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Shared molecular characteristics of successfully transformed mitochondrial genomes in *Chlamydomonas reinhardtii*

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Abstract

Three types of respiratory deficient mitochondrial strains have been reported in *Chlamydomonas reinhardtii*: a deficiency due to (i) two base substitutions causing an amino acid change in the apocytochrome *b* (COB) gene (i.e., strain named *dum-15*), (ii) one base deletion in the COXI gene (*dum-19*), or (iii) a large deletion extending from the left terminus of the genome to somewhere in the COB gene (*dum-1*, *-14*, and *-16*). We found that these respiratory deficient strains of *C. reinhardtii* can be divided into two groups: strains that are constantly transformable and those could not be transformed in our experiments. All transformable mitochondrial strains were limited to the type that has a large deletion in the left arm of the genome. For these mitochondria, transformation was successful not only with purified intact mitochondrial genomes but also with DNA-constructs containing the compensating regions. In comparison, mitochondria of all the non-transformable strains have both of their genome termini intact, leading us to speculate that mitochondria lacking their left genome terminus have unstable genomes and might have a higher potential for recombination. Analysis of mitochondrial gene organization in the resulting respiratory active transformants was performed by DNA sequencing and restriction enzyme digestion. Such analysis showed that homologous recombination occurred at various regions between the mitochondrial genome and the artificial DNA-constructs. Further analysis by Southern hybridization showed that the wild-type genome rapidly replaces the respiratory deficient monomer and dimer mitochondrial genomes, while the *E. coli* vector region of the artificial DNA-construct likely does not remain in the mitochondria.

Abbreviations: aad, aminoglycoside adenine transferase; *cob*, apocytochrome b; *cox1*, subunit 1 of cytochrome c oxidase; DIG, digoxigenin; *nd*, NADH dehydrogenase; non-WT, non-wild type; PCR, polymerase chain reaction; *rtl*, reverse transcriptase-like protein

Introduction

Artificial stable incorporation of foreign DNA into the mitochondrial genome has previously been demonstrated only in two species, the yeast *Saccharomyces cerevisiae* (Johnston *et al*., 1988) and the unicellular green alga *Chlamydomonas reinhardtii* (Randolph-Anderson *et al*., 1993). However, in the latter case, successful mitochondrial transformation has been demonstrated only

by introducing the entire wild-type mitochondrial genome into a respiratory deficient mutant *dum-1* strain (Randolph-Anderson *et al.*, 1993), and rescue of strains containing other respiratory deficiencies (e.g., *dum-14*, *-15*, *-16*, and *-19*) were not attempted.

Curiously, no follow-up paper has been published, in contrast to the many papers that have been published since the first report of successful *C. reinhardtii* chloroplast transformation in 1988 (Boynton *et al.*, 1988). Transformation of the *C. reinhardtii* chloroplast seems much easier than the mitochondria, because only a single large chloroplast exists in the cell (for a review, Harris, 2001). In addition to this, another significant reason for underutilization of this mitochondrial transformation system is probably due to the lack of a convenient transformation vector that can be amplified in *E. coli*. Therefore, we developed three kinds of artificial DNA-constructs to transform respiratory deficient *C. reinhardtii* strains. Various combinations of the DNA-constructs and the *dum* strains were tried to find out the most efficient set.

The mitochondrial genome of *C. reinhardtii* is a linear 15.8 kb molecule (Gray and Boer, 1988). A 0.5 kb inverted repeat sequence is present at the right and left termini of the linear mitochondrial DNA. It remains unclear what DNA configuration and mechanisms are involved in DNA replication. However, the replication origin (*ori*) of the *Chlamydomonas* mitochondrial genome is thought to be located in this inverted repeat sequence, because A + T rich clusters and numerous short direct and inverted repeats are detected in this region (Gray and Boer, 1988; Nedelcu and Lee, 1998). Both strands of mitochondrial DNA encode genetic information, and each strand may be organized into a single transcriptional unit, suggested by the mapping of two separate promoters in the gap between the *nd5* and *cox1*, one pointing in each direction.

We discovered that only a limited number of respiratory deficient strains are competent for mitochondrial transformation, and mitochondrial genome transitions were analyzed by Southern hybridization and restriction enzyme digestion. Here, we describe the common characteristics of the competent mitochondrial genomes, and suggest a plausible reason for their successful transformation.

Experimental procedures

Respiratory deficient strains

Respiratory deficient C. reinhardtii *strains used in this study are as follows*

dum-1 CC-2654 was obtained from the *Chlamydomonas* Genetic Center (c/o Dr. Elizabeth Harris, Department of Botany, Duke University, Durham, NC 27706, USA) and is a subclone of the original *dum-1* CC-2255 (mt^-) strain; wall-less strain *cw-15* CC-277 (mt^+) was also obtained from *Chlamydomonas* Genetic Center; *dum-14* (mt^-), *dum-15* (mt^-), *dum-16* (mt^-), and *dum-19* (mt^-) strains were kindly provided by Dr. R.F. Matagne (University of Liege, Belgium). The gene organization of wild-type mitochondria and respiratory deficient strains are shown schematically in Figure 1.

Preparation of recipient cells

Respiratory deficient *Chlamydomonas* strains were cultured under constant cool white fluorescent light (84 μmol m^{-2} s^{-1}) in Tris–acetate–phosphate (TAP) medium (Gorman and Levine, 1965) at 25 °C with vigorous shaking. About 7 ml of mid-log phase cultures (2–3 $\times 10^6$ cells/ml) were collected on a 9 cm diameter membrane (Hybond-N^+, Amersham, Burkinghamshire, England) by filtration, and transferred to a TAP-1% agar-plate containing 50 μg/ml ampicillin to reduce the possibility of bacterial contamination.

Conditions for biolistic bombardment

We used a PDS-1000/He particle delivery system (Bio-Rad, California, USA) to transform the mitochondria and chloroplast, following the standard procedures designed for bacteria (Smith *et al.*, 1992). We tested gold particles of 40 nm (G-40, E-Y Laboratories, Inc. California, USA), 100 nm (EM. GC 100, British Biocell International, England), 200 nm (EM. GC 200, British Biocell International), 600 nm (Bio-Rad catalogue #165–2262), and also a mixture of 100 and 600 nm gold particles. Gold particle mixtures were prepared as follows: 3 mg of 600 nm gold particles and the sediment of the 100 nm gold particles (prepared from 1 ml colloidal suspension) were suspended together in 50 μl of 50% glycerol. Five μl of donor DNA (1 μg/μl), 50 μl of 2.5 M $CaCl_2$,

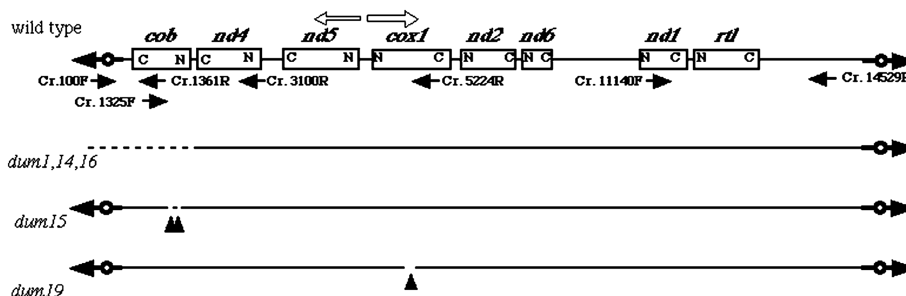

Figure 1. The relative gene organization and positions of mutations or deleted regions of respiratory deficient mitochondrial strains. Horizontal thick arrows indicate the long terminal inverted repeat, while open arrows designate the direction of transcription. Vertical arrow-heads identify the location of mutations. Open-circle indicate the expected replication origin (see text). C; C-terminus of the gene product. N; N-terminus of the gene product. Relative position and the direction of PCR primers are shown by short arrows.

and 20 μl of freshly prepared 0.1 M spermidine solutions (S-0266, Sigma, St. Louis, USA) were added to coat the gold particles with the DNA. The *Chlamydomonas* strains were bombarded with the gold particles using He at a pressure of 1100 pounds per square inch (1 psi = 6895 kPa) in a pressure reduction chamber at 25 inches Hg. After bombardment, respiratory competent transformants were selectively grown on a TAP-1% agar-plate and incubated under limited lighting condition [a 2:22 hour light (17 μmol m^{-2} s^{-1}): dark regimen] at 25 °C for 4 weeks. The exposure to limited light stimulates the growth of transformed strains.

Organization of transformed mitochondrial genomes

Culture growth conditions and total DNA preparation

Four weeks after biolistic-bombardment, individual colonies were inoculated into 20 ml (to prepare DNA for Southern hybridization) or 5 ml (to prepare DNA for PCR) of TAP medium. The cultures were grown for an additional 2 weeks under limited lighting (a 2:22 hour light (17 μmol m^{-2} s^{-1}): dark regimen) with shaking (Phase I culture). About 50 μl of the Phase I culture was inoculated into 20 ml of new TAP-medium (alternatively, 12 μl was inoculated into 5 ml of culture). The resulting culture (Phase II culture) was grown under the same conditions as the Phase I culture. This operation was repeated once more to obtain the Phase III culture.

The CTAB method (Murray and Thompson, 1980) was applied to separate total DNA from 20 ml cultures, and the Plant DNeasy mini kit (Qiagen, GmbH, Germany) was used for DNA isolation from the 5 ml cultures after disrupting the cells with glass beads.

Southern hybridization

One μg of intact DNA or *Sac*I-digested DNA were electrophoresed on a 0.8% agarose gel. The gel was transferred onto a nylon membrane and hybridized with alkali-unstable digoxigenin (DIG)-labeled probes, which were prepared following the manufacturer's recommendations (Roche, Mannheim, Germany). Four kinds of DIG-probes were used to analyze the mitochondrial genome. A 108 bp COB-probe, hybridizing to the middle of the COB gene (bases 1215–1323; numbering follows Gray and Boer 1988; Genbank acc. no. NC_001638), a 173 bp ND4-probe hybridizing to the middle part of the ND4 gene (2613–2786), and T7-promoter region and U19 primer region probes (120 bp each) were used to determine whether the pT7Blue-2 vector is retained in the transformants.

All hybridizations described above were performed at 44 °C for 18 hours. The membrane was washed once at 44 °C in a solution containing 2XSSC-0.1% SDS, followed by two consecutive washes in 0.1XSSC-0.1% SDS at 44 °C. The image was detected using a LAS-1000 (Fuji film Co., Tokyo, Japan).

Artificial DNA-constructs used for respiratory deficient mitochondrial transformation

5.0-kb construct

This construct consists of three PCR fragments, or elements, all of which were amplified from the total DNA of wild-type *C. reinhardtii* (CC-277,

cw-15 mt$^+$). The first element corresponds to bases 1–1780 of the *C. reinhardtii* mitochondrial genome, which contains the left inverted repeat sequence, the COB gene, and the C-terminal 67 bp of the NADH-cytochrome *c* reductase subunit 4 (*nd4*) gene [hereafter, C(*nd4*); Figures 1 and 2A]. The second element corresponds to bases 4780–6620, which carries the N-terminal 180 bp of the ND5 gene [hereafter, N(*nd5*)], along with the two expected promoters (one in each direction) and the entire COXI gene. The final element corresponds to bases 14400–15758, and contains the right inverted repeat sequence of the genome, expected region for DNA replication (*ori*), and modules of the fragmented rRNA genes. These three elements were assembled into one continuous DNA fragment (totally about 5.0-kb), whose gene organization is: left terminus-*cob*-C(*nd4*)/(*nd5*)N-two promoters-*cox1*/right terminus (Figure 2A), and ligated into the *Eco*RV site of the *E. coli* pT7Blue-2 T-vector (Novagen, Milwaukee, USA). This plasmid is designated p5.0 kb. The plasmid p5.0 kb was digested with *Not*I or *Bam*HI to linearize it, when it is required. This construct has potential to compensate the deficiencies of all *dum* strains used in this study, because this bears the left arm to the entire *cob* region and also the wild type COXI gene.

3.8-kb construct

This construct shares the first and the third elements of the 5.0-kb construct, while the middle element is 1.2-kb shorter than that for p5.0 kb, is comprised of bases 4780–5400 (Figure 2B), and contains the N-terminal region of the ND5 gene and the N-terminal 358 bp sequence of the COXI gene [hereafter N(*cox1*)]. These fragments were assembled as follows: left terminus-*cob*-C(*nd4*)/(*nd5*)N-two promoters-N(*cox1*)/right terminus, and ligated into the *Eco*RV site of the pT7Blue-2

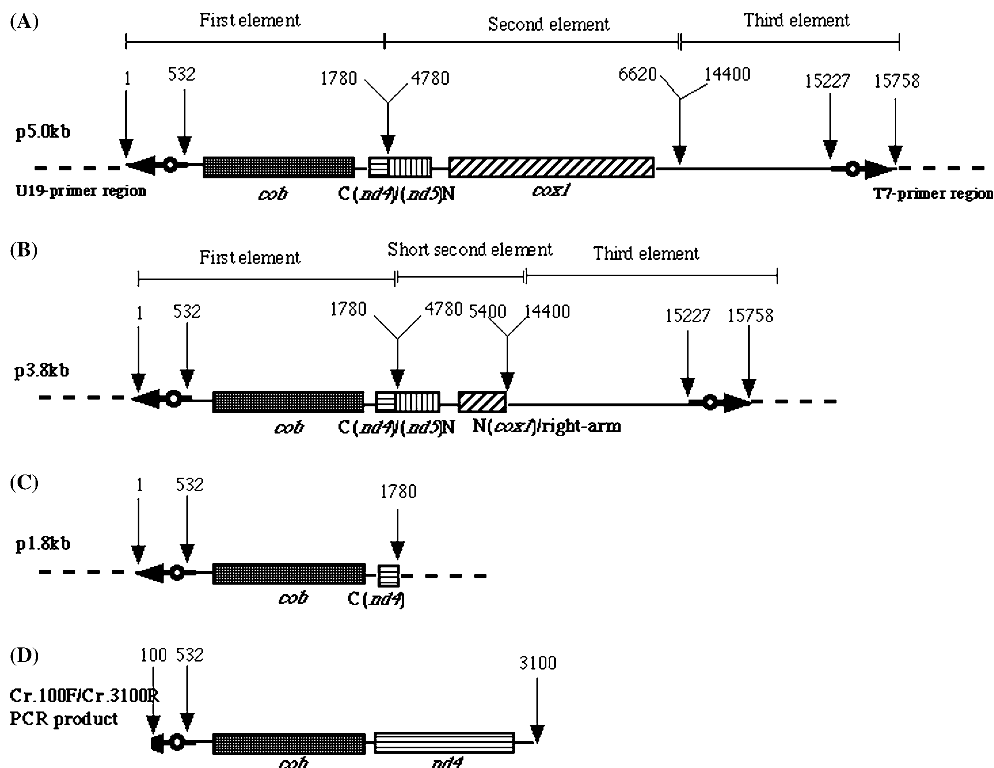

Figure 2. The gene organization of the DNA-constructs. Horizontal arrows show the repeated sequence of the mitochondrial genome at the termini, while vertical arrows show the boundary between the elements. The number refers to the nucleotide position from the mitochondrial sequence of *C. reinhardtii* (GenBank accession number NC_001638). (A) 5.0-kb DNA-construct, p5.0 kb. (B) 3.8-kb DNA-construct, p3.8 kb. (C) 1.8-kb DNA-construct, p1.8 kb. (D) PCR product obtained using a primer set, Cr.100F and Cr.3100R. Open-circles indicate the expected replication origin (see text). Dotted line shows pT7Blue-2 vector region.

Table 1. Transformation of *Chlamydomonas reinhardtii dum-1* using various sizes of gold particles.

Transformation of the chloroplast by pEX-50-AADA					
Gold particle size	600 + 100 nm	600 nm	200 nm	100 nm	40 nm
No. of colonies per plate	156, 103	79, 79	22, 22	5, 3	0, 0
Transformation of the mitochondria by p5.0 kb					
Gold particle size	600 + 100 nm	600 nm	200 nm	100 nm	40 nm
No. of colonies per plate	14, 11, 10, 9	9, 6, 5, 4	N/D[a]	0, 0, 0, 0	0, 0, 0, 0

[a] not determined.

T-vector. This plasmid is designated p3.8 kb. This construct has potential to compensate the deficiencies of *dum-1, -14, -15*, and *-16*, but not *dum-19*.

1.8-kb construct

This construct contains bases 1–1780 of the mitochondrial genome (i.e., the first element of the 5.0-kb construct itself; Figure 2C). This construct was ligated into the *Eco*RV site of the pT7Blue-2 T-vector, and is referred to as p1.8 kb. Basic potency of this construct is same to the p3.8 kb.

Cr.100F/Cr.3100R-RCR product

This PCR product (3.0 kb long) was obtained using a primer set (Cr.100F and Cr.3100R) and the *cw-15* mitochondrial genome as a template. This contains bases 100–3100 of the mitochondrial genome (Figure 2D), i.e., most part of the left terminal inverted repeat region (left arm; bases 1–545), whole COB and ND4 genes.

Results

Requirements for successful transformation of C. reinhardtii *mitochondria*

We attempted to transform five respiratory deficient strains of *C. reinhardtii* (*dum-1*, *-14*, *-15*, *-16*, and *-19*) (Figure 1) with DNA-constructs that contain compensatory regions (Figure 2). The strains *dum-1*, *-14*, and *-16* contain large deletions in the left terminus of their mitochondrial genome and showed no significant difference in the frequency of mitochondrial transformation (only the data for *dum-1* is shown in Table 1). Colonies active in respiration began to appear after 2 weeks of incubation in the dark with the latest appearing 4 weeks after bombardment (an example of respiration active transformants is shown in Figure 3). In contrast, no transformants were obtained for *dum-15* even after 20 attempts (4 plates per attempt), and only one actively respirating colony was yielded for *dum-19* through 22 attempts. However, DNA sequencing of the COXI gene revealed that the colony resulted from non-canonical back mutation accompanied by complex base changes, GGT/TG/AGC to GGT/GCA/AGC, which rescued the frame shift mutation in the *cox1* of *dum-19*. Consequently, no transformants due to homologous recombination were obtained from the *dum-15* and *dum-19* strains.

On the other hand, chloroplast transformation for all of these five strains was repetitively successful using the plasmid pEX-50-AAD (Takahashi *et al.*, 1996), which contains the bacterial aminoglycoside adenine transferase (*aadA*) gene conferring spectinomycin resistance (Goldschmidt-Clermont, 1991; Takahashi *et al.*, 1996). Moreover, the frequency of the chloroplast transformation was

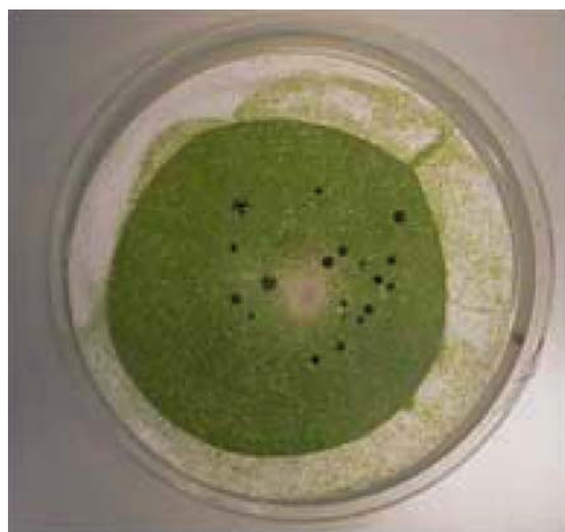

Figure 3. Appeared respiratory active transformants after 4 weeks of biolistic bombardment.

almost the same among the strains, i.e., 100–150 colonies per plate (a frequency of about 1×10^{-5} per cell) (data for *dum-1* is shown in Table 1).

Variable transformation efficiency depends on the gold particle size

Randolph-Anderson *et al.* (1995) suggest that 600 nm gold micro-projectiles give the highest transformation frequency for *C. reinhardtii* chloroplast. However, these particles are apparently too large to penetrate the mitochondria, which have a much smaller average diameter, estimated at 200–300 nm (Sager and Palade, 1957), therefore, we tried 40 and 100 nm gold particles for mitochondrial bombardment. Preliminary tests on chloroplast bombardment using 40, 100, or 200 nm gold particles resulted in a much lower frequency of transformation than the 600 nm particles, and no mitochondrial transformants were observed when bombarded with the 40 or 100 nm particles (Table 1). The highest frequency for chloroplast transformation was observed using a mixture of 100 and 600 nm particles, so we tested this mixture for mitochondrial transformation as well. The mixed projectiles increased the frequency about two-fold for mitochondrial and chloroplast transformations compared to the bombardment with the 600 nm particles alone (Table 1).

Variable transformation efficiency depends on the DNA-constructs

The closed-circular plasmid p5.0 kb (Figure 2A) showed the highest transformation efficiency (11 ± 5.1 transformants per $14–21 \times 10^6$ cells, ca. $0.5–0.8 \times 10^{-6}$ transformants per cell) (see Experimental procedures and Table 2), similar to that achieved by Randolph-Anderson *et al.* (1993) using the entire 15.8-kb mitochondrial genome (0.7×10^{-6} transformants per cell).

The transformation efficiencies using closed-circular p3.8 kb (Figure 2B) and p1.8 kb (Figure 2C) were about 50% and 20% of that for p5.0 kb, respectively (Table 2). Considering that mitochondrial genome in the *dum-1* mutant, with deletion ranging in size from ca. 1.7 to 1.5 kb (Randolph-Anderson *et al.*, 1993), then, the homologous region between plasmid p5.0 kb and the *dum-1* mitochondrial genome is 3.3–3.5 kb long, compared to 2.1–2.3 kb for p3.8 kb and 0.1–0.3 kb for p1.8 kb. The comparative ratio of the transformation efficiency between p5.0 kb and p3.8 kb was about 2:1 (Table 2), which is close to the differences in the length of the two construct's regions of homology (3.3–3.5 kb vs. 2.1–2.3 kb) with *dum-1*. Far reduced transformation efficiency of the p1.8 kb than the other two constructs is also in consistent with the difference of homologous regions (Table 2).

The closed-circular plasmid p5.0 kb, showed about 5 times higher transformation efficiency than the linear form prepared by digestion with *Not*I or *Bam*HI (choice of a restriction enzyme had no significant effect, Table 2). Even attempting transformation using the PCR product (3 kb long), corresponding to bases 100–3100 and containing the most part of left arm, the entire COB and ND4 genes (Figure 2D), resulted in transformants (Table 2), Sequencing of the PCR product transformed colonies indicated the homologous recombination most probably at the ND4 gene (also see below).

Table 2. Transformation of *Chlamydomonas reinhardtii dum-1* using various donor DNA-constructs and mixed gold particles of 100 and 600 nm.

DNA Construct	c.c-p5.0 kb[a]	c.c-p3.8 kb[b]	linear-p5.0 kb[c]	3kb-PCR product[d]	c.c-p1.8 kb
Experiment 1	18, 16, 16, 13	11, 9, 9, 7	6, 3, 1, 1	3, 2, 1, 1	1, 0, 1, 0
Experiment 2	11, 6, 2, 3	6, 3, 3, 2	3, 1, 1, 1	N/D[e]	N/D
Experiment 3	14, 11, 10, 9	13, 3, 3, 1	3, 2, 2, 1	N/D	N/D
Average (colonies per plate)	10.8 ± 5.1	5.8 ± 3.9	2.1 ± 1.5	N/D	N/D

[a] Closed circular form of 5.0 kb construct ligated into the pT7Blue-2 T-vector.
[b] Closed circular form of 3.8 kb construct ligated into the pT7Blue-2 T-vector.
[c] p5.0 kb linearized by *Bam*HI was used for experiment 1 and 2, while *Not*I was used for experiment 3.
[d] PCR product obtained using a primer set, Cr.100F and Cr.3100R.
[e] not determined.

Analysis of the mixed population of mitochondrial genomes in transformed cells

Preliminary characterization of transformants showed a mixed population of mitochondrial genomes. Therefore, we investigated the transition of heterogeneous populations of mitochondrial genomes associated with successive mitotic cell divisions as described below.

Analysis of the Phase I culture of dum-14/ *p5.0 kb transformants*

A mixed population of mitochondrial genomes was detected from some *dum-14*/p5.0 kb transformants of the Phase I culture (see Experimental procedures), and their representative gene organization was investigated. The mitochondrial gene organization was analyzed for *dum-14* transformants with restored respiration activity by the plasmid p5.0 kb. Total DNA was isolated from 14 *dum-14* transformants of the Phase I culture. PCR amplification was performed using the seven primers shown in Figure 1 [each primer name is composed of the number showing its 5′-base position and direction of extension, forward (F) or reverse (R)].

PCR amplification performed using the primer set, Cr.1325F/Cr.5224R, produced two kinds of PCR products 3.9 kb and 0.9 kb in length (Figure 4). DNA sequencing and restriction enzyme digestion of the 3.9 kb fragment showed that it contained a wild type segment, *cob-nd4*, which did not originally exist in the respiratory deficient *dum-1*, *-14*, *-16* strains (Figure 1). Further analysis of other PCR products by similar methods and by Southern hybridization (see below) suggested that gene organization of this mitochondrial genome segment is: left arm-*cob-nd4-nd5-cox1-nd2-nd6-nd1-rtl*-right arm, i.e., wild-type (Figure 5A). This is a result of homologous recombination between the first element of plasmid p5.0 kb and the *dum-14* mitochondrial genome. On the other hand, DNA sequencing of the 0.9 kb fragment reveled that this fragment contains the non-wild type (non-WT) segment (Figure 5B), *cob*-C(*nd4*)/(*nd5*)N-*cox1* ("/", "N", and "C" represent a direct fusion of truncated genes or elements, intact N-terminus of the C-terminus truncated gene, and intact C-terminus of the N-terminus truncated gene, respectively), which was derived from the plasmid p5.0 kb DNA.

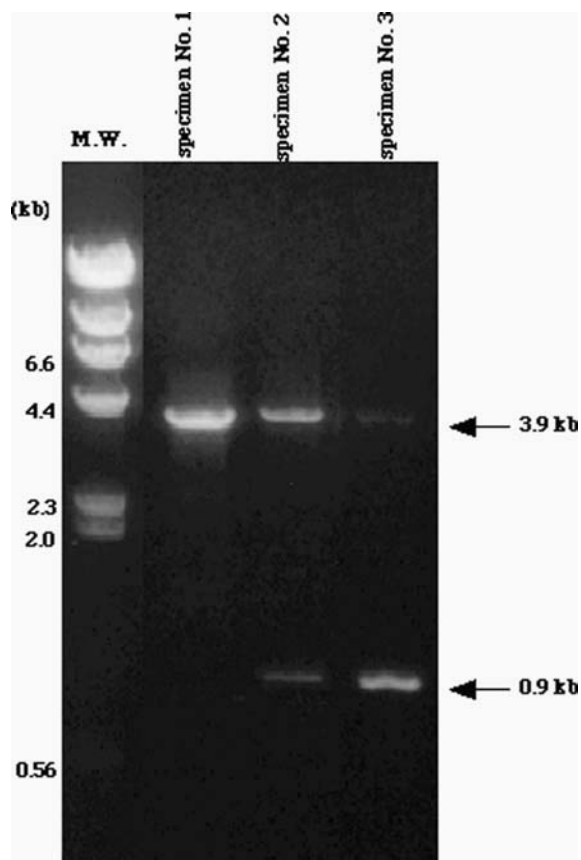

Figure 4. Mitochondrial genome analysis of transformants yielded by p5.0 kb. For PCR amplification, total DNA prepared from the Phase I culture of specimen Nos. 1, 2, and 3, and the primer set, Cr.1325F and Cr.5224R were used. A single band of 3.9-kb long was detected from specimen, No. 1, while 3.9-kb and 0.9-kb products were detected from Nos. 2 and 3. The 3.9-kb band was more intense than the 0.9-kb band for specimen No. 2, while the 0.9-kb band is more intense than the 3.9-kb band in the case of No. 3.

A 2.3 kb PCR product was amplified from one of the 14 examined *dum-14* transformants using the primer set: Cr.1325F/Cr.14529R (Figure 1). DNA sequencing reveled that this PCR product has a non-WT gene organization, *cob*-C(*nd4*)/(*nd5*)N-N(*cox1*)/right arm (Figure 5C), which likely is a result of homologous recombination between the 3rd element of plasmid p5.0 kb (Figure 2A) and the *dum-14* mitochondrial genome. No PCR product is expected when the wild-type genome is used as a template, because the two PCR primers (Cr.1325F and Cr.14529R) are positioned beyond the amplification limit of ExTaq polymerase (expected fragment size:

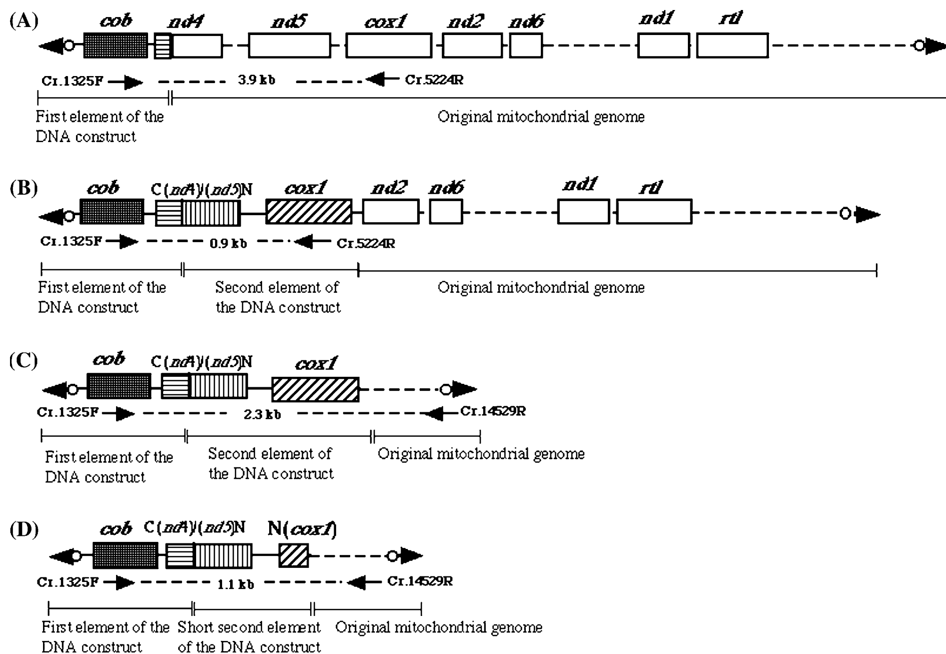

Figure 5. Structures of the recombinant mitochondrial genomes produced by biolistic bombardment. (A) Wild-type genome resulting from recombination with the first element of the DNA-construct p5.0 kb, p3.8 kb, or p1.8 kb. (B) Non wild-type genome resulting from recombination with the second element of the DNA-constructs p5.0 kb or the short second element of the p3.8 kb. (C) Non wild-type genome resulting from recombination with the third element of the p5.0 kb construct. (D) Non wild-type genome resulting from recombination with the third element of p3.8 kb. The relative position of each PCR primer used to analyze the gene organization is shown by an arrow. Genes or elements probably originated from *dum* mitochondrial genome are shown by dotted lies or plain boxes.

13 kb, ExTaq limit: ~8 kb; Takara Bio, Kyoto, Japan) (Figure 1).

Altogether, all of the examined *dum-14*/p5.0 kb transformants had their mutant mitochondrial genomes restored to wild type. In addition to the wild-type, four of the 14 transformants contained a non-WT genome that has the gene organization of: left arm-C(*cob*)N-C(*nd4*)/(*nd5*)N-*cox1*-*nd2*-*nd6*-*nd1*-*rtl*-right arm (Figure 5B), and one transformant has another non-WT gene organization, left arm-*cob*-C(*nd4*)/(*nd5*)N-N(*cox1*)/right arm (Figure 5C). Probably nine of the examined 14 transformants contained only the wild type, because no additional products besides the 3.9 kb fragment were detected by the PCR method, even after 40 cycles.

No PCR product was amplified using a primer that hybridizes to the ND4 gene and one that hybridizes to the U19 primer region of pT7Blue-2 vector (see Figure 2A). This shows that vector DNA is not maintained in the mitochondrial, and was also supported by the Southern hybridization result (see below).

(Analysis of the Phase I culture of *dum-1*/p3.8 kb transformants): Additionally, we detected three types of mitochondrial genomes in the mixed population of cells derived from transformants with p3.8 kb (Figure 2B). In all of the examined eight *dum-1* transformants, part of the wild-type specific gene organization, left arm-*cob*-*nd4*-*nd5*-*coxI* (Figure 5A), was confirmed by DNA sequencing of the Cr.1325F/Cr.5224R PCR product. In addition to this type, we detected a non-WT gene organization, left arm-*cob*-C(*nd4*)/(*nd5*)N-*cox1*, from all of the analyzed specimens (Figure 5C), and another non-WT organization, left arm-*cob*-C(*nd4*)/(*nd5*)N-N(*cox1*)/right arm (Figure 5D), from half of the specimens transformed with the plasmid p3.8 kb.

Analysis of the Phase II and III cultures

We performed similar PCR analysis using DNA prepared from the Phase II and III cultures (see Experimental procedures) of p5.0 kb transformants. Three of the five transformants that contained the non-WT segment, C(*nd4*)/(*nd5*)N, in the

Phase I culture DNA did not contain the corresponding PCR band in this phase. Furthermore, the PCR band corresponding to C(*nd4*)/(*nd5*)N-N(*cox1*)/right arm was also not detected from all cultures of p3.8 kb transformants. Using DNA prepared from the Phase III cultures, no non-WT mitochondrial genomes were detected in the culture by the PCR method using several sets of primers.

Analysis of mitochondrial genome transitions by Southern hybridization

We performed Southern hybridization to estimate the comparative quantities of wild-type vs. non-WT genomes detected by PCR amplification. As expected, no clear band was detected from untransformed *dum-1* and *-14* strains using a probe for the COB gene (Figure 6A and C). This is because most of the COB gene is absent in these *dum* mitochondrial genomes. On the other hand, two bands were detected using a probe for the ND4 gene irrespective of whether the genome was intact or digested by *Sca*I (Figure 6B and D). The lower band corresponds to the expected size of the left arm and most of the COB gene lacking *dum* mitochondrial genome, while the upper band corresponds to mitochondrial genome dimers that are fused head-to-head (Figure 6B, and D).

In contrast, a membrane blotted with DNAs prepared from the transformants (intact or *Sca*I digested) showed a single band in a position is similar to that for CC-124 wild-type DNA, while the band corresponding to the dimer mitochondrial genome was not detected (Figure 6A and C). We could not detect any clear specific band that corresponds to non-WT mitochondrial genomes even from the Phase I DNA using probes for the COB or ND4 genes (Figure 6). This is probably due to the lower sensitivity of the Southern hybridization method in relation to the PCR method.

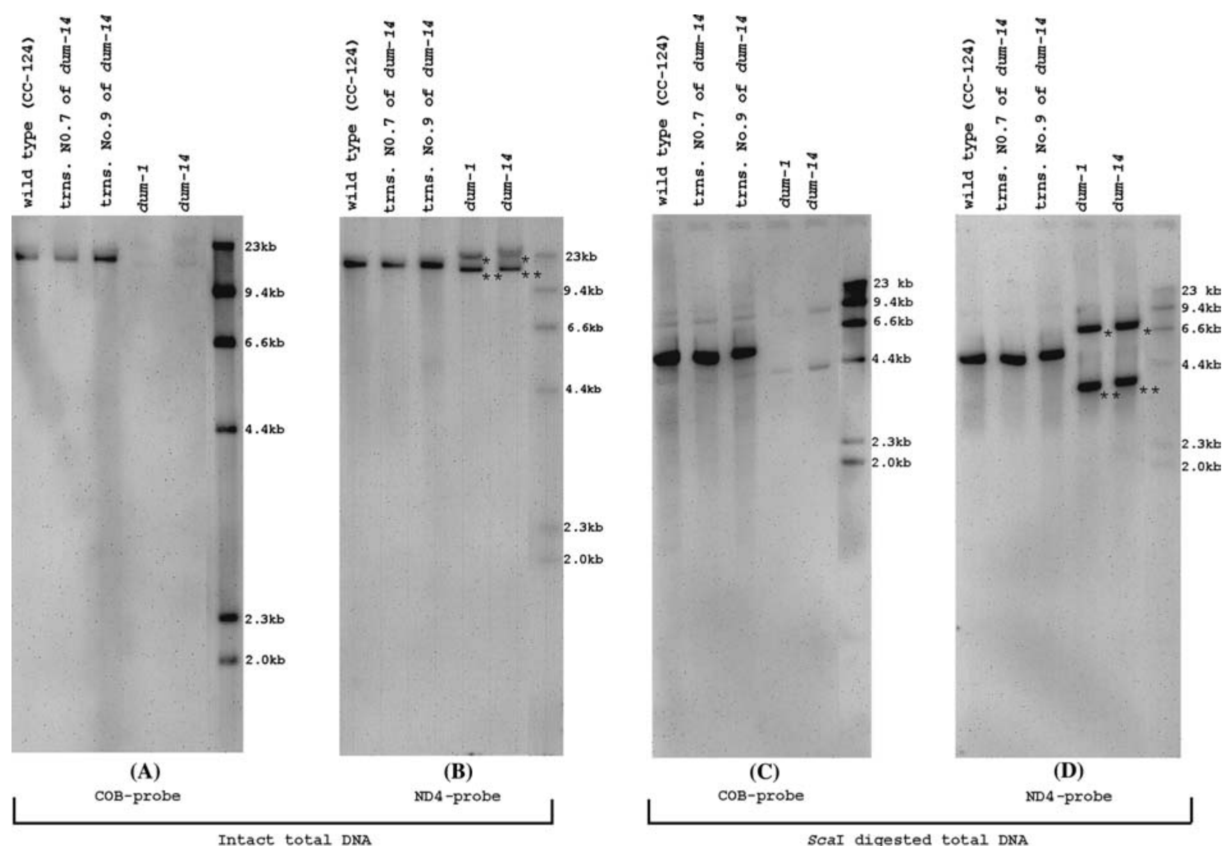

Figure 6. Mitochondrial genome analysis by Southern hybridization. *: band corresponding to head-to-head fused mitochondrial genome; **: band corresponding to the mitochondrial DNA that has a deletion of its left-arm and most of the COB gene. trans.: transformant.

No clear band was detected by hybridization using T7- or U19-probes for intact DNA blotted membranes (data not shown). This suggests that plasmid vector-region is not retained, even in the transformants of the Phase I culture.

Discussion

The ability to transform the *C. reinhardtii* mitochondrial genome using cloned DNA opens up the possibility of reverse genetic studies. Transformation of a DNA construct containing changes in the NADH dehydrogenase subunit genes (i.e., *nd1, nd2, nd4*, *nd5*, and *nd6*) would be extremely valuable to study the functions of the respiratory complex I (Cardol *et al*., 2002), because another species of which mitochondria are transformable, *Saccharomyces cerevisiae*, lacks this complex but instead has rotenone-insensitive NADH-quinone oxidoreductases (Seo *et al*., 1998). The COB gene of *C. reinhardtii* contains a target sequence of the intronic homing enzyme I-*Csm*I encoded in the ORF of a *C. smithii* group I intron named 'alpha' (Colleaux *et al*., 1990; Kurokawa *et al*., 2005). Mitochondrial transformation will also allow directed mutagenesis of the target sequence to investigate the recognition properties of the I-*Csm*I *in vivo*, because *C. reinhardtii* and *C. smithii* are interfertile and intron-invasion into the target site has been observed through mating. Moreover, the ability to transform both chloroplast (Boynton *et al*., 1988) and mitochondria provides a unique opportunity to explore the genetic interaction between these two organelles.

Effect of particle size on DNA delivery

Each series of experiments were performed using the same procedures, although we observed some variation in transformation efficiency between multiple plates even in a single experiment (Table 2), possibly because of poor distribution of the DNA-coated particles on each round plastic-disk (macrocarrier-disk). Irrespective of this, the overall transformation efficiency was consistently dependent on the key conditions described below.

Direct delivery of the donor DNA into the mitochondrial matrix appears essential for successful transformation, because it is likely that mitochondria cannot transport free cytosolic DNA into the matrix. We believe that this may also be true for delivering DNA into chloroplasts. In our experiments, the highest frequency of *C. reinhardtii* mitochondrial transformation was limited to about one-tenth of that for the chloroplast transformation (Table 1). This ratio may reflect the smaller volume of the mitochondria compared to the chloroplast.

Biolistic bombardment using the 40 or 100 nm gold particles was not successful for transforming the mitochondria, and using the 100 nm only slightly successful for the chloroplast, while the 600 nm particles were efficient for both organelles (Table 1). This might reflect the difference of physical energy among three kinds of particles, with only the 600 nm particles effective at 1100 psi, while the smaller particles may require a higher gas pressure to penetrate the *C. reinhardtii* cell wall. When the 100 and 600 nm gold particles were mixed, we achieved about two-fold higher transformation efficiency than using the 600 nm particles alone for the mitochondria as well as the chloroplast transformation (Table 1). This increase in efficiency may be cooperative, with the 600 nm particles perforating the cell wall, allowing some of the 100 nm particles to enter the cell and collide with the organelles, delivering the DNA into the organelle matrices. If the size of the mitochondrial cross section is constantly 200–300 nm in diameter, as previously reported by Randolph-Anderson *et al*. (1993), then even the 100 nm gold particles may not be small enough to enter the matrix without significant damage to the mitochondria. A recent report demonstrated that the mitochondria undergo a dynamic morphological change during the cell cycle, resulting in a fluctuating diameter of approximately 200 nm to 2.0 μm (Ehara *et al*., 1995). In our experiments the host cells were not synchronized, as they were cultured under continuous exposure to light preceding the bombardment, and it is possible that only the larger sizes of mitochondria survived the collision with the 100 nm gold particles.

It is known that the original 600 nm gold particles are contaminated up to 15% with 100–200 nm size particles (Randolph-Anderson *et al*., 1995), potentially reducing the differences in the observed transformation efficiencies between the "600 nm" particles and the 600 nm/100 nm particle mixture. Additionally, the closed-circular

DNA-construct may be more resistant to cellular degradation, in comparison to the linear form, and may explain the reproducibly higher transformation efficiency.

Characteristics of the successfully transformed strains

The five respiratory deficient mutants used in this study originally had an identical nuclear genetic background, because they were all developed from the same wild-type parent strain, *C. reinhardtii* 137C (Matagne *et al.*, 1989). Respiratory mutants were obtained after growing a culture in medium containing acriflavine or ethidium bromide as a mutagen, which enhances mutations in organelle genomes predominantly over nuclear genomes. Moreover, it has been shown that respiratory deficiency in the mutants used is due to alternations in the mitochondrial genome (Matagne *et al.*, 1989; Dorthu *et al.*, 1992; Colin *et al.*, 1995). All the transformable strains (*dum-1*, *-14*, and *-16*) have deletions in their mitochondrial genomes, ranging from the left terminus to somewhere within the neighboring COB gene (Dorthu *et al.*, 1992). On the other hand, the strains that could not be transformed do not share this deletion in their genome: *dum-15* carries two base substitutions in the COB gene, which results in an amino acid change, serine (UCU) to tyrosine (UAC) (Colin *et al.*, 1995), and *dum-19* has a one base deletion in the COXI gene, which introduces a UGA stop codon into the middle of the gene (Colin *et al.*, 1995). This suggests that determinant of the successful mitochondrial transformation is highly likely the large deletion at the left terminus.

The mitochondrial genomes carrying deletions in their left termini are known to be quite unstable, possibly because of the absence of a terminal inverted repeat region (Randolph-Anderson *et al.*, 1993). Such a wounded genome might induce the expression of required enzymes for homologous recombination in the mitochondria. This might explain why only the terminus-deleted strains are successful in transformation by homologous recombination between the delivered DNA and the mitochondrial genome. However in the case of the chloroplast, proteins that are required for homologous recombination seem to be abundant even when the entire genome is intact. This is supported by our data that shows wild-type *C. reinhardtii* and also these five respiratory deficient strains were easily transformed with pEX-50-AAD, and the construct was integrated into the chloroplast genome by homologous recombination (data not shown).

Conversion of a left arm deleted genome into a wild-type mitochondrial genome

How the linear *Chlamydomonas* mitochondrial genome replicates is not known, however two models have been proposed, a recombination-mediated model and a reverse-transcriptase model (Vahrenholz *et al.*, 1993). A replication origin-like (OL) structure has been suggested in the terminal inverted sequences based on similarities to the human mitochondrial OL sequence (Nedelcu and Lee, 1998). Unfortunately, we do not have solid experimental data that proves whether the circular 5.0-kb or 3.8-kb DNA-constructs, which has OL-like sequences in their right and left termini, are able to replicate in the mitochondrial matrix or not. However, we could not detect the vector region of the p5.0 kb molecules within the *dum-14* transformants by Southern hybridization (Figure 6) or by PCR, therefore homologous recombination might occur immediately after introduction of the DNA-construct.

To restore the wild-type genome, homologous recombination between the mitochondrial genome and somewhere within the first element of the structure in Figure 5, followed by successful elimination of the *E. coli* vector region appears to be the simplest mechanism. The appearance of non-WT genomes detected by PCR must be the result of homologous recombination occurring in the other regions of the DNA-constructs. For example, the genome structure shown in Figure 5B is probably the result of homologous recombination with the second element of p5.0 kb or the shorter second element of p3.8 kb, while that in Figure 5C is the result of recombination with the third element of p5.0 kb. The structure of Figure 5D is probably the result of recombination between the *dum* genome and the third element of p3.8 kb. It is possible that even these non-WT genomes can compensate the respiratory deficiency of the *dum* mutants, because they have a promoter to transcribe the COB gene and a ribosomal binding signal for its translation. Probably even these non-WT mitochondrial genomes can be converted to

the wild-type genome through a secondary homologous recombination with the newly replicated wild-type genomes.

Most of the *dum* mutants obtained to date have deficient activities in respiratory complex III or IV, and very rarely in the complex I, because almost all of the *dum* mutants result from deletion or point mutation of the COB gene (subunit of complex III) or COXI gene (subunit of complex IV), respectively (Remacle and Matagne, 1998). In addition, a mixed population of mitochondrial genomes in the original *dum*-1 mutant eventually stabilized, with fragments never exceeding 1.7 kb in deletion size, which is the maximum permissible size to avoid the loss of ND4 (one of the subunits of complex I) (Randolph-Anderson *et al*., 1993). These results indicate that complex I activity is significant, if not essential, for survival of *C. reinhardtii*. On the other hand, homologous recombination within the second or third elements of the donor DNA-constructs leads to the loss of active ND4 and ND5 genes in the mitochondrial genome and acquisition of the defective C(*nd4*)/(*nd5*)N-gene from the donor construct. Therefore, the negative effect on formation of active complex I seems serious in such a recombinant. In contrast, mitochondrial DNA that recombined within the first element of the DNA-construct retains active ND4 and 5 genes and also restores the COB gene. Therefore, this recombinant DNA molecule, i.e., a wild-type genome, will be selected strongly over the non-WT genes. This is the most probable reason for the high frequency of the wild-type mitochondrial genome observed in the Phase I culture, despite the very short homologous region (100–200 bp) for recombination with *dum* mitochondrial genomes. We observed rapid disappearance of head-to-head ligated dimer and other non-WT mitochondrial genomes from the culture. This may be due to the faster division of the cells that have a larger number of the wild-type mitochondrial genome.

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