electrophoresis, blotted to nylon filters, and hybridized with the coding sequence of the chloroplast *chlL* gene, according to standard procedures (15, 53). The distribution of ^{32}P radioactivity in each lane was quantified with the PhosphorImager System (Molecular Dynamics).

Generation of *C. reinhardtii* strains with a disrupted *chlL* gene and determination of chloroplast DNA recombination. The chloroplast *chlL* gene encodes a subunit of the light-independent protochlorophyllide oxidoreductase, required for the synthesis of chlorophyll in the dark (4, 61). A 3.8-kb *Eco*RI-*Sca*I fragment containing the 3′ end of the *chlL* gene and flanking region (61) was placed upstream of the *atpA* promoter in the pUC8 vector containing the 5′-*atpA*::*aadA*::*rbcL*-3′ selectable marker (24). This plasmid was linearized downstream from the *rbcL* terminator to insert a 1.2-kb *Csp*45I-*Hin*dIII fragment containing the 5′ end of *chlL* and flanking region (61). In the final construct, the *atpA*::*aadA*::*rbcL* marker is flanked by a 216-bp repeat (from *Sca*I to *Csp*45I [61]) in the coding sequence of the *chlL* gene. This construct was transformed into wild-type *C. reinhardtii* and the different *recA* transgenics by microprojectile bombardment (40). Homoplasmic chloroplast transformants were obtained by selection in liquid TAP medium containing spectinomycin (200 to 800 μg/ml) and verified by Southern analysis. Under selective conditions, the transformants are yellow in the dark. In the absence of selection, recombination between the direct repeats regenerates a functional *chlL* gene, giving rise to viable green colonies or green sectors in yellow colonies (see Fig. 6A). The frequency of recombination per cell per generation was determined by subtracting the frequency of green colonies [(number of green colonies)/(number of yellow plus sectored colonies)] after 3 days of nonselective photoheterotrophic growth from that frequency after 6 days of growth and dividing by the number of generations between the two time points, as previously described for a similar system in *E. coli* (41). Approximately 10,000 cells for each time point were spread on TAP plates (27) and incubated in the dark at 25°C for 20 days before the different colonies were counted.

RESULTS

Expression of chimeric *E. coli recA* genes in the *C. reinhardtii* chloroplast. We made four constructs expressing the wild-type *E. coli* RecA protein (WtRecA), two amino-terminally truncated RecA proteins (ΔN33RecA or ΔN42RecA, missing 33 or 42 amino acids, respectively), or an internally deleted RecA protein (ΔIntRecA, lacking 24 amino acids from positions 180 to 203 [30, 54]). Nearly identical mutant proteins lack any of the typical RecA activities when expressed in a *recA*-deleted *E. coli* strain (31). Moreover, two amino-terminally truncated RecA proteins, missing 28 or 41 residues, behaved as dominant negative mutants in *E. coli* $recA^+$ cells, significantly reducing the frequency of recombination and resistance to UV irradiation (31). Since the ΔIntRecA protein only slightly inhibited conjugational recombination in this $recA^+$ host (31), it was included as a negative control.

The coding sequences of the different *E. coli recA* alleles were placed under the control of plastid regulatory elements and integrated in the inverted repeat (IR) region of the chloroplast genome by microprojectile bombardment of cells with a partially deleted *atpB* gene (CC-373) (9). Stable homoplasmic transformants, isolated after several cycles of single-cell cloning, contained the chimeric *recA* genes in both copies of the IR, presumably because of copy correction by homologous recombination (9, 10). When total-cell DNA was digested with *Bam*HI and probed with a chloroplast segment containing the *atpB* gene and the flanking IR region, hybridizing fragments of 7.6 kb (*Bam*10) and 8.4 kb (*Bam*9) were seen in wild-type cells

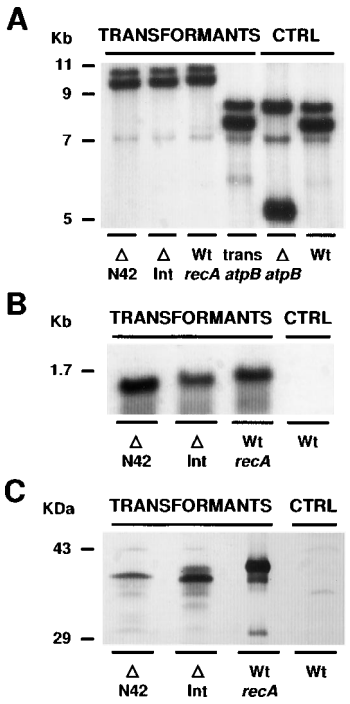

FIG. 1. Characterization of homoplasmic chloroplast transformants of *C. reinhardtii* expressing chimeric *E. coli recA* genes. Abbreviations: Wt, wild type; Δ*atpB*, *atpB* deletion mutant (CC-373); trans *atpB*, CC-373 transformed with the wild-type *atpB* gene; W*trecA*, CC-373 transformed with the wild-type *E. coli recA* construct; ΔInt, CC-373 transformed with the internally deleted *E. coli recA* construct; ΔN42, CC-373 transformed with the amino-terminally truncated *E. coli recA* construct lacking 42 residues. (A) Southern blot analysis of *Bam*HI-digested total-cell DNA probed with a 5.3-kb chloroplast fragment containing the *atpB* gene and part of the flanking inverted repeat region. The faint bands are presumably due to cross-hybridization with short dispersed repeats located in many intergenic regions of the chloroplast genome (9, 10). (B) Northern blot analysis of total-cell RNA probed with the *E. coli recA* coding sequence. (C) Immunoblot analysis of total-cell protein probed with an affinity-purified polyclonal antibody raised against the *E. coli* RecA protein. Relative lane loadings for the ΔN42, ΔInt, and W*trecA* transformants, and Wt *C. reinhardtii* were 40, 16, 1, and 50, respectively. Note that at this level of loading the chloroplast RecA homolog of *C. reinhardtii* is not detectable; the observed faint bands are unrelated cross-reacting proteins (12, 13).

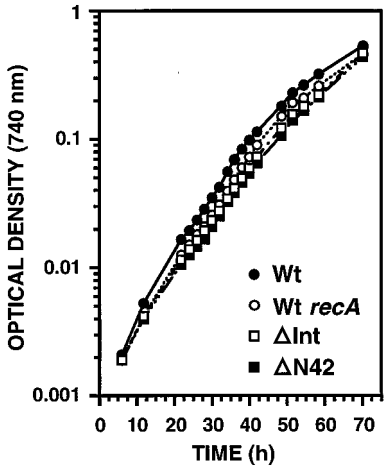

FIG. 2. Photoautotrophic growth of homoplasmic chloroplast transformants of *C. reinhardtii* expressing chimeric *E. coli recA* genes. Abbreviations are as in the legend to Fig. 1. Each time point is the average of eight replicates (two independent experiments). Error bars have been omitted for reasons of clarity. The exponential phase of the growth curve was used to calculate the following doubling times (in hours) ± standard deviations: Wt, 6.79 ± 0.09; W*trecA*, 6.61 ± 0.36; ΔInt, 7.32 ± 0.24; and ΔN42, 7.16 ± 0.44.

and in CC-373 cells transformed with wild-type DNA (Fig. 1A). In the *recA* transformants, these fragments were replaced by two larger segments (Fig. 1A) corresponding to the insertion of the chimeric *recA* genes into *Bam*10 and *Bam*9. This was verified by probing with the *recA* coding sequence (12).

Expression of the W*trecA*, ΔInt*recA*, and ΔN42*recA* genes in homoplasmic chloroplast transformants was analyzed by Northern (RNA) blotting and immunoblotting. Similar steady-state levels of *recA* transcripts were seen in all transformants grown under photoautotrophic conditions (Fig. 1B), suggesting similar rates of transcription and mRNA degradation for the different chimeric genes. However, accumulation of the different RecA proteins varied greatly (Fig. 1C). The ΔIntRecA protein was present at approximately 5%, and the ΔN42RecA protein was present at less than 1% of the WtRecA protein level (Fig. 1C). Since the different *recA* coding sequences were under the control of the same regulatory elements, their efficiencies of translation should be similar. Thus, the variation observed in the steady-state level of the chloroplast-expressed RecA proteins probably reflects their differential stability in *C. reinhardtii.*

Effect of the *E. coli* RecA proteins on cell growth and survival. Growth rates of all *recA* transformants were similar to that of wild-type *C. reinhardtii*, under both photoautotrophic and photoheterotrophic conditions (Fig. 2). Thus, expression of the different alleles of *E. coli recA* in the chloroplast does not appear to interfere with any essential function for cell growth and survival under normal conditions. In contrast, the different *recA* transformants varied noticeably in their response to DNA-damaging agents such as UV-C irradiation (<280 nm), MMS, or FdUrd (Fig. 3). Survival of the ΔN42*recA* transformants was significantly reduced in the presence of any of these genotoxic agents (Fig. 3). This effect was particularly severe with FdUrd (Fig. 3C and D), which selectively affects chloroplast DNA in *C. reinhardtii* by inducing thymidine starvation (66). A similar outcome was observed with the ΔN33*recA* transformants (Fig. 3D), but they showed lower sensitivity to MMS and, particularly, to UV-C irradiation (12). The ΔInt*recA* transformants behaved like wild-type *C. reinhardtii* when treated with UV-C light or MMS (Fig. 3A and B), whereas their growth rate was moderately reduced by FdUrd (Fig. 3D). On the other hand, transformants expressing the WtRecA protein grew slightly better than the wild type in the presence of FdUrd (Fig. 3D). These results indicated a detrimental effect of the dominant negative mutants of the *E. coli* RecA protein on the survival of *C. reinhardtii* exposed to genotoxic agents.

Effect of the *E. coli* RecA proteins on plastid DNA repair. In alkaline gels, an enhanced mobility of DNA is indicative of single- and double-strand breaks and alkali-labile lesions (25, 44). A similar extent of MMS-induced chloroplast DNA damage was observed in all the *recA* transformants and in wild-type *C. reinhardtii*, by denaturing gel electrophoresis of DNA isolated immediately after the treatment (Fig. 4 and 5A). In the W*trecA* and ΔInt*recA* transformants, as in wild-type *C. reinhardtii*, most of this damage was repaired by 12 h after the removal of MMS (Fig. 4 and 5A). In the ΔN42*recA* transformants, however, the reductions in the amount detectable by hybridization (Fig. 5A) and average molecular mass (Fig. 4) of chloroplast DNA persisted for a longer period. However, the mass distribution of plastid DNA became virtually identical to that of the untreated control by 24 h after the treatment (12).

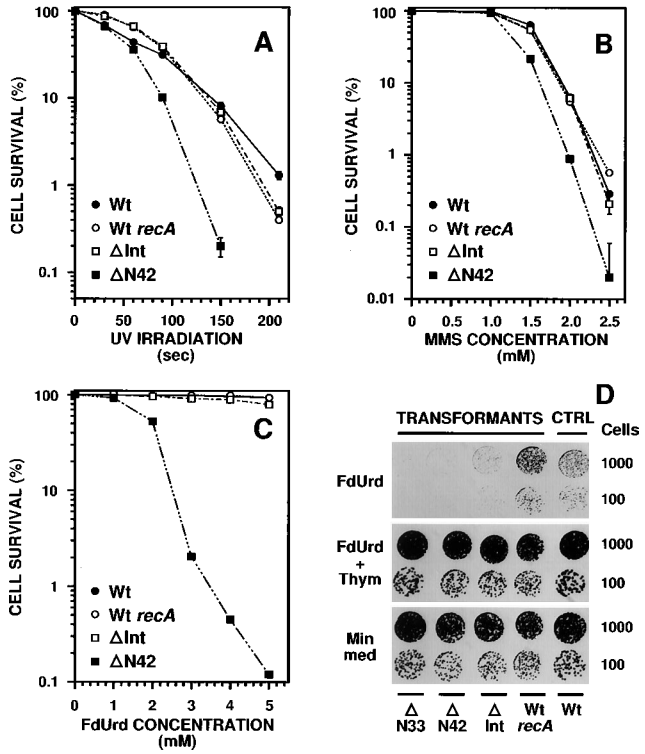

FIG. 3. Effects of DNA-damaging agents on the survival and growth of homoplasmic chloroplast transformants of *C. reinhardtii* expressing chimeric *E. coli recA* genes. Abbreviations are as in the legend to Fig. 1; ΔN33, CC-373 transformed with the amino-terminally truncated *E. coli recA* construct missing 33 residues. (A, B, and C) Each graph point (± standard deviation) is the average of nine replicates (three independent experiments). Where the error bars are not visible, they are smaller than the symbols. (A) Survival of the *recA* transformants and wild-type *C. reinhardtii* exposed to increasing levels of UV-C irradiation. (B) Survival of the *recA* transformants and wild-type *C. reinhardtii* grown on minimal medium containing increasing concentrations of MMS. (C) Survival of the *recA* transformants and wild-type *C. reinhardtii* grown on minimal medium containing increasing concentrations of FdUrd. Note that the symbols corresponding to Wt are completely overlapped by those of Wt*recA*. (D) Growth of the *recA* transformants and wild-type *C. reinhardtii* on HS plates (27) without (Min med) or with either 5 mM FdUrd (FdUrd) or 5 mM FdUrd plus 5 mM thymidine (FdUrd + Thym). Cells grown photoautotrophically to logarithmic phase were diluted in minimal medium to the indicated numbers per 10 μl and spotted on HS plates. The panels show the growth of the colonies after 10 days of incubation as described in Materials and Methods.

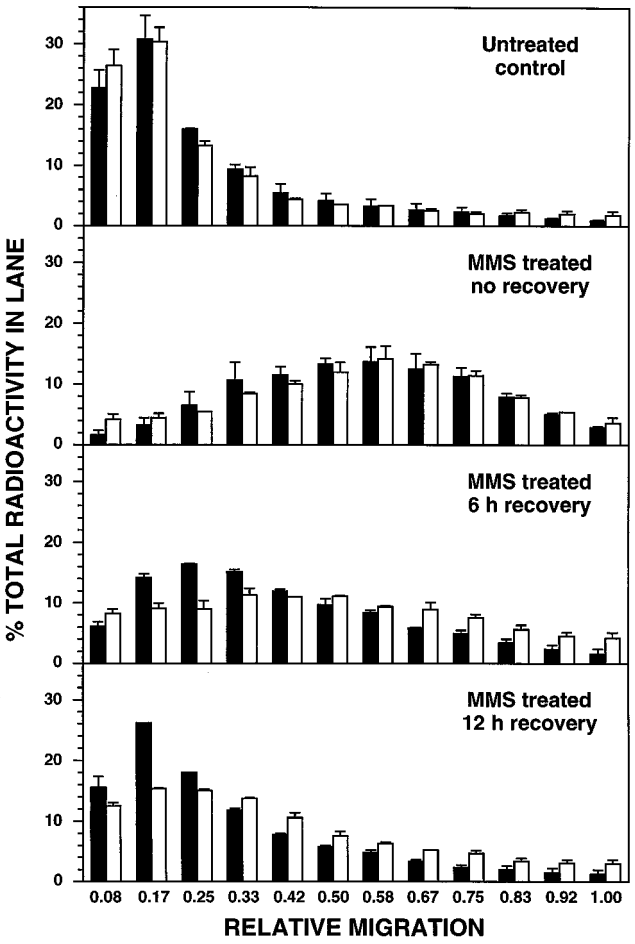

FIG. 4. Repair of plastid DNA after MMS-induced damage in the wild type (closed bars) and in homoplasmic chloroplast transformants of *C. reinhardtii* expressing the ΔN42*recA* gene (open bars). DNA was isolated from untreated control cells and from cells treated with 25 mM MMS for 30 min, either immediately after the treatment or after MMS was washed out and the cells were allowed to recover for 6 or 12 h. Purified DNA was separated by alkaline gel electrophoresis, blotted to nylon membranes, and probed with the coding sequence of the *chlL* gene. The relative distribution of radioactivity in each lane, analyzed with a Molecular Dynamics PhosphorImager, is plotted as a function of the distance of migration. Each bar is the average (± standard deviation) of three independent experiments. In some cases the error bars are too small to be visible.

These results indicated that the ΔN42*recA* transformants have a diminished capacity to repair plastid DNA. Moreover, when cells were grown in minimal medium containing 1 mM FdUrd, the specific decrease in plastid DNA level (66) was approximately twofold greater in the ΔN42*recA* transformants than in the other genotypes (Fig. 5B). As expected (66), the addition of an equimolar amount of thymidine to the growth medium completely reversed the effects of FdUrd on cell survival and plastid DNA level in all the *recA* transformants and in wild-type *C. reinhardtii* (Fig. 3D and 5B).

The primary DNA lesions caused by UV-C light, MMS, and FdUrd are most likely repaired by several different enzymatic pathways in chloroplasts of *C. reinhardtii* (13, 37, 43, 57). However, these genotoxic agents also induce secondary lesions, such as double-strand breaks, interstrand cross-links, and/or damage in single-stranded regions, that require recombination for their repair (7, 18, 25, 37, 43, 44, 58). In fact, recombinational repair in *E. coli* seems to overcome, at least partially, the damage induced by many different genotoxic compounds (7, 18, 58). Thus, the deleterious effects of the ΔN42RecA protein on cell survival as well as on the integrity and steady-state level of plastid DNA are consistent with an inhibition of recombinational repair in the chloroplast of *C. reinhardtii.*

Effect of the *E. coli* RecA proteins on plastid DNA recombination. To evaluate whether expression of the different *E. coli* RecA proteins affects chloroplast DNA recombination, we designed a visual in vivo assay based on the capability of *C. reinhardtii* to synthesize chlorophyll in the dark (4, 61). We made a construct containing the 5′ end of the chloroplast *chlL* gene separated from its 3′ end by the chimeric *atpA*::*aadA*::*rbcL* selectable marker, conferring resistance to spectinomycin (24). The truncated portions of the *chlL* gene have an overlapping region of homology of 216 bp, flanking the selectable marker as direct repeats (Fig. 6A). The wild-type *chlL* gene was replaced with this construct in the different *recA* transgenics and in wild-type *C. reinhardtii* by biolistic transformation (40) and selection on spectinomycin-containing medium. Since

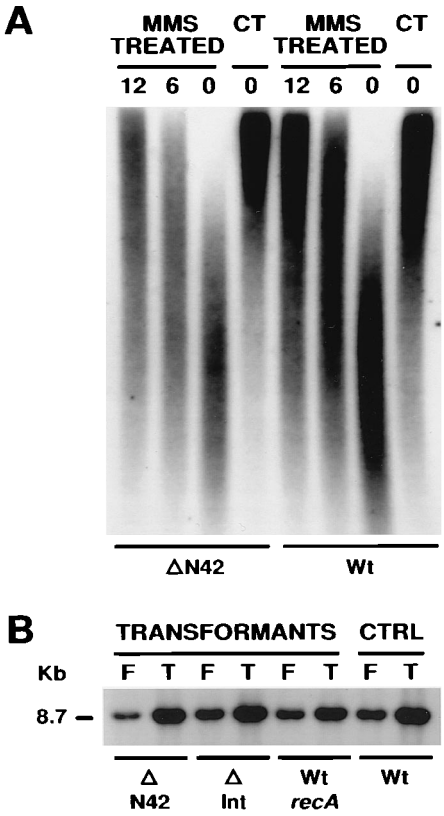

FIG. 5. Effects of MMS and FdUrd on the integrity and amount of plastid DNA in homoplasmic chloroplast transformants of *C. reinhardtii* expressing chimeric *E. coli recA* genes. Abbreviations are as in the legend to Fig. 1. (A) Southern blot showing the repair of chloroplast DNA after exposure to 25 mM MMS for 30 min. Total-cell DNA was isolated from untreated control cells (CT) and MMS-incubated cells, either immediately after the treatment (0) or after recovery for 6 or 12 h in the absence of MMS. The DNA was separated by denaturing gel electrophoresis and hybridized with the coding sequence of the chloroplast *chlL* gene. Note that some regions in the Wt lanes are overexposed in the autoradiograph, making the comparison of DNA mass distribution with the ΔN42 lanes difficult. This problem of nonlinearity was overcome with the PhosphorImager analysis of the blots, as shown in Fig. 4. (B) Southern blot showing the chloroplast DNA level in cells grown in the presence of FdUrd. Cells were grown photoautotrophically in minimal medium containing 1 mM FdUrd (F) or 1 mM FdUrd plus 1 mM thymidine (T) for approximately seven generations. Total-cell DNA digested with *Eco*RI was probed with the coding sequence of the chloroplast *atpE* gene.

the *chlL* gene product is a component of the light-independent protochlorophyllide oxidoreductase (4, 61), colonies of transformants homoplasmic for the disrupted gene are yellow in the dark when grown under selective conditions. However, under nonselective conditions, excision of the chimeric *aadA* gene by an inter- or intramolecular recombination event between the direct repeats is not lethal and regenerates a functional *chlL* gene (Fig. 6A). According to the timing of this event, green or green and yellow sectored colonies are produced (Fig. 6B). By Southern analysis, these colonies were found to be heteroplasmic, containing copies of both the wild-type and the disrupted *chlL* genes (12).

The frequency of chloroplast DNA recombination per cell per generation was determined by counting the number of green colonies relative to the number of yellow and sectored colonies as a function of the age of the cultures. Whereas the recombinational activity of the ΔInt*recA* transformants was similar to that of wild-type *C. reinhardtii*, the ΔN42*recA* transformants showed a fivefold reduction (Table 1). In contrast, the Wt*recA* transformants displayed an over-15-fold increase in the frequency of plastid DNA recombination (Table 1). The recombinational activity of the different *recA* transformants was also reflected in the frequency of green sectors observed in individual colonies (Fig. 6B). Since recombination between directly repeated sequences, separated by a nonhomologous region, has been shown to be *recA* dependent in eubacteria (8, 41, 42), these results strongly suggest that the ΔN42RecA protein inhibits a RecA-mediated recombination system in the chloroplast of *C. reinhardtii.*

DISCUSSION

Our results indicate that expression of dominant negative mutants of the *E. coli* RecA protein in the chloroplast of *C. reinhardtii* has a deleterious effect on cell survival under genotoxic stress, plastid DNA repair, and homologous DNA recombination. Since there is no significant reduction in survival, growth, and chloroplast DNA integrity under normal conditions, these effects are almost certainly not due to general toxicity by the ΔN42RecA or ΔN33RecA proteins. In addition, the steady-state levels of these mutant proteins are not enhanced by genotoxic stress, making an increased nonspecific toxicity unlikely (12). Moreover, the ΔIntRecA protein had no effect or only a mildly detrimental one on the response of *C. reinhardtii* to DNA-damaging agents and the frequency of plastid DNA recombination. In contrast, transformants expressing the WtRecA protein behaved as well as wild-type *C. reinhardtii* in response to genotoxic stress and showed increased chloroplast recombination activity. Similar results, in terms of sensitivity to UV irradiation and reduction in recombination frequency, were obtained with nearly identical mutant RecA proteins expressed in wild-type *E. coli* (31). Thus, the simplest interpretation of our results is that the dominant negative mutants of *E. coli* RecA interfere with the activity of their homolog in the *Chlamydomonas* chloroplast, inhibiting DNA recombination, repair, and possibly replication (see below) under genotoxic stress. The ΔIntRecA protein lacks a β-strand in its hydrophobic core and part of a loop implicated in DNA binding (59, 60). This presumably alters the structural conformation of the mutant protein (31), preventing its efficient interaction with the endogenous chloroplast RecA protein. On the other hand, the WtRecA protein is functionally competent and appears to be able to complement positively its chloroplast homolog.

A dominant negative mutant of *E. coli* RecA could exert its effect by competition for DNA binding sites, by the formation of mixed multimers defective in one or several RecA activities, or by preventing the formation of an active filament. Cleavage of the λ repressor, necessary for λ prophage induction, has been shown in vitro to require the formation of a ternary complex composed of RecA protein, single-stranded DNA, and ATP (11, 39). Truncated RecA proteins, nearly identical to ΔN42RecA and ΔN33RecA, are unable to induce λ prophage upon UV irradiation when expressed in a *recA*-deleted *E. coli* host (31). These proteins lack some residues involved in subunit-subunit interactions in the filament and in interfilament contacts, but possess an intact ATP binding site (59, 60). Thus, the ΔN42RecA and ΔN33RecA proteins are most likely defective in their ability to polymerize on single-stranded DNA. Moreover, a RecA protein lacking 32 amino acid residues at the N terminus forms oligomers very poorly, as shown by gel filtration analysis (48). However, when the equivalent of ΔN42RecA was expressed in *recA*$^+$ *E. coli* cells, it inhibited conjugational recombination and increased UV light sensitivity without affecting λ prophage induction (31). This

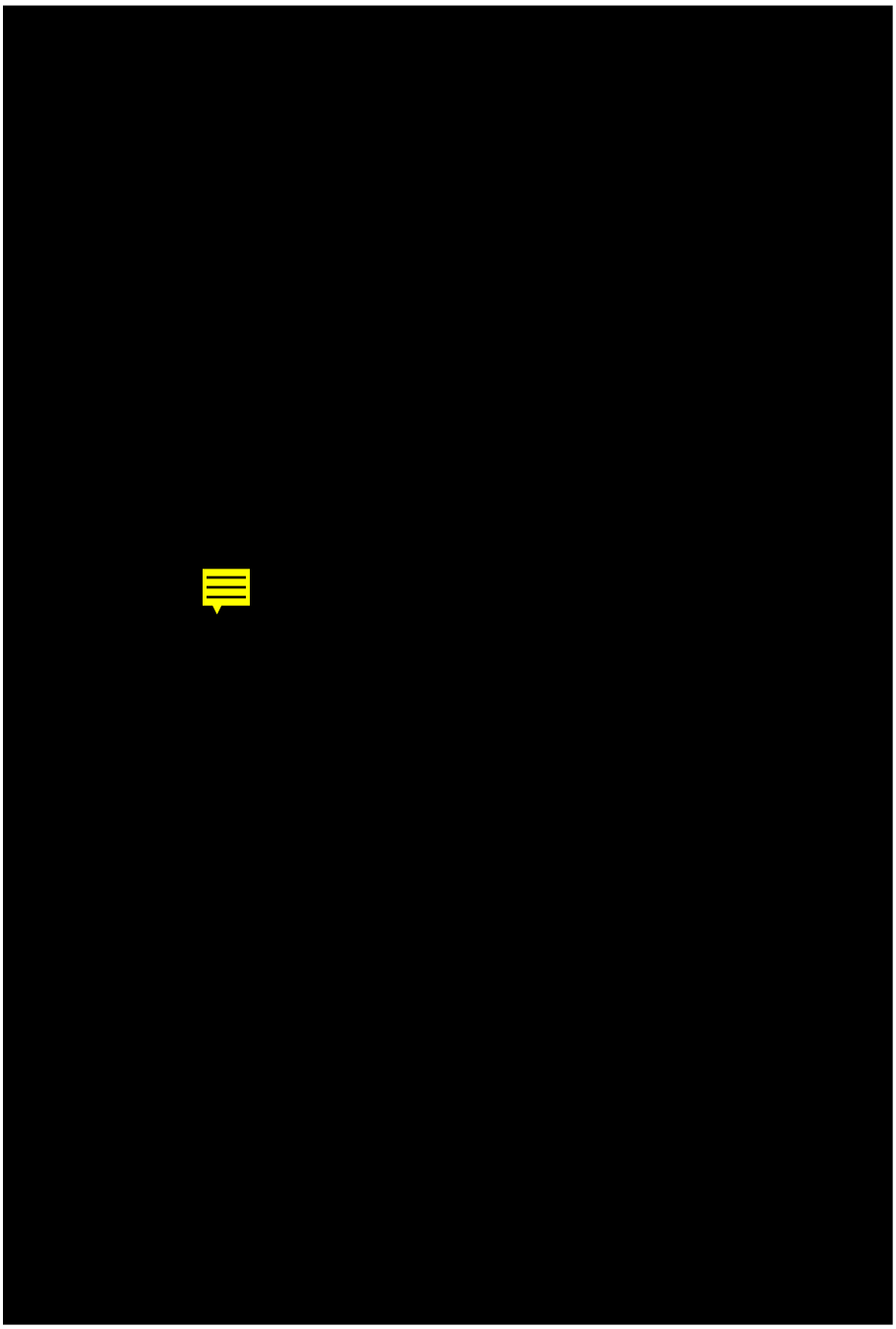

FIG. 6. In vivo detection of chloroplast DNA recombination between directly repeated sequences. Abbreviations are as in the legend to Fig. 1. (A) Scheme illustrating the assay used to measure plastid DNA recombination. The chloroplast *chlL* gene (open box with closed arrow), required for the synthesis of chlorophyll in the dark, was disrupted with a chimeric *aadA* gene (cross-hatched) conferring resistance to spectinomycin (Spec^r). The selectable marker is flanked by a 216-bp duplication (closed arrows), corresponding to part of the coding sequence of *chlL*. Transformants homoplasmic for the disrupted *chlL* gene, kept under selective conditions, are yellow in the dark. Under nonselective conditions, a recombination event between the repeats (intramolecularly, as depicted, or intermolecularly) regenerates a functional *chlL* gene giving rise to a viable green colony or green sectors in yellow colonies. For reasons of simplicity, chloroplast DNA recombination is depicted as reciprocal (conservative). However, we have not examined whether a closed circle containing the selectable marker is produced. (B) Colonies of wild-type *C. reinhardtii*, Wt*recA* transformants, and ΔN42*recA* transformants containing the *chlL* construct described above and grown under nonselective conditions. Note the almost complete absence of green sectors in colonies of the ΔN42*recA* transformants and, conversely, the higher number of green sectors and overall green color in colonies of the Wt*recA* transformants. Completely green colonies are most likely formed by cells in which the recombination event occurred either before plating or immediately thereafter.

suggests that the ΔN42RecA protein either forms mixed multimers with the wild-type protein, which are defective for some activities, or precludes the wild-type protein from forming long filaments, since repressor cleavage can be promoted by relatively short multimers unable to catalyze strand exchange (11, 39). In any case, the inhibitory effects appear to be due to direct interaction between the wild-type and mutant RecA proteins, rather than competition for DNA binding sites. The

TABLE 1. Frequency of plastid DNA recombination in homoplasmic chloroplast transformants of *C. reinhardtii* expressing chimeric *E. coli recA* genes

Genotype[a]	Recombination frequency (cell^{-1} · generation^{-1} [10^{-5}])	Relative frequency
Wt	22.8[b]	1.0
Wt*recA*	405.1[c]	17.8
ΔInt	12.5[c]	0.6
ΔN42	4.3[c]	0.2

[a] Abbreviations are given in the legend to Fig. 1.
[b] Value shown is the average of six independent experiments (standard deviation = 8.0×10^{-5}).
[c] Values shown are the averages of two independent experiments.

formation of defective mixed multimers between a mutant (RecA56) and the wild-type RecA proteins has recently been demonstrated in vitro (39). Although we cannot rule out the possibility that the inhibitory effects of the dominant negative mutants of *E. coli* RecA on plastid DNA metabolism may be indirect, the evidence discussed above strongly suggests that they are due to direct interaction of the mutant proteins with a RecA homolog in the *Chlamydomonas* chloroplast. In addition, the wild-type *E. coli* RecA protein, which would be expected to have a higher DNA binding affinity than any of the mutant proteins, actually increases rather than inhibits the frequency of plastid DNA recombination compared with that in control cells.

Since the ΔN42*recA* transformants are defective in chloroplast DNA repair and recombination, the marked reduction in hybridizable plastid DNA induced by MMS and FdUrd might reflect increased degradation of damaged genomes, as observed in *recA* mutants of *E. coli* exposed to DNA-damaging agents (37, 56, 63). However, the ΔN42RecA protein may also affect the replication of chloroplast DNA under genotoxic stress. In *E. coli*, recovery of DNA replication following its inhibition by UV irradiation (induced replisome reactivation) requires a functional RecA protein (33, 64). In addition, the initiation of DNA synthesis is altered upon DNA damage, becoming independent of transcription, translation, DnaA, and the normal origin (*oriC*) (2, 3). This induced stable DNA replication requires the RecA and RecBC(D) functions, and initiation is thought to occur via the formation of an intermediate of recombination (2, 3). Intriguingly, chloroplast DNA replication in *C. reinhardtii*, inhibited by sublethal doses of novobiocin, reinitiates preferentially near a hot spot of recombination (46) rather than from the normal replication origins (65). Recombination intermediates have been postulated to be involved in this process (65). An alternative mode of replication involving recombination could also account for the observation of rolling-circle intermediates and linear multimers of the chloroplast genome (5, 35), as well as partially deleted linear plastid DNA having a hairpin structure at both termini, as found in plants derived from anther cultures (17, 26).

Overexpression of the wild-type RecA protein does not increase markedly the frequency of homologous recombination in *E. coli* (18, 19, 31, 34). In fact, there is no correlation between the level of RecA protein in the cell and the recombination frequency (18, 19). Moreover, several lines of evidence point to DNA damage as the precipitating event in homologous recombination in eubacteria (except during conjugation or transduction) (2, 18, 19), suggesting that the starting DNA substrates, presumably generated by DNA damage, are rate limiting. In contrast, we observed an over-15-fold enhancement in the frequency of chloroplast DNA recombination in *Chlamydomonas* transformants expressing the WtRecA protein. Although several alternative explanations are possible, we speculate that plastid DNA recombination might be limited by the availability of enzymes involved in strand exchange rather than by the level of initiating DNA substrates. The polyploid nature of the chloroplast genome may allow a slower repair or even the degradation of some damaged DNA molecules without compromising cell survival. This interpretation is supported by experiments with FdUrd, which damages chloroplast DNA and markedly reduces its level in *C. reinhardtii* without affecting growth and survival (12, 32, 66).

Our results also revealed that excess RecA activity is only marginally beneficial for the survival of *C. reinhardtii*, even under conditions of genotoxic stress. Accordingly, the repair of chloroplast DNA is not substantially increased by overexpression of the wild-type *E. coli* RecA protein (12). Since the steady-state level of the endogenous plastid RecA protein is enhanced by exposure to genotoxic agents (12, 13), the amount of recombinase in chloroplasts is very likely modulated according to the DNA damage that needs to be repaired. Moreover, the observation that the plastid RecA activity seems to be much more limiting for DNA recombination than for DNA repair suggests that, during the course of evolution, the level of recombinase has been optimized to cope with DNA damage to the polyploid genome that could compromise cell survival. This strongly supports the interpretation that, at least in vegetative cells of *C. reinhardtii*, a primary biological role of the chloroplast recombination system is in DNA repair. Since transformants expressing the dominant negative mutants of *E. coli* RecA are photobleached by exposure to light of high intensity (12), whereas wild-type *C. reinhardtii* is not, the repair of chloroplast DNA appears to be required for proper maintenance of the photosynthetic apparatus under photoinhibitory conditions. In an organelle subject to significant photooxidative damage, the role of the chloroplast recombination system in DNA repair would be sufficient to explain its evolutionary conservation.

In summary, the results reported here strongly support the existence of a RecA-mediated recombination system in chloroplasts. They also provide evidence for a role of this system in repair and, possibly, replication of plastid DNA, a function that becomes essential for cell survival under genotoxic stress. To our knowledge, this is the first time that DNA recombination in a highly polyploid genome has been studied in any molecular detail, and our results strongly suggest that the system has been optimized for DNA repair rather than recombination. This hypothesis might also explain the conservation of DNA recombination systems in the mitochondria of fungi and plants, since their genomes are most likely subject to extensive oxidative damage. In addition, homologous recombination in chloroplasts presumably affects the sequence evolution of the genome. The low synonymous substitution rate, particularly in the inverted repeat regions (49), might be explained at least partially by (i) reduction in the frequency of mutagenic lesions due to recombinational repair combined with enzymatic reversal of damage and excision repair, and/or (ii) biased (in favor of the wild type) repair of mispaired bases in heteroduplex DNA formed during recombination between mutant and wild-type copies of the polyploid genome. Mechanisms of biased mismatch repair have been found in eubacteria and several eukaryotes (21, 45, 47). However, further work will be necessary to elucidate whether similar pathways operate in chloroplasts. Although our results emphasize the similarity between the chloroplast and the eubacterial recombination systems,

there are also intriguing differences such as the apparent limitation in the level of a plastid strand-exchange protein(s) possibly associated with the more relaxed requirements involved in the repair of a highly polyploid genome.

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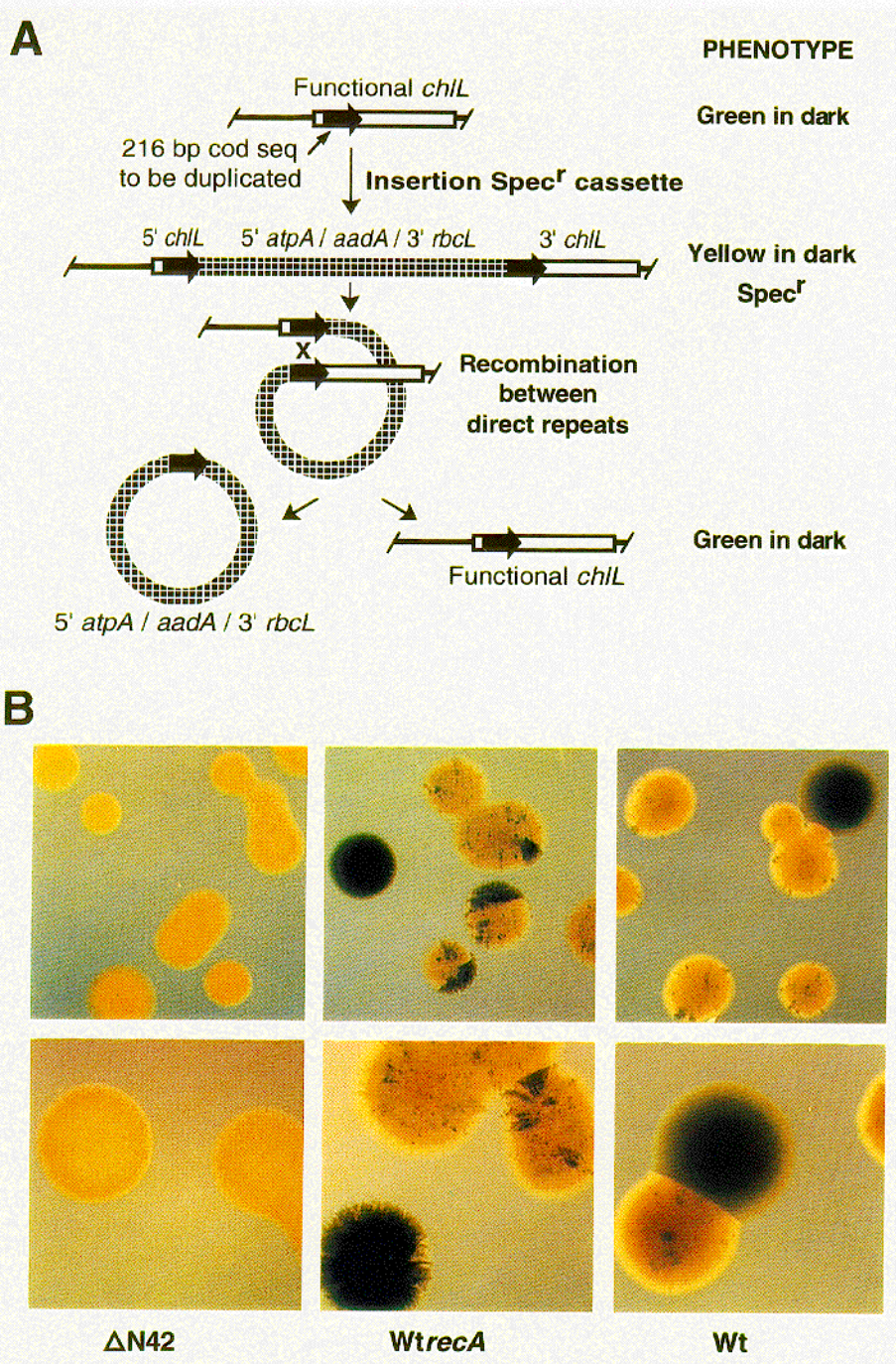
REFERENCES

1. **André, C., A. Levy, and V. Walbot.** 1992. Small repeated sequences and the structure of plant mitochondrial genomes. Trends Genet. **8:**128–132.
2. **Asai, T., D. B. Bates, and T. Kogoma.** 1994. DNA replication triggered by double-stranded breaks in E. coli: dependence on homologous recombination functions. Cell **78:**1051–1061.
3. **Asai, T., and T. Kogoma.** 1994. D-loops and R-loops: alternative mechanisms for the initiation of chromosome replication in *Escherichia coli*. J. Bacteriol. **176:**1807–1812.
4. **Bauer, C. E., D. W. Bollivar, and J. Y. Suzuki.** 1993. Genetic analyses of photopigment biosynthesis in eubacteria: a guiding light for algae and plants. J. Bacteriol. **175:**3919–3925.
5. **Bendich, A. J., and S. B. Smith.** 1990. Moving pictures and pulsed-field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria. Curr. Genet. **17:**421–425.
6. **Bennett, C. B., A. L. Lewis, K. K. Baldwin, and M. A. Resnick.** 1993. Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid. Proc. Natl. Acad. Sci. USA **90:**5613–5617.
7. **Bernstein, C., and H. Bernstein.** 1991. Aging, sex, and DNA repair. Academic Press, Inc., San Diego, Calif.
8. **Bi, X., and L. F. Liu.** 1994. *recA*-independent and *recA*-dependent intramolecular plasmid recombination. Differential homology requirement and distance effect. J. Mol. Biol. **235:**414–423.
9. **Boynton, J. E., N. W. Gillham, E. H. Harris, J. P. Hosler, A. M. Johnson, A. R. Jones, B. L. Randolph-Anderson, D. Robertson, T. M. Klein, K. B. Shark, and J. C. Sanford.** 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science **240:**1534–1538.
10. **Boynton, J. E., N. W. Gillham, S. M. Newman, and E. H. Harris.** 1992. Organelle genetics and transformation of *Chlamydomonas*, p. 3–64. *In* R. G. Hermann (ed.), Cell organelles. Springer-Verlag, Vienna.
11. **Cazaux, C., A. M. Mazard, and M. Defais.** 1993. Inducibility of the SOS response in a *recA730* or *recA441* strain is restored by transformation with a new *recA* allele. Mol. Gen. Genet. **240:**296–301.
12. **Cerutti, H., J. E. Boynton, and N. W. Gillham.** Unpublished data.
13. **Cerutti, H., H. Z. Ibrahim, and A. T. Jagendorf.** 1993. Treatment of pea (*Pisum sativum* L.) protoplasts with DNA-damaging agents induces a 39-kilodalton chloroplast protein immunologically related to *Escherichia coli* RecA. Plant Physiol. **102:**155–163.
14. **Cerutti, H., and A. T. Jagendorf.** 1993. DNA strand-transfer activity in pea (*Pisum sativum* L.) chloroplasts. Plant Physiol. **102:**145–153.
15. **Cerutti, H., M. Osman, P. Grandoni, and A. T. Jagendorf.** 1992. A homolog of *Escherichia coli* RecA protein in plastids of higher plants. Proc. Natl. Acad. Sci. USA **89:**8068–8072.
16. **Clark, A. J., and S. J. Sandler.** 1994. Homologous genetic recombination: the pieces begin to fall into place. Crit. Rev. Microbiol. **20:**125–142.
17. **Collin, S., and T. H. N. Ellis.** 1991. Evidence for the presence of hairpin chloroplast DNA molecules in barley cultivars. Curr. Genet. **20:**253–258.
18. **Cox, M. M.** 1993. Relating biochemistry to biology: how the recombinational repair function of RecA protein is manifested in its molecular properties. Bioessays **15:**617–623.
19. **Feng, W. Y., E. Lee, and J. B. Hays.** 1991. Recombinagenic processing of UV-light photoproducts in nonreplicating phage DNA by the *Escherichia coli* methyl-directed mismatch repair system. Genetics **129:**1007–1020.
20. **Foster, P. L.** 1993. Adaptive mutation: the uses of adversity. Annu. Rev. Microbiol. **47:**467–504.
21. **Fox, M. S., J. P. Radicella, and K. Yamamoto.** 1994. Some features of base mismatch repair and its role in the formation of genetic recombinants. Experientia **50:**253–260.
22. **Freitag, N., and K. McEntee.** 1988. Affinity chromatography of RecA protein and RecA nucleoprotein complexes on RecA protein-agarose columns. J. Biol. Chem. **263:**19525–19534.
23. **Gillham, N. W., J. E. Boynton, and E. H. Harris.** 1991. Transmission of plastid genes. Cell Cult. Somatic Cell Genet. Plants **7A:**55–92.
24. **Goldschmidt-Clermont, M.** 1991. Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of chlamydomonas. Nucleic Acids Res. **19:**4083–4089.
25. **Hall, R. D., G. J. A. Rouwendal, and F. A. Krens.** 1992. Asymmetric somatic cell hybridization in plants. I. The early effects of (sub)lethal doses of UV and gamma radiation on the cell physiology and DNA integrity of cultured sugarbeet (*Beta vulgaris* L.) protoplasts. Mol. Gen. Genet. **234:**306–314.
26. **Harada, T., R. Ishikawa, M. Niizeki, and K. Saito.** 1992. Pollen-derived rice calli that have large deletions in plastid DNA do not require protein synthesis in plastids for growth. Mol. Gen. Genet. **233:**145–150.
27. **Harris, E. H.** 1989. The Chlamydomonas sourcebook. Academic Press, Inc., San Diego, Calif.
28. **Harris, R. S., S. Longerich, and S. M. Rosenberg.** 1994. Recombination in adaptive mutation. Science **264:**258–260.
29. **Heifetz, P. B., J. E. Boynton, and N. W. Gillham.** Unpublished data.
30. **Horii, T., T. Ogawa, and H. Ogawa.** 1980. Organization of the *recA* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**313–317.
31. **Horii, T., N. Ozawa, T. Ogawa, and H. Ogawa.** 1992. Inhibitory effects of N- and C-terminal truncated *Escherichia coli recA* gene products on functions of the wild-type *recA* gene. J. Mol. Biol. **223:**105–114.
32. **Hosler, J. P., E. A. Wurtz, E. H. Harris, N. W. Gillham, and J. E. Boynton.** 1989. Relationship between gene dosage and gene expression in the chloroplast of *Chlamydomonas reinhardtii*. Plant Physiol. **91:**648–655.
33. **Khidhir, M. A., S. Casaregola, and I. B. Holland.** 1985. Mechanism of transient inhibition of DNA synthesis in ultraviolet-irradiated *E. coli*: inhibition is independent of *recA* whilst recovery requires RecA protein itself and an additional, inducible SOS function. Mol. Gen. Genet. **199:**133–140.
34. **Kolodner, R., R. A. Fishel, and M. Howard.** 1985. Genetic recombination of bacterial plasmid DNA: effect of RecF pathway mutations on plasmid recombination in *Escherichia coli*. J. Bacteriol. **163:**1060–1066.
35. **Kolodner, R. D., and K. K. Tewari.** 1975. Chloroplast DNA from higher plants replicates by both the Cairns and the rolling circle mechanism. Nature (London) **256:**708–711.
36. **Kowalczykowski, S. C., and A. K. Eggleston.** 1994. Homologous pairing and DNA strand-exchange proteins. Annu. Rev. Biochem. **63:**991–1043.
37. **Kunz, B. A.** 1985. Thymineless mutagenesis in bacteria, p. 189–209. *In* F. J. de Serres (ed.), Genetic consequences of nucleotide pool imbalance. Plenum Press, New York.
38. **Kuroiwa, T.** 1991. The replication, differentiation, and inheritance of plastids with emphasis on the concept of organelle nuclei. Int. Rev. Cytol. **128:**1–62.
39. **Lauder, S. D., and S. C. Kowalczykowski.** 1993. Negative co-dominant inhibition of recA protein function. Biochemical properties of the recA1, recA13 and recA56 proteins and the effect of recA56 protein on the activities of the wild-type recA protein function *in vitro*. J. Mol. Biol. **234:**72–86.
40. **Lers, A., P. B. Heifetz, J. E. Boynton, N. W. Gillham, and C. B. Osmond.** 1992. The carboxyl-terminal extension of the D1 protein of photosystem II is not required for optimal photosynthetic performance under CO_2- and light-saturated growth conditions. J. Biol. Chem. **267:**17494–17497.
41. **Louarn, J. M., J. Louarn, V. François, and J. Patte.** 1991. Analysis and possible role of hyperrecombination in the termination region of the *Escherichia coli* chromosome. J. Bacteriol. **173:**5097–5104.
42. **Mahan, M. J., and J. R. Roth.** 1989. Role of *recBC* function in formation of chromosomal rearrangements: a two-step model for recombination. Genetics **121:**433–443.
43. **McLennan, A. G.** 1988. DNA damage, repair, and mutagenesis, p. 135–186. *In* J. A. Bryant and V. L. Dunham (ed.), DNA replication in plants. CRC Press, Boca Raton, Fla.
44. **Mirzayans, R., M. Liuzzi, and M. C. Paterson.** 1988. Methylmethanesulfonate-induced DNA damage and its repair in cultured human fibroblasts: normal rates of induction and removal of alkali-labile sites in xeroderma pigmentosum (group A) cells. Carcinogenesis **9:**2257–2263.
45. **Modrich, P.** 1991. Mechanisms and biological effects of mismatch repair. Annu. Rev. Genet. **25:**229–253.
46. **Newman, S. M., E. H. Harris, A. M. Johnson, J. E. Boynton, and N. W. Gillham.** 1992. Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: evidence for a hotspot and an adjacent cold region. Genetics **132:**413–429.
47. **Nicolas, A., and T. D. Petes.** 1994. Polarity of meiotic gene conversion in fungi: contrasting views. Experientia **50:**242–252.
48. **Ogawa, T., A. Shinohara, H. Ogawa, and J. Tomizawa.** 1992. Functional structures of the RecA protein found by chimera analysis. J. Mol. Biol. **226:**651–660.
49. **Palmer, J. D.** 1992. Comparison of chloroplast and mitochondrial genome evolution in plants, p. 99–133. *In* R. G. Hermann (ed.), Cell organelles. Springer-Verlag, Vienna.
50. **Pang, Q., J. B. Hays, and I. Rajagopal.** 1993. Two cDNAs from the plant *Arabidopsis thaliana* that partially restore recombination proficiency and DNA-damage resistance to *E. coli* mutants lacking recombination-intermediate-resolution activities. Nucleic Acids Res. **21:**1647–1653.
51. **Piskur, J.** 1994. Inheritance of the yeast mitochondrial genome. Plasmid **31:**229–241.
52. **Puchta, H., P. Swoboda, and B. Hohn.** 1994. Homologous recombination in plants. Experientia **50:**277–284.

53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Sancar, A., C. Stachelek, W. Konigsberg, and W. D. Rupp.** 1980. Sequences of the *recA* gene and protein. Proc. Natl. Acad. Sci. USA **77:**2611–2615.
55. **Sedgwick, S. G., and P. A. Goodwin.** 1985. Differences in mutagenic and recombinational DNA repair in enterobacteria. Proc. Natl. Acad. Sci. USA **82:**4172–4176.
56. **Skarstad, K., and E. Boye.** 1993. Degradation of individual chromosomes in *recA* mutants of *Escherichia coli*. J. Bacteriol. **175:**5505–5509.
57. **Small, G. D.** 1987. Repair systems for nuclear and chloroplast DNA in *Chlamydomonas reinhardtii*. Mutat. Res. **181:**31–35.
58. **Smith, K. C., and T. V. Wang.** 1989. *recA*-dependent DNA repair processes. Bioessays **10:**12–16.
59. **Story, R. M., D. K. Bishop, N. Kleckner, and T. A. Steitz.** 1993. Structural relationship of bacterial RecA proteins to recombination proteins from bacteriophage T4 and yeast. Science **259:**1892–1896.
60. **Story, R. M., I. T. Weber, and T. A. Steitz.** 1992. The structure of the *E. coli recA* protein monomer and polymer. Nature (London) **355:**318–325.
61. **Suzuki, J. Y., and C. E. Bauer.** 1992. Light-independent chlorophyll biosynthesis: involvement of the chloroplast gene *chlL* (*frxC*). Plant Cell **4:**929–940.
62. **Turmel, M., C. Lemieux, and R. W. Lee.** 1981. Dispersive labelling of *Chlamydomonas* chloroplast DNA in ^{15}N-^{14}N density transfer experiments. Curr. Genet. **4:**91–97.
63. **Willetts, N. S., and A. J. Clark.** 1969. Characteristics of some multiply recombination-deficient strains of *Escherichia coli*. J. Bacteriol. **100:**231–239.
64. **Witkin, E. M., V. Roegner-Maniscalco, J. B. Sweasy, and J. O. McCall.** 1987. Recovery from ultraviolet light-induced inhibition of DNA synthesis requires *umuDC* gene products in *recA718* mutant strains but not in *recA*$^+$ strains of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**6805–6809.
65. **Woelfle, M. A., R. J. Thompson, and G. Mosig.** 1993. Roles of novobiocin-sensitive topoisomerases in chloroplast DNA replication in *Chlamydomonas reinhardtii*. Nucleic Acids Res. **21:**4231–4238.
66. **Wurtz, E. A., J. E. Boynton, and N. W. Gillham.** 1977. Perturbation of chloroplast DNA amounts and chloroplast gene transmission in *Chlamydomonas reinhardtii* by 5-fluorodeoxyuridine. Proc. Natl. Acad. Sci. USA **74:**4552–4556.
67. **Zweifel, S. G., and W. L. Fangman.** 1991. A nuclear mutation reversing a biased transmission of yeast mitochondrial DNA. Genetics **128:**241–249.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PHENOTYPE
Functional chlL
Green in dark
216 bp cod seq
to be duplicated
Insertion Spec^r cassette
5' chlL
5' atpA / aadA / 3' rbcL
3' chlL
Yellow in dark
Spec^r
X
Recombination
between
direct repeats
Green in dark
Functional chlL
5' atpA / aadA / 3' rbcL
B
ΔN42
WtrecA
Wt