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Double strand break-induced recombination in *Chlamydomonas reinhardtii* chloroplasts

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ABSTRACT

The mechanisms of chloroplast recombination are largely unknown. Using the chloroplast-encoded homing endonuclease I-*Cre*I from *Chlamydomonas reinhardtii*, an experimental system is described that allows the study of double strand break (DSB)-induced recombination in chloroplasts. The I-*Cre*I endonuclease is encoded by the chloroplast ribosomal group I intron of *C.reinhardtii* and cleaves specifically intronless copies of the large ribosomal RNA (23S) gene. To study DSB-induced recombination in chloroplast DNA, the genes encoding the I-*Cre*I endonuclease were deleted and a target site for I-*Cre*I, embedded in a cDNA of the 23S gene, was integrated at an ectopic location. Endonuclease function was transiently provided by mating the strains containing the recombination substrate to a wild-type strain. The outcome of DSB repair was analyzed in haploid progeny of these crosses. Interestingly, resolution of DSB repair strictly depended upon the relative orientation of the ectopic ribosomal cDNA and the adjacent copy of the 23S gene. Gene conversion was observed when the 23S cDNA and the neighbouring copy of the 23S gene were in opposite orientation, leading to mobilization of the intron to the 23S cDNA. In contrast, arrangement of the 23S cDNA in direct repeat orientation relative to the proximal 23S gene resulted in a deletion between the 23S cDNA and the 23S gene. These results demonstrate that *C.reinhardtii* chloroplasts have an efficient system for DSB repair and that homologous recombination is strongly stimulated by DSBs in chloroplast DNA.

INTRODUCTION

The importance of DNA repair and recombination for the maintenance of genome integrity is well documented (1–3). Repair of DNA double strand breaks (DSBs) has been studied in detail in the yeast *Saccharomyces cerevisiae.* In *S.cerevisiae* DSBs are predominantly repaired through recombination between the broken DNA and an intact homologous sequence (reviewed in 4). Two distinct mechanisms, DSB repair (5) and single strand annealing (6–9), have been proposed for the repair of DSBs. In addition, it has been demonstrated that certain hot spots for meiotic recombination are associated with DSBs, suggesting that meiotic recombination is initiated by DSBs (10,11).

The chloroplast genome of the green unicellular alga *Chlamydomonas reinhardtii* is composed of ~80 copies of a 195 kb circular DNA molecule (12,13). An inverted repeat of 19 kb which includes the ribosomal RNA genes divides the chloroplast genome of *C.reinhardtii* into two large single copy regions (Fig. 1). A characteristic feature of mutant strains with deletions or point mutations within the inverted repeat is that they are transmitted to both repeated segments, presumably through an efficient gene conversion mechanism (14). Each 23S ribosomal RNA gene of *C.reinhardtii* contains an intron with an internal open reading frame encoding I-*Cre*I, a homing endonuclease (15,16). This enzyme has previously been shown to recognize a sequence of at least 19 bp and to cleave at the intron insertion site in 23S DNA lacking the ribosomal intron (17,25). In addition, integration of a ribosomal cDNA containing the I-*Cre*I target site into the *C.reinhardtii* chloroplast genome results in efficient transfer of the ribosomal intron to that ectopic homing site (15).

Although chloroplasts fuse in zygotes of *C.reinhardtii*, one of the parental chloroplast genomes is selectively degraded. This process is genetically controlled by the nuclear mating type loci and results in mostly uniparental inheritance of chloroplast genes. However, the fact that 1–10% of meiotic zygotes transmit chloroplast markers from both parents permits the genetic analysis of chloroplast DNA recombination (13). Due to the low frequency of biparental transmission of chloroplast genes it seems unlikely that the main function of the chloroplast recombination system is promoting genetic diversity. Furthermore, studies of *C.reinhardtii* chloroplast transformants expressing dominant negative versions of the *Escherichia coli* RecA protein indicate that a RecA-mediated recombination system exists in chloroplasts. These experiments lead to the suggestion that in chloroplasts, which have to cope with significant photooxidative stress, the primary role of DNA recombination is in DNA repair (18).

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The occurrence of a rare-cutting homing endonuclease in the chloroplast of *C.reinhardtii* presents an excellent opportunity to study DSB-induced recombination in chloroplasts (15). Several studies, mainly in yeast and bacteriophages, have revealed that homing endonucleases are paramount for the genetic mobility of introns which they inhabit. These intron-encoded endonucleases cleave both strands of the recipient (intronless) DNA duplex at a specific site close to the intron insertion site. Subsequent repair of the DSB in the recipient allele using the intron-containing allele as a template is thought to proceed by a mechanism similar to the DSB repair mechanism originally proposed for recombination of nuclear DNA in yeast (5). In genetic terms, the net result of intron-homing is the unidirectional gene conversion of an allele lacking a given intron to the corresponding intron-containing form (reviewed in 19,20).

To gain more insight into the molecular mechanisms underlying chloroplast recombination, we have established a system in *C.reinhardtii* which allows us to induce transient DSBs with the I-*Cre*I endonuclease at a specific target site within the chloroplast genome and to assess the consequences of these breaks on homologous recombination between partially repeated ribosomal sequences. We discuss our findings with respect to current models for DSB-induced recombination.

MATERIALS AND METHODS

Construction of the ΔI-*Cre*I c23S recombination tester strains

In a first step, FuD50, a *C.reinhardtii* strain carrying a deletion of the 3′ end of the chloroplast *atp*B gene and some downstream sequences (21), was transformed with pES7.2 with a microprojectile gun as described previously (16). pES7.2 contains a deletion in the 23S intron that removes 360 bp of the 489 bp I-*Cre*I coding sequence. In addition, the flanking 16S and 23S ribosomal sequences present in pES7.2 contain point mutations that confer resistance to spectinomycin and erythromycin, respectively (16). Thus, chloroplast transformants were selected on TAP plates (22) containing spectinomycin and erythromycin. Homoplasmicity for the I-*Cre*I deletion was tested by Southern blotting. The deletion was present in both segments of the inverted repeat, most likely as the result of an efficient copy correction mechanism (14). One of the resulting Δ*atp*B ΔI-*Cre*I transformants was subjected to a second round of chloroplast transformation with plasmids pINTc23S.2 and pINTc23S.3 in order to provide the I-*Cre*I target site contained within the 600 bp 23S cDNA, as described previously (15). Site-specific integration of the 23S cDNA at the *atp*B locus is accompanied by replacement of the mutant *atp*B gene with the functional *atp*B gene delivered by the transforming pINT:c23S plasmids, thereby restoring photoautotrophic growth. Photosynthetic proficient ΔI-*Cre*I *c23S* transformants were recovered on HSM plates (22). Precise insertion of the 23S cDNA either in the same or in the opposite orientation relative to the proximal 23S gene was verified by Southern blotting.

Crosses

Crosses were performed as described (13). The wild-type *C.reinhardtii* strain was 137c (13). Strains 2A1 and 4C2, harbouring the 23S cDNA in inverted and direct orientation, respectively, were used as the recombination tester strains. The structure of the 23S cDNA was analyzed only in tetrads showing 2:2 segregation for the nuclear mating type loci. Segregant B9A was employed as the mt^- parent for the control crosses in which both mating partners carried the deletion in the I-*Cre*I gene. B9A (mt^- ΔI-*Cre*I) was isolated from the cross between strains 2A1 and wild-type, and had the 23S cDNA with flanking sequences precisely replaced with the corresponding allelic wild-type *atp*B locus, whereas the deletion in the endonuclease gene was maintained (based on Southern analysis and resistance to spectinomycin and erythromycin).

Southern analysis

Total DNA was prepared from 10 ml cultures of light grown *C.reinhardtii* cells using a previously published protocol (22) with modifications: cell pellets were resuspended in 350 µl TEN (50 mM EDTA, 20 mM Tris–HCl, pH 8.0, 100 mM NaCl). After addition of 25 µl proteinase K (5 mg/µl) and 50 µl 10% SDS, the lysates were incubated for 2 h at 55°C. After two phenol–chloroform extractions, 70 µl 5 M NaCl were added to the aeqeous phase and gently mixed. Sixty-five microlitres of 10% CTAB (Hexadecyltrimethylammoniumbromide, Sigma) solution prepared in 0.7 M NaCl was added and the mixtures were incubated for 10 min at 65°C. After a final chloroform extraction, the nucleic acids were precipitated by addition of 1 ml ethanol, recovered by a 1 min centrifugation in a microcentrifuge, washed with 70% ethanol, dried, and resuspended in 50 µl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.2 mg/µl RNase A). Total DNA (1–2 µl) was digested with restriction enzymes, fractionated on agarose gels and transferred to Hybond N^+ (Amersham) membrane. Double-stranded DNA probes for Southern hybridizations were labeled by random priming with [α-^{32}P]dATP (23). The c23S probe was the 0.6 kb *Bam*HI fragment of pc23S (15). The ribosomal intron probe was the 0.2 kb *Hin*fI fragment from pKS:HB100 (15) which maps outside of the deletion in the I-*Cre*I gene. The IR probe was a 1.2 kb *Bam*HI–*Sph*I fragment isolated from the p*atp*B-INT chloroplast transformation vector and corresponds to a *Bam*HI–*Kpn*I fragment of the same size in wild-type chloroplast DNA (24). The *atp*B probe was a 0.5 kb *Pst*I–*Kpn*I fragment isolated from p*atp*B3′NT (J.-D. Rochaix, unpublished results) corresponding to the coding region of the *atp*B gene deleted in FuD50. The HB2.5 probe was isolated from pCWHB2.5, a plasmid carrying the HB150 subclone of the R24 chloroplast restriction fragment (24).

PCR analysis

Selective amplification of the 23S cDNA was carried out as described previously (15). One primer hybridized to the *psb*D sequence present at the 3′ end of the *atp*B gene (primer 3′psbD). The second primer was specific either for the 5′ end of the 23S cDNA (primer 5′23S.1) in amplifications involving transformants carrying the 23S cDNA in inverted orientation, or for the 3′ end (primer 3′23S.2) when transformants were analyzed with the 23S cDNA in direct repeat orientation.

RESULTS

Experimental system for the study of DSB repair and recombination in chloroplast DNA

To study DSB-induced homologous recombination of chloroplast DNA, we decided to establish an experimental system that permits the induction of a site-specific DSB within one repeat of a pair of homologous sequences in *C.reinhardtii* chloroplast DNA.

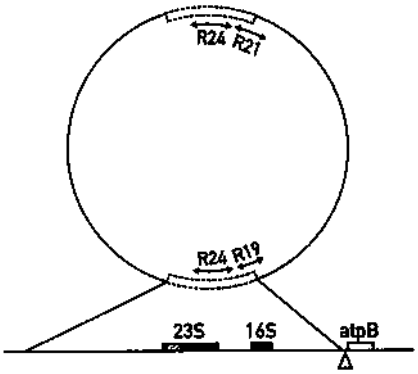

Figure 1. Chloroplast genome of *C.reinhardtii* with the two segments of the ribosomal inverted repeat (indicated by dotted boxes). An enlargement of one segment with the 16S and 23S ribosomal RNA genes including the intron (striped box) is shown below. The *atp*B gene located outside the inverted repeat is indicated. R24, R21 and R19 are *Eco*RI fragments which span partly the inverted repeat. The site of integration of the ectopic c23S fragment in which the DSB occurs is marked with an arrow.

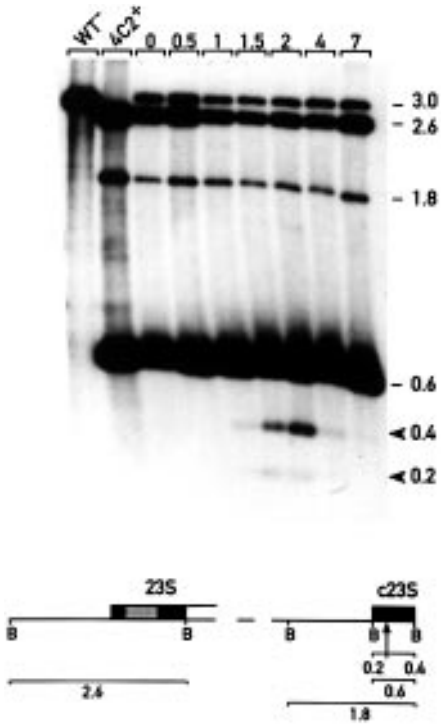

Figure 2. DSBs at the intron insertion site in young ΔI-*Cre*I c23S/I-*Cre*I$^+$ zygotes. A mating was performed between the ΔI-*Cre*I c23S^{direct}*mt*$^+$ double transformant 4C2 and an *mt*$^-$ wild-type (I-*Cre*I$^+$) strain. Gametes of both strains were mixed and samples were taken after the indicated time (in hours). Total DNA was prepared, digested with *Bam*HI and analyzed using the 23S cDNA as a probe. Samples of total DNA isolated from vegetative haploid *mt*$^-$ wild-type cells (WT$^-$) and double transformant 4C2 (4C2$^+$) are also shown. The additional DNA fragments generated by cleavage at the intron insertion site are indicated by arrow heads. Sizes in bp of DNA markers are indicated to the left.

The ~20 bp recognition sequence of the *C.reinhardtii* chloroplast homing endonuclease I-*Cre*I spans the intron insertion site of the ribosomal intron (17,25) and accordingly is disrupted in wild-type chloroplast DNA of *C.reinhardtii* in which both 23S genes carry the ribosomal intron. Hence, we expected the 195 kb chloroplast DNA circles of *C.reinhardtii* to be cut by this rare cutting endonuclease only if a I-*Cre*I target site is introduced via chloroplast transformation. Therefore, strains were constructed carrying a 0.6 kb cDNA of the 23S gene either as an inverted or a direct repeat relative to the proximal 23S gene located 8 kb away from the 23S cDNA (Figs 3 and 7, respectively). The 23S cDNA (*c23S*) contains the target site for I-*Cre*I and has 0.4 and 0.2 kb of perfect homology to upstream and downstream flanking sequences, respectively, of the ribosomal intron in the 23S genes located in both large inverted repeats of the chloroplast DNA. However, previous experiments disclosed that targeted integration of the 23S cDNA into wild-type chloroplast DNA of *C.reinhardtii* led in all cases to mobilization of the ribosomal intron to the ectopic cDNA and therefore to disruption of the I-*Cre* I recognition sequence (15). Thus, a strain was constructed with a deletion in the intron open reading frame encoding I-*Cre*I (ΔI-*Cre*I). Subsequently the 23S cDNA was introduced into the ΔI-*Cre*I strain (Materials and Methods).

We verified that no gene conversion occurred between the 23S genes and the 23S cDNA in the absence of I-*Cre*I function in 14 independent ΔI-*Cre*I c23S double transformants using the polymerase chain reaction with appropriate primers specific to the c23S cDNA and Southern analysis (data not shown). Hence, no recombination occurs between the 23S genes and the 23S cDNA in vegetatively growing I-*Cre*I deletion strains. As a next step we asked whether DSBs at the I-*Cre*I target site can be induced by mating of ΔI-*Cre*I c23S strains to wild-type.

ΔI-*Cre*I c23S strains 4C2 and 2A1, having the 23S cDNA integrated in direct and inverted repeat orientation with respect to the proximal 23S gene, respectively, were crossed to a wild-type strain. In order to detect DSBs within the 23S cDNA, cells were harvested at different time points after mixing of the gametes, total DNA was extracted, digested with *Bam*HI and hybridized to a 23S DNA probe. The only DNA fragment detected in wild-type is the 3.0 kb Ba1 chloroplast restriction fragment (Fig. 2, WT) which contains part of the 23S gene with the ribosomal intron (24). Note that the Ba1 fragment from the ΔI-*Cre*I c23S strain 4C2 is smaller than the corresponding wild-type fragment due to a 0.4 kb deletion in the I-*Cre*I coding sequence. In addition, DNA from 4C2 shows a 0.6 kb fragment which corresponds to the 23S cDNA (Fig. 2, 4C2). The 1.8 kb fragment, detected in DNA from 4C2 and from the mixed gametes, results from partial digestion at the *Bam*HI site between the 23S cDNA and the proximal 23S gene. As expected, additional fragments of 0.4 and 0.2 kb in size are detected between 1 and 4 h after mixing of the gametes (Fig. 2, lanes 1–4). These additional fragments are predicted from a DSB at the I-*Cre*I cleavage site (17,25). The same results were obtained for the mating of wild-type to the ΔI-*Cre*I c23S strain 2A1 carrying the 23S cDNA in inverted repeat orientation (data not shown). These results show that I-*Cre*I endonuclease—expressed from the wild-type chloroplast genome—is functional in chloroplasts of young zygotes and cleaves its target site within the 23S cDNA. Thus, DSBs can be induced specifically via mating of the recombination tester strains (4C2, 2A1) to a wild-type strain. To determine the type of recombination events that result from repair of DSBs in chloroplast DNA, these zygotes were sporulated and the structure of the *c23S* locus was analyzed in their haploid progeny.

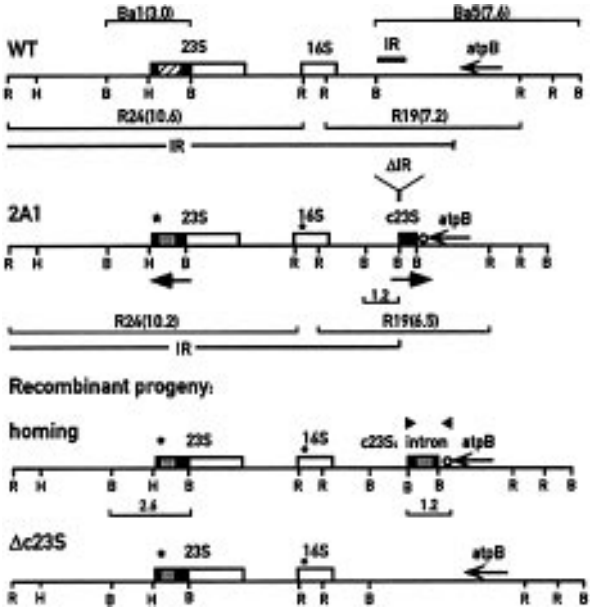

Figure 3. 23S cDNA in inverted repeat orientation: DSB-dependent intron homing and DSB-independent loss of the 23S cDNA. Restriction maps of the *C.reinhardtii* chloroplast DNA showing part of the large inverted repeat (IR) with adjacent *atp*B gene. Maps are depicted for the parental strains (WT, 2A1) and the two major types of recombinant progeny (homing, Δc23S) obtained from the cross 2A1 × WT. The locations of the 16S and 23S ribosomal RNA genes are indicated. The 0.6 kb 23S cDNA (c23S) integrated between the IR and the *atp*B gene is shown. Homologous sequences between the 23S gene and the 23S cDNA (filled rectangle), wild-type ribosomal intron (cross hatched rectangle), intron with the deletion in I-*Cre*I (stippeled rectangle), deletion of the terminal part of the IR (ΔIR), IR probe (thick horizontal bar), the 3′ end of the *psb*D gene fused to the *atp*B gene (open circle) and primers (filled arrow heads) used for PCR are indicated. The *er-u-11* (∗), *spr-u-1-6-2* (filled circle) markers, which confer resistance to erythromycin and spectinomycin, respectively are shown. Only relevant restriction sites are indicated: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. *Bam*HI (Ba1, Ba5) and *Eco*RI (R19, R24) restriction fragments (12) with their sizes in kb are indicated. 'Homing' results from DSB-dependent mobilization of the ribosomal intron (with the deletion in the I-*Cre*I gene) to the 23S cDNA. The predominant type of Δc23S progeny is shown, in which the 23S cDNA with flanking IR deletion and 3′ *psb*D sequences has been replaced (via a DSB-independent mechanism) with the corresponding wild-type (*mt*⁻) region.

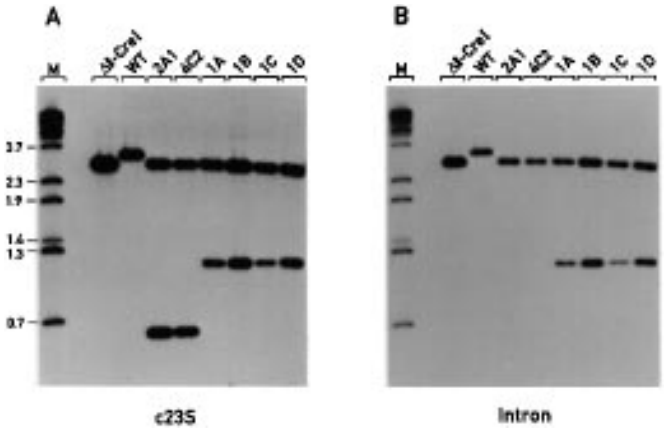

Figure 4. Southern analysis of one representative homing-type tetrad. Total DNA was prepared from the ΔI-*Cre*IΔ*atp*B recipient strain for transformation with the 23S cDNA, the parental ΔI-*Cre*I c23S *mt*⁺ recombination tester strains 2A1 (inverted repeat orientation of the 23S cDNA) and 4C2 (direct orientation), the parental *mt*⁻ wild-type strain (WT) and from the four members of one representative homing-type tetrad derived from cross 2A1 × WT (1A, 1B, 1C, 1D). DNAs were digested with *Bam*HI and hybridized (**A**) to the 23S cDNA probe (c23S) and (**B**) to the ribosomal intron probe (intron). DNA sizes are indicated in kb.

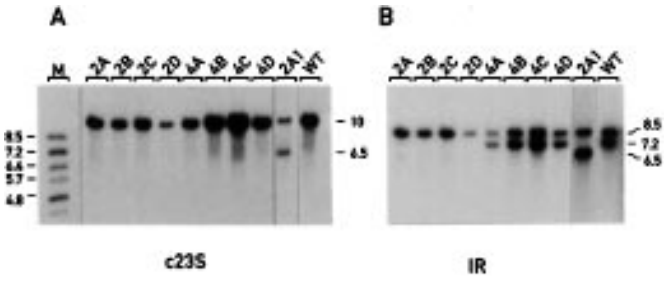

Figure 5. Southern analysis of Δc23S progeny from cross 2A1 × WT (inverted orientation of the 23S cDNA). Total DNA was isolated from the recombination tester strain 2A1 (inverted repeat orientation of the 23S cDNA), from the parental *mt*⁻ wild-type strain (WT), from the members of the two different Δc23S tetrads. Tetrad 4A–D is one of the two tetrads that show the precise replacement of the 23S cDNA and flanking sequences with allelic wild-type DNA. Tetrad 2A–2D has the deletion of the 23S cDNA with additional rearrangements. DNAs were digested with *Eco*RI and hybridized (**A**) to the 23S cDNA probe (c23S) and (**B**) to the IR probe (Fig. 3). DNA sizes are indicated in kb.

I-*Cre*I-mediated intron-homing to the 23S cDNA in inverted repeat orientation

In *C.reinhardtii* the chloroplast genome is inherited in a uniparental fashion from the mating-type plus (*mt*⁺) parent (13). The ΔI-*Cre*I strains containing the 23S cDNA as a recombination substrate were therefore constructed in a *mt*⁺ nuclear background to transmit the modified chloroplast DNA to most of the progeny. The outcome of DSB repair was analyzed in the haploid progeny from the cross between the ΔI-*Cre*I strain carrying the 23S cDNA in inverted orientation (2A1) and a wild-type strain (*mt*⁻). Eight tetrads were analyzed by Southern blotting and PCR (data not shown) for the fate of the 23S cDNA. Figure 3 shows restriction maps of part of the large inverted repeat proximal to the 23S cDNA for 2A1, wild-type and their progeny.

Four tetrads had the ribosomal intron integrated at the 23S cDNA (Fig. 3, 'homing'). Probing of *Bam*HI-digested DNA isolated from the members of these tetrads with the 23S cDNA revealed that the fragment corresponding to the 23S cDNA has increased in size from 0.6 to 1.2 kb. One representative of the homing type tetrads is shown in Figure 4A (lanes 1A–1D). The same 1.2 kb fragments also hybridized to an intron probe (Fig. 4B). Homing of the wild-type intron to the 23S cDNA results in a *Bam*HI fragment of 1.5 kb (15), whereas a 1.2 kb *Bam*HI fragment is expected if homing of the ΔI-*Cre*I intron occurred. Hence, among the analyzed progeny only homing of the intron with the I-*Cre*I deletion was observed. Only one tetrad had the majority of the homing sites unoccupied by the ribosomal intron, as shown by weak hybridization to the 1.2 kb fragment diagnostic for homing (data not shown).

Table 1. I-CreI promotes homing and deletion formation

Cross		**Progeny**							
Orientation of c23S	I-CreI	Homing		Δc23S[a]		c23S unchanged		ΔR24-R19	
inverted	+	16	50	8 (4)	25 (12)	4	12	0	0
inverted	–	0	0	6[b]	21	22[b]	79	0	0
direct	+	0	0	0	0	0	0	32	100
direct	–	0	0	4[b]	14	24[b]	86	0	0

Crosses are as described in the text. Classification of progeny is as shown in Figures 3 and 7. Numbers of haploid progeny of the same class are shown on the left of each column. Relative values in percent are given on the right of each column.

[a]Data are presented for the deletion of the 23S cDNA accompanied by precise restoration of the *atp*B locus. Values for the single tetrad that showed additional rearrangements besides deletion of the 23S cDNA are presented in parenthesis.

[b]Several haploid segregants were heteroplasmic for the loss of the 23S cDNA (6/28 in inverted orientation and 4/28 in direct orientation). The heteroplasmic progeny was scored as Δc23S if >50% recombination occurred, as judged by the relative intensities of bands on Southern blots.

Three tetrads were found in which all their members have lost the 23S cDNA (Fig. 3, Δc23S). Figure 5A shows a Southern blot with *Eco*RI digests of DNA from the members of two such tetrads, hybridized to the 23S cDNA probe. Δc23S progeny lacks the 6.5 kb fragment, which corresponds to the modified R19 *Eco*RI chloroplast restriction fragment (12) containing the 23S cDNA. The 10 kb fragment, present in all DNA samples shown in Figure 5A, corresponds to the R24 *Eco*RI fragment from the large inverted repeats (12) which contains the 23S gene (Fig. 3). Consistently, also the 0.6 kb *Bam*HI fragment containing the 23S cDNA was absent in DNA samples of these tetrads (data not shown). As shown in Figure 3, the flanking sequences of the 23S cDNA in the parental 2A1 strain can be distinguished from the corresponding allelic wild-type locus. Since the 23S cDNA is bound by *Bam*HI sites, the recombination tester strain has a characteristic 1.2 kb *Bam*HI fragment at the end of the large inverted repeat (IR) neighboring the 23S cDNA, whereas in wild-type DNA this 1.2 kb fragment is contained within the 7.6 kb *Bam*HI fragment Ba5 (12). Southern analysis, using a probe hybridizing to this 1.2 kb interval of the IRs (Fig. 3, IR probe) revealed that the 1.2 kb *Bam*HI fragment is absent in Δc23S tetrads, indicating that the left *Bam*HI site of the 23S cDNA is missing (data not shown). Consistently, when the IR probe was hybridized to *Eco*RI digested DNA of this type of tetrad, a 7.2 kb *Eco*RI fragment with the same size as the wild-type R19 restriction fragment was detected in two of the three Δc23S tetrads (Fig. 5B, 4A–4D). However, the 7.2 kb *Eco*RI fragment was missing in one of the Δc23S tetrads (Fig. 5B, 2A–2D), and only the 8.5 kb R21 fragment containing part of the other large inverted repeat (12) was detected, suggesting that in this tetrad additional rearrangements occurred besides deletion of the 23S cDNA. Furthermore, the 3′ end of the *atp*B gene next to the 23S cDNA in the ΔI-*Cre*I *c23S* strains differs from wild-type in that it has been replaced with the 3′ untranslated region of the *psb*D gene (Fig. 3). As expected, hybridization to an oligonucleotide, specific for the 3′ untranslated region of *psb*D, revealed that these *psb*D sequences were not present in the R19 fragment from the tetrads that had regained the wild-type structure at the *atp*B locus (data not shown). Yet, the Δc23S tetrad that lacked the 7.2 kb R19 fragment (2A–2D) showed hybridization of the *psb*D oligo to an 8.5 kb *Eco*RI fragment (data not shown), indicating that recombination occurred between the *psb*D sequence at the 3′ end of *atp*B and the 23S cDNA and that the wild-type configuration at the *atp*B locus has not been restored.

To determine the requirement for I-*Cre*I in intron homing and deletion of the 23S cDNA, control crosses were performed between strain 2A1 (carrying the 23S cDNA in inverted orientation) and a compatible ΔI-*Cre*I strain (Materials and Methods). Seven tetrads were analyzed for the fate of the 23S cDNA and flanking sequences by Southern blotting (data not shown). The results are summarized in Table 1 and compared to the results obtained with the analogous cross in which I-*Cre*I function was provided by the wild-type partner. As anticipated, intron-homing was not detected at all in progeny from the cross in which both parents carried the ΔI-*Cre*I allele, confirming that I-*Cre*I mediates mobilization of the ribosomal intron to the 23S cDNA during matings. In contrast, recombination leading to loss of the 23S cDNA and to precise restoration of the wild-type structure at the *atp*B locus was observed with comparable frequencies, 25 and 21%, in the presence and in the absence of I-*Cre*I function, respectively. Hence, loss of the 23S cDNA seems to be a DSB-independent process. No Δc23S tetrads displaying additional rearrangements at the *atp*B locus were detected in the control. Due to the low frequency of this recombination event and the limited sample size of control cross progeny, it is not quite clear whether loss of the cDNA with associated rearrangements was initiated by I-*Cre*I. From these experiments we conclude that DSBs at the 23S cDNA oriented as an inverted repeat are repaired using the 23S genes as a template and result in conversion of the 23S cDNA to its intron-containing form.

I-*Cre*I-mediated formation of a deletion between the 23S gene and the 23S cDNA in direct repeat orientation

To determine whether repair of DSBs at the 23S cDNA is dependent on the orientation of the 23S cDNA, we also analyzed tetrad progeny derived from a cross between a wild-type strain and the ΔI-*Cre*I c23S recombination tester strain 4C2 which has the 23S cDNA integrated in direct repeat orientation. Initially, the members of eight tetrads were subjected to PCR analysis using primers which selectively amplify the 23S cDNA and not the 23S genes. In all cases PCR products of 1.2 kb were obtained (Fig. 7 and data not shown), indicating very efficient homing of the ΔI-*Cre*I intron to the 23S cDNA when present as a direct repeat. However, Southern analysis did not confirm the PCR results, since the characteristic 1.2 kb *Bam*HI fragment containing the 23S cDNA with an integrated ΔI-*Cre*I intron was not detected (data not shown). This suggested that a deletion between the 23S

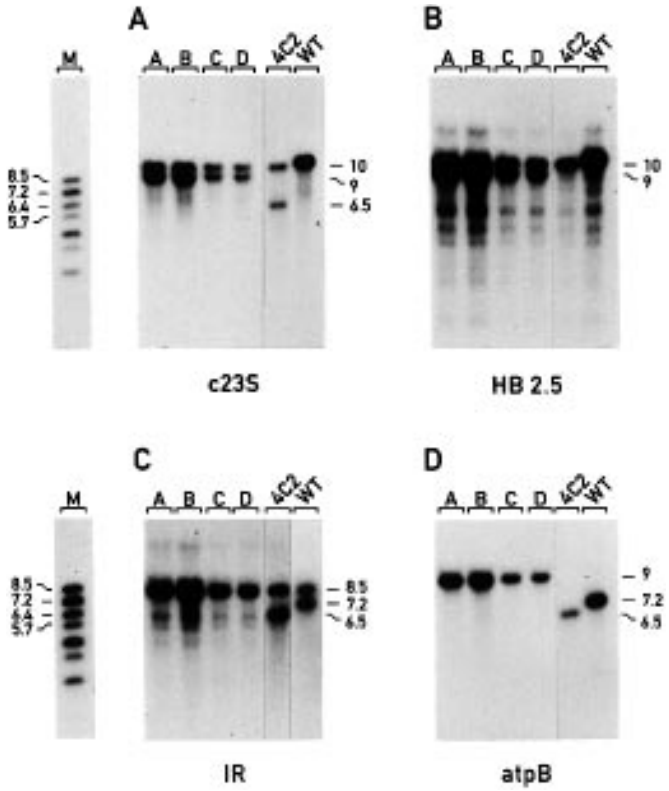

Figure 6. Formation of a deletion between the 23S gene and the 23S cDNA in direct repeat orientation. Analysis is shown for one representative tetrad from the progeny of the cross 4C2 (ΔI-*Cre*I c23S^{direct} *mt*$^{+}$) × WT. Total DNA was isolated from the members of the tetrad progeny (A–D) and their parents (4C2, WT), digested with *Eco*RI and probed with (**A**) the 23S cDNA (c23S); (**B**) the HB2.5 subclone of R24; (**C**) the IR probe and (**D**) the *atp*B probe. The same DNA size markers are used as in Figures 5 and 6. See Figure 7 for location of the probes.

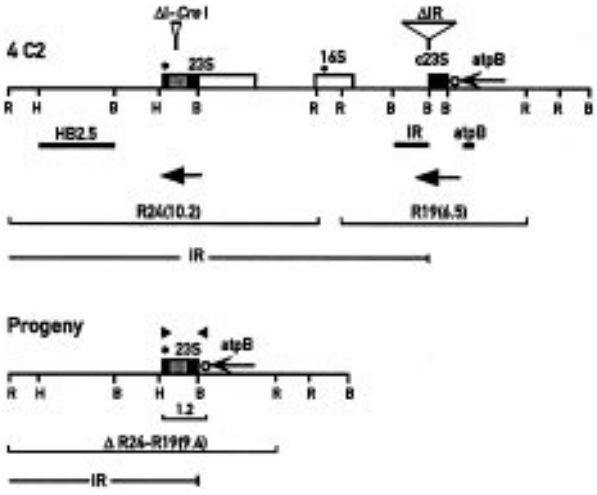

Figure 7. 23S cDNA in direct repeat orientation: DSB-dependent formation of a deletion between the 23S gene and the 23S cDNA. Structure of the recombination tester strain 4C2 (ΔI-*Cre*I c23S^{direct}) and of the resulting progeny from the cross 4C2 × WT. Thick horizontal bars indicate the probes used for Southern analysis shown in Figure 6. Other symbols as in Figure 3.

gene and the 23S cDNA had occurred, joining the R24 and R19 fragments and placing the *atp*B gene next to the remaining 23S gene (Fig. 7). To test this hypothesis, Southern analysis was carried out with *Eco*RI digested DNA from all the progeny with different probes. As an example, Figure 6 shows the results obtained with one representative tetrad. The 23S cDNA probe revealed, in addition to the 10 kb R24 fragment, a 9 kb *Eco*RI fragment which was present neither in the wild-type nor in the 4C2 parent. As expected, no *Eco*RI fragment of 7 kb, the size of the modified R19 fragment containing the 23S cDNA with the ΔI-*Cre*I intron inserted, is detected in DNA from the progeny. When the HB 2.5 probe, which maps to the left end of the R24 fragment (Fig. 7), was hybridized to DNA of the tetrad progeny, essentially the same result was obtained as for the 23S cDNA probe, i.e. the 9 kb fragment was detected only in the tetrad progeny (Fig. 6B). Hybridization with the IR probe (Fig. 7) revealed in all strains the 8.5 kb R21 fragment from the other large inverted repeat region, whereas the modified 6.5 kb R19 fragment harboring the 23S cDNA was almost completely absent in the tetrad progeny (Fig. 6C), corroborating that a deletion has occured in this part of the R19 fragment. The faint bands, which were detected in the tetrad samples at the same position as the R19 fragment of the 4C2 parent, showed that the tetrad progeny is not 100% homoplasmic. Figure 6D shows that the *atp*B probe, which maps to the right end of the 9 kb *Eco*RI fragment (Fig. 7) hybridized to the 9 kb fragment in DNA from the tetrad progeny and to the R19 fragments of 4C2 and wild-type. Taken together, these results are in agreement with the formation of a deletion between the 23S gene and the 23S cDNA in direct repeat orientation. This deletion removes 8 kb of the chloroplast DNA containing most of the 23S gene and the entire 16S gene in one of the large inverted repeats. Interestingly, loss of the 23S cDNA in direct repeat orientation was never accompanied with restoration of the allelic wild-type structure at the *atp*B locus, which occurred in 25% of the cases when the 23S cDNA was present in inverted repeat orientation.

To establish the role of I-*Cre*I in the formation of the deletion between the 23S gene and its cDNA in direct repeat orientation, a control cross was performed in which both parents lacked I-*Cre*I function. Analysis of seven tetrads derived from this control cross disclosed that deletion formation was completely dependent on I-*Cre*I function in the *mt*$^{-}$ parent (Table 1). The question arises whether deletion formation was preceded by an intron homing event, which would considerably increase the size of the duplicated sequence. No evidence for the transient appearance of a 1.2 kb *Bam*HI fragment indicative of a homing event could be obtained in young zygotes (Fig. 2) and attempts to detect such an event by PCR were unsuccessful.

Loss of the 23S cDNA without deletion formation accompanied by restoration of the allelic wild-type configuration was again observed among the progeny from the control cross, although the frequency of this recombination event was somewhat lower in direct orientation compared with the analogous cross involving the 23S cDNA in inverted orientation (14 versus 21%, respectively). Taken together, these data demonstrate that DSB repair at the 23S cDNA is strongly dependent on the orientation of the cDNA relative to its proximal homologous gene. DSB repair at the 23S cDNA in direct repeat orientation leads in all cases to deletion of the DNA between the cDNA and

the proximal 23S gene, whereas repair of DSBs at the 23S cDNA in inverted orientation results in intron-homing to the cDNA.

DISCUSSION

Group I introns are a prominent feature of *Chlamydomonas* chloroplast genomes where they are frequently encountered in LSU rRNA genes (26). Besides I-*Cre*I, two additional homing endonucleases have been identified in chloroplasts of the genus *Chlamydomonas*. Both, I-*Ceu*I and I-*Chu*I are encoded by ribosomal introns of *C.eugametos* and *C.humicola*, respectively (27,28). Unidirectional gene conversion in interspecific crosses has been reported for the *C.eugametos* intron encoding I-*Ceu*I (29). Dependence of intron-homing on endonuclease function has been demonstrated for mobile group I introns from T4 phage and yeast mitochondria (30–33). However, the role of intron-encoded endonucleases in mobility of chloroplast introns has not been directly assessed so far. Here we present two lines of experimental evidence that intron mobility in chloroplasts requires homing endonuclease function. First, mobility of the *C.reinhardtii* ribosomal intron to an ectopic homing site upon transformation of chloroplasts with the 23S cDNA is abolished in strains carrying deletions in their I-*Cre*I genes. Secondly, the ribosomal intron can be mobilized during crosses to its artificial homing site only if endonuclease function is provided by the mating partner. Considering only progeny that had maintained the 23S cDNA, the efficiency of intron homing was 80% (Table 1). Surprisingly, intron mobility did not occur if the 23S cDNA had the same orientation as the proximal ribosomal gene, suggesting that recombination mechanisms for the repair of DSBs at the homing site differ for the two orientations of the 23S cDNA (see below).

Recombination functions required for mobility of the phage *td* intron have been described in some detail. The recombinases and nucleases required for efficient mobility of this prokaryotic group I intron are consistent with the functions predicted by the DSB repair (DSBR) pathway for intron inheritance (34). Assuming that mobility of eukaryotic group I introns proceeds by a similar DSBR mechanism as described for their prokaryotic counterparts, our results for intron mobility to the 23S cDNA in inverted repeat orientation are most readily explained by the DSBR model for intron-homing. The single-stranded 3′ ends of the 23S cDNA generated by cleavage of I-*Cre*I (17,25), and presumably further exposed by subsequent recession of the 5′ termini, invade homologues stretches of the 23S gene flanking the ribosomal intron. DNA repair synthesis using the 23S gene as a template and subsequent resolution leads to copying of the ribosomal intron to the cDNA. Since in all examined cases intron mobility was not associated with inversions between the 23S cDNA and the 23S gene, DSBR was not accompanied by crossing over of flanking DNA (data not shown). The reason could be that such an inversion might have deleterious effects. First, the segment of chloroplast DNA bordered by the 23S cDNA and the proximal 23S gene contains the strong promoter of the ribosomal RNA unit which would lead to increased transcription outside of the large inverted repeat and might thereby interfere with expression of nearby essential genes. Second, the inversion would create a chloroplast genome with two ribosomal direct repeats which may undergo recombination with each other and compromise the stability of the chloroplast genome.

Interestingly, only homing of the intron carrying the deleted I-*Cre*I gene was observed among the analyzed progeny. This is in contrast with typical (allelic) intron-homing, where both endonuclease and intron are contributed in *trans*. The difference might be due to the fact that in *C.reinhardtii* the chloroplast DNA from the mt^- mating partner is degraded in the zygote (35), thereby increasing the number of ΔI-*Cre*I introns which can be used as donors for conversion of the 23S cDNA to the intron-containing form. Alternatively, this result might indicate a preference of the system for intramolecular gene conversion, if the choice between inter- and intramolecular reaction is offered.

Chloroplast genes are mainly uniparentally transmitted to meiotic progeny of *C.reinhardtii* such that most of the progeny has the same chloroplast genotype as the mt^+ parent. However, between 1 and 10% of meiotic zygotes transmit chloroplast markers from the mt^- parent (13). Surprisingly, up to 25% of the meiotic progeny derived from crosses involving the mt^+ chloroplast recombination tester strain carrying the 23S cDNA in inverted orientation (strain 2A1) had lost the cDNA and acquired the mt^- (i.e. wild-type) configuration at the *atp*B locus (Table 1). These recombination events were mostly independent of I-*Cre*I endonuclease function, since similar frequencies were obtained whether or not the mt^- parent provided endonuclease function (25 and 21%, respectively, Table 1). They appear to be induced during the meiotic cycle because they were never observed in cells grown vegetatively. However, endonuclease-independent loss of the 23S cDNA seems to be orientation-dependent, since this recombination event was detected in only 14% of the progeny derived from the cross between the recombination tester strain carrying the 23S cDNA in direct repeat orientation (4C2) and an mt^- strain deficient in I-*Cre*I . In addition, several *aad*A reporter gene fusion constructs integrated at the same site of the *C.reinhardtii* chloroplast genome (*atp*B locus) were transmitted with >95% frequencies to meiotic progeny, and showed therefore little recombination with mt^- chloroplast DNA (36,37). Thus, the relatively high frequency of I-*Cre*I-independent loss of the 23S cDNA appears to be caused by the nature and orientation of the inserted ribosomal cDNA rather than the site of insertion.

It is intriguing that intron homing during genetic crosses was not observed when the 23S cDNA was in direct repeat orientation. Instead, repair of DSBs at the 23S cDNA in direct orientation led in all cases to deletion of the DNA flanked by the cDNA and the neighbouring 23S gene. On the basis of the proposed DSBR pathway for intron-homing the formation of a deletion between the 23S gene and the 23S cDNA arranged in the same orientation can be explained as resolution of the canonical recombination intermediate in the crossing-over configuration. However, if formation of these deletions follows the classical DSBR mechanism, it has to be postulated that resolution of the Holliday junctions is nonrandom, since no intron-homing to the 23S cDNA in direct repeat orientation was detected, which corresponds mechanistically to gene conversion without associated crossing-over (Fig. 8).

Interestingly, a similar orientation dependence for DSB-induced recombination between repeated sequences in the nuclear genome of *S.cerevisiae* has been reported (6). Plasmids containing two nonfunctional copies of the *lacZ* gene either in direct or inverted orientation were used as substrates for DSB-induced recombination. $LacZ^+$ plasmids were generated by gene conversion promoted by a DSB introduced by the mating-type switching HO endonuclease at a specific site created by insertion of the HO target site into one of the two copies of the *lacZ* gene. In direct orientation, repair of the DSB resulted in 80%

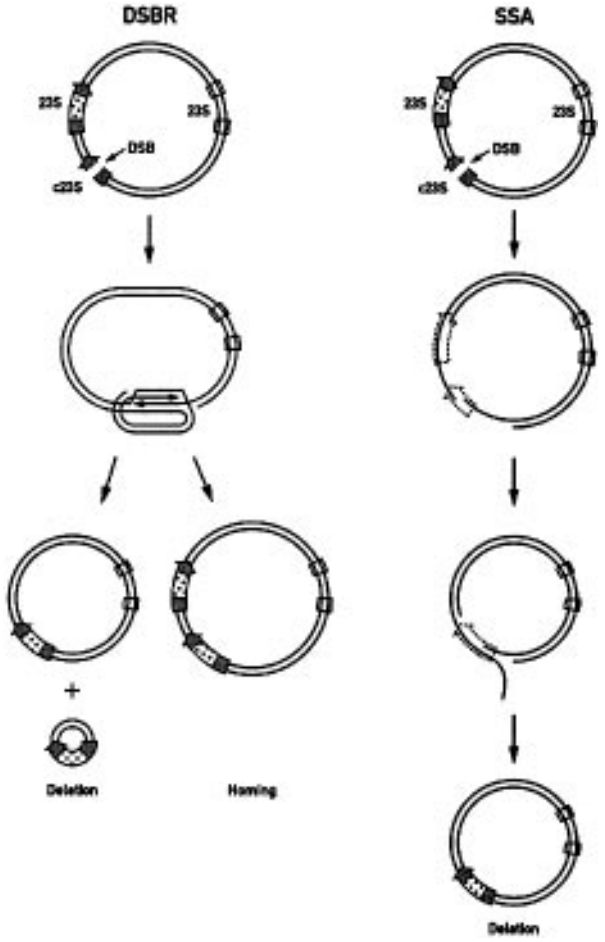

Figure 8. DSBR and SSA models for DSB-initiated recombination between the 23S gene and the 23S cDNA in direct repeat orientation. (**A**) The double-strand break repair (DSBR) mechanism invokes 5′→3′ exonucleases to render the 3′ ends of the double-strand break (DSB) single-stranded. The single-stranded ends invade the homologous proximal 23S duplex and the gap is filled by DNA synthesis, resulting in copying of the ribosomal intron into the 23S cDNA. Resolution of the Holliday junction intermediate results either in intron homing for resolution without associated crossing-over, or in the formation of a deletion for resolution with crossing-over. The deleted sequence transiently accumulates as a circular molecule. (**B**) In the SSA (single-stranded annealing) mechanism, 5′→3′ degradation starting at the ends of the DSB continues until homologous complementary regions are single-stranded. Annealing of the complementary single-strands is followed by trimming of the protruding single-strands and repair DNA synthesis. This pathway yields only one type of product, the deletion, and the DNA between the direct repeats is removed. Homologous stretches between the 23S cDNA and the proximal 23S gene present in the same orientation are designed by large arrows shaded in gray, and the intron as stippled region within these arrows; ribosomal sequences homologous to the 23S cDNA that are present in the distal ribosomal unit are indicated by open boxes. This figure is adapted from ref. 8.

of the cases in deletion of the region between the *lacZ* repeats, whereas in inverted orientation only 50% of the gene conversion events to LacZ$^+$ were associated with inversion of the sequence flanked by the repeats (6). For the *lacZ* repeat plasmid system in yeast, it has been shown that formation of the observed deletions in direct orientation proceeds via a single-stranded annealing (SSA) mechanism (8). SSA recombination has been proposed on the basis of studies of plasmid recombination in mammalian cells and is mechanistically distinct from the DSBR pathway for homologous recombination (38). Although SSA recombination has so far been described exclusively for nuclear recombination, this mechanism could readily explain why homing of the ribosomal intron to the 23S cDNA in direct repeat orientation was not observed. As shown in Figure 8, the SSA mechanism involves extensive 5′→3′ single-stranded degradation, proceeding in both directions from the DSB, followed by annealing of the 23S cDNA and complementary sequences of the proximal 23S gene. Subsequent trimming and DNA repair synthesis removes the 23S cDNA.

The presumed prokaryotic ancestry of chloroplasts predicts that DNA recombination systems of plastids are related to those of eubacteria. Indeed, chloroplast homologs of the *E.coli* RecA protein have been identified in *Arabidopsis thaliana* and pea (39). In addition, evidence has been obtained for a functional homolog of RecA in *C.reinhardtii* chloroplasts by demonstrating that expression in chloroplasts of dominant negative mutants of the *E.coli* RecA protein interferes with repair of plastid DNA and diminishes chloroplast DNA recombination (18). Moreover, an *E.coli ruvC recG* double mutant, deficient in resolution of crossed-strand recombination intermediates, could be complemented with two *Arabidopsis* cDNAs that encode predicted chloroplast-targeted proteins (40). There are, however, differences between eubacterial and chloroplast recombination systems. Here we show that *C.reinhardtii* chloroplasts are competent to repair DSBs via recombination with intact homologous sequences even if the terminal homologies at the DSB are only 200 and 400 bp. In contrast, the wild-type *E.coli* recombination machinery is incapable to support recombinational repair of DSBs that have only short (1–2 kb) terminal homologies to intact sequences (34,41).

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