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Homologous recombination in the nuclear genome of *Chlamydomonas reinhardtii*

(transformation/gene targeting/nitrate reductase)

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ABSTRACT **Nuclear transformation of the unicellular green alga *Chlamydomonas reinhardtii* has thus far been characterized by integration of the introduced DNA into nonhomologous sites. In this study, the occurrence of homologous recombination events during transformation was investigated with the intent of developing strategies for gene targeting and gene disruption. Homologous recombination was monitored by using nonfunctional 5′ and 3′ deletion derivatives of the wild-type *C. reinhardtii nit1* gene (encodes nitrate reductase) as selectable markers (p5′Δ and p3′Δ respectively) and the low reverting *nit1-305* strain as the transformation recipient. After introduction of the DNA into the cell, intermolecular recombination between p5′Δ and p3′Δ occurs at a high frequency to restore a functional *nit1* gene, indicating the presence of homologous recombination machinery in mitotic cells. Gene-targeting events at the *nit1* locus were selected by restoring *nit1-305* cells to prototrophy after transformation with only p5′Δ and were confirmed by analysis of genomic DNA. By comparing the number of transformants obtained after transformation with p5′Δ to the number obtained after transformation with a functional *nit1* gene, the frequency of homologous-to-random integration events ranged between 1:1000 after glass bead-mediated transformation and 1:24 after bombardment with DNA-coated tungsten microprojectiles.**

A powerful approach for the analysis of gene function involves cloning DNA sequences, modifying them *in vitro*, and then introducing the altered sequences into the genome by DNA-mediated transformation. In organelles (1, 2), prokaryotes and lower eukaryotes such as budding yeast (3), filamentous fungi (4), and slime molds (5), transformation results predominantly from homologous recombination events between the introduced DNA and the endogenous gene. In contrast, transformation of higher eukaryotic nuclear genomes is characterized by illegitimate recombination events, resulting in ectopic integration of the introduced DNA.

Homologous gene replacement has significant advantages over random integration for many molecular genetic experiments. With random integration events, the expression of ectopically integrated genes may be subject to gene dosage or position effects and often must be detected against a background of endogenous gene activity. In contrast, homologous replacement events do not have these drawbacks and, furthermore, provide a means of determining gene function by introducing *in vitro* generated mutations into the genomic copy of a cloned gene (targeted gene disruption).

Several approaches for identifying, selecting, and/or enriching for rare homologous recombination events have been developed in both plant and animal cells (6–16). The ratio of homologous to nonhomologous recombination events varies between 10^{-2} and 10^{-5} in animal cells and between 10^{-4} and 10^{-5} in plant cells.

Transformation of the nuclear genome of *Chlamydomonas reinhardtii* occurs efficiently when using *Chlamydomonas* genes as selectable markers. As is typical of mammalian and plant cell transformation, the introduced DNA is integrated at apparently random sites in the nuclear genome (17–20). As a prelude to developing methods for targeted gene disruption, we have investigated the occurrence of homologous recombination during nuclear transformation in *Chlamydomonas*. We have used truncated derivatives of the wild-type *C. reinhardtii nit1* gene (encodes nitrate reductase) to restore a *C. reinhardtii nit1* mutant to growth on nitrate as a sole nitrogen source after transformation. In this paper, we present evidence for (*i*) efficient homologous recombination between two introduced DNA fragments and (*ii*) homologous recombination events at the *nit1* locus of *C. reinhardtii*.

MATERIALS AND METHODS

Strains and Plasmids. Plasmids were propagated in *Escherichia coli* K-12 DH5α or *E. coli* K-12 GM2929 (F⁻ *dam13*::Tn*9*, *dcm6*, *hsdR2*, *recF143*, *mcrA*, *mcrB*), obtained from M. Marinus (University of Massachusetts Medical School, Worcester).

Cloning vectors were pBluescript II KS(−) (Stratagene) and pBR-Δ1, a derivative of pBR322 (21) prepared for this study by deleting the 1.1-kilobase (kb) *Pvu* II/*Nru* I fragment. pnit1 contains the wild-type genomic *nit1* sequence marked with a small insertion into intron 9 and was constructed as follows. An 11-kb *Sph* I/*Eco*RI fragment from pMN24 (22) was subcloned into the cognate sites of pBR-Δ1 (see Fig. 1). An 84-base-pair (bp) *Pvu* II/*Ssp* I fragment from pBluescript II KS(−) was then inserted into the *Pvu* II site located within intron 9 of the *nit1* gene (D. Zhang and P. Lefebvre, personal communication); the *Pvu* I site in this fragment provides a molecular marker for the introduced *nit1* gene(s). To construct p5′Δ, pnit1 was digested with *Sal* I, filled in with the Klenow fragment of DNA polymerase I, and partially digested with *Pvu* II; the resulting fragment was self-ligated. This eliminated the promoter and a portion of the first exon of the *nit1* gene; the *Sal* I site was restored at the ligation junction. To create p3′Δ, pnit1 was digested with *Sph* I, trimmed with phage T4 DNA polymerase, and then digested with *Stu* I; this fragment was ligated into the filled-in *Sal* I site of pBluescript II KS(−). p3′Δ lacks the four 3′-terminal exons and the 3′ untranslated region of pnit1; *Sal* I sites were restored at the ligation junctions.

A *Chlamydomonas* strain carrying the *nit1-305* mutation (23) and a cell-wall-deficient derivative (*nit1-305 cw15*) (19) were used as recipients for transformations. Both harbor an unmapped mutation in the *nit1* gene (reversion frequency $<10^{-8}$).

Media and Growth Conditions. *E. coli* strains were grown at 37°C in Luria broth supplemented with ampicillin to a final concentration of 50 μg/ml. *Chlamydomonas* strains were

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cultured in air under a 14/10-hr light (24 W/m^2)/dark cycle at 22°C in Sager–Granick medium II (24) containing either ammonium nitrate (SGII-NH_4; nonselective conditions) or potassium nitrate (SGII-NO_3; selective conditions).

Transformations. *E. coli* strains were transformed either by electroporation (25) or the $CaCl_2$/heat shock method (26). The cell-wall-deficient *Chlamydomonas* strain was transformed by agitating cells with glass beads in the presence of plasmid DNA and polyethylene glycol as described (19). The 3×10^7 cells used for each transformation were spread on a single selective plate. In later experiments, cells were incubated in SGII-NO_3 medium for 2–4 hr prior to transformation. We have found that this increases nonhomologous nuclear transformation as much as 10-fold. For the cell-wall-containing strain, 3×10^7 cells were spread on SGII-NO_3 plates and bombarded without further preincubation with 0.5 mg of tungsten microprojectiles coated with 1 μg of plasmid DNA by using the Helium upgrade of the DuPont/Bio-Rad PDS 1000 particle-delivery system (27). When using either method, Nit$^+$ colonies appeared within 1–2 weeks.

Preparation and Analysis of Nucleic Acids. Small-scale isolation of plasmid DNA was performed by the boiling method of Holmes and Quigley (28). Large-scale plasmid preparations were performed by a modified alkaline lysis procedure (29). *Chlamydomonas* genomic DNA was isolated as described (18).

Restriction endonuclease digestion of *Chlamydomonas* genomic DNA was performed in the presence of 4 mM spermidine (30). Digested DNA was resolved in 0.7% Tris acetate/agarose gels, and transferred to nylon membranes, which were hybridized with [α-^{32}P]dATP-labeled DNA probes (31) and washed at 65°C (32).

RESULTS

Homologous Recombination in Vegetative Cells. The aim of our initial experiments was to investigate the occurrence of nuclear homologous recombination events in mitotic cells. A stable nitrate reductase mutant strain carrying the *nit1-305* mutation was transformed with a pair of *nit1* plasmids that share a region of homology but harbor a deletion at the 5′ or 3′ end of *nit1* that inactivates the gene. Complementation of the *nit1* mutation could result from homologous recombination between the two deletion plasmids to produce an intact *nit1* gene. Such a recombined gene would be expected to integrate randomly into the nuclear genome, as previously observed in nuclear transformation experiments with intact selectable markers (17–20). Nit$^+$ colonies could also arise as a result of reversion (occurs at $<10^{-8}$) or by recombination between one of the plasmids and the endogenous gene.

Fig. 1 shows the deletion plasmids derived from pnit1, which includes the entire *nit1* gene marked by the insertion of an 84-bp sequence tag into intron 9. This tag contains a unique *Pvu* I site that can be used to distinguish the introduced *nit1* gene(s) from the endogenous gene. This insertion does not affect transformation efficiency when compared with an identical plasmid lacking the sequence tag (data not shown). The *nit1* coding region was deleted at the 5′ and 3′ ends to generate p5′Δ and p3′Δ, respectively (see *Materials and Methods*); these plasmids share a 4.7-kb overlap of *nit1* sequence.

p5′Δ and p3′Δ were digested with *Sal* I/*Eco*RI or *Sal* I, respectively, to separate the *nit1* portion of the plasmids from vector sequences. Transformation of the *nit1-305* mutant with either of these *nit1* fragments alone did not yield transformants, confirming that the deletions had inactivated the *nit1* gene. In contrast, transformations with both p5′Δ and p3′Δ resulted in a significant number of transformants, yielding ≈10% of the number of transformants obtained with the intact *nit1* gene (pnit1 digested with *Sph* I/*Eco*RI). This suggested that a functional *nit1* gene had resulted from

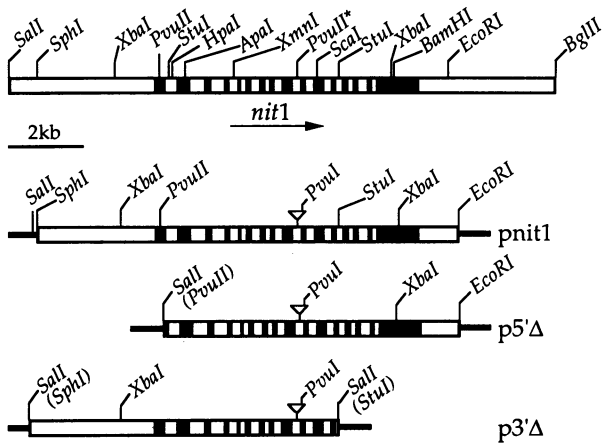

FIG. 1. Restriction map of the *C. reinhardtii nit1* locus (*Top*) and transforming plasmids. The arrow indicates the direction of transcription. Filled boxes represent exons, and open boxes represent introns and 5′ and 3′ noncoding regions; the line represents vector sequences in pnit1, p5′Δ, and p3′Δ. Restriction sites in parentheses represent sites in the *nit1* gene that were destroyed during the creation of p5′Δ and p3′Δ (see *Materials and Methods*). The inverted triangle represents an 84-bp sequence tag introduced into the *Pvu* II site of intron 9 (marked with an asterisk).

recombination between the introduced nonfunctional *nit1* fragments. Genomic DNA from representative transformants was analyzed to test this prediction.

Fig. 2*A* illustrates the results expected from DNA blot analysis of a cotransformant harboring a randomly integrated functional *nit1* gene derived from homologous recombination between p5′Δ and p3′Δ. A tag-specific probe should not hybridize with DNA isolated from the recipient strain. However, in cotransformants containing an integrated copy of the recombined gene, the tag-specific probe should hybridize with a 7.4-kb *Xba* I fragment and with *Xba* I/*Pvu* I fragments of 5.0 and 2.4 kb. A *nit1*-specific probe that spans the site in which the tag was inserted should hybridize with a 7.4-kb fragment in both *Xba* I and *Xba* I/*Pvu* I digests of the recipient DNA. In cotransformants, this probe should hybridize with 7.4-kb *Xba* I fragments derived from both the endogenous and recombined genes and with *Xba* I/*Pvu* I fragments of 7.4 kb (from the endogenous gene) and 5.0 and 2.4 kb (from the recombined gene). Fig. 2*B* shows the results of one such experiment, which are in complete agreement with these predictions. We conclude that homologous recombination between introduced DNA molecules occurs efficiently during nuclear transformation of *C. reinhardtii*.

Gene Targeting at the *nit1* Locus. The above experiments demonstrated efficient homologous recombination machinery in the nucleus of *Chlamydomonas*. Subsequent experiments investigated the possibility of homologous recombination between introduced DNA and an endogenous nuclear gene—i.e., gene targeting. In these experiments, only p5′Δ was used to transform the *nit1-305* strain. Experiments in yeast and mammalian cells have demonstrated that linearizing plasmids within a region of homology shared with a genomic locus markedly stimulates the frequency of homologous integration (9, 16, 33). Consequently, *Chlamydomonas* cells were transformed with p5′Δ linearized at the unique restriction site for *Apa* I or *Hpa* I (Fig. 1).

Since the deletion in p5′Δ eliminates the promoter and a portion of the first coding exon of the *nit1* gene, colonies arising from transformation of the *nit1-305* strain with p5′Δ are most likely to be either products of homologous recombination at the *nit1* locus or reversion of the *nit1-305* mutation. Although it is formally possible that control elements at a nonhomologous integration site could direct expression of

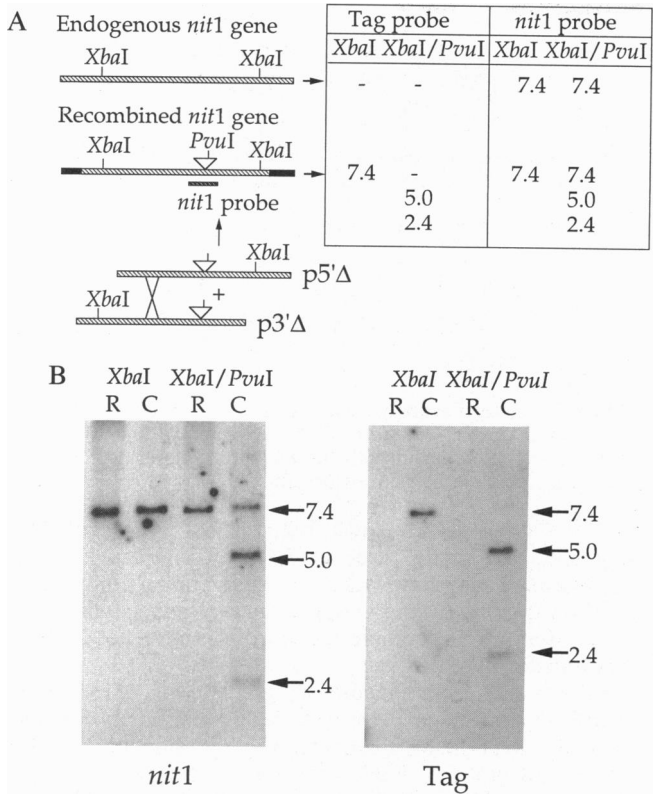

FIG. 2. Intermolecular homologous recombination. (*A*) Structures expected from intermolecular recombination between p3′Δ and p5′Δ followed by random integration into the genome. The inverted triangle represents the unique sequence tag introduced into the plasmids. The *nit1* probe is a 2.3-kb *Xmn* I/*Sca* I fragment that spans the site of the tag insertion; the tag probe is the 84-bp *Pvu* II/*Ssp* I fragment from pBluescript II KS(−). The hatched bar represents sequences at the *nit1* locus, and the solid bar represents DNA from an unlinked genomic location. (*B*) Southern blot analysis of genomic DNA from the *nit1-305* recipient (lanes R) and a representative p3′Δ + p5′Δ cotransformant (lanes C) probed with *nit1* and the tag-specific probes. Although the 5.0- and 2.4-kb fragments are equimolar, the 2.4-kb fragment is less intense because of a shorter region of homology with the probe.

a fusion protein capable of complementing the *nit1-305* mutation, we have seen no evidence for such an event in any of the Nit$^+$ colonies we have analyzed.

Fig. 3 illustrates the results predicted from several types of homologous recombination events between p5′Δ and the endogenous *nit1* gene. A single crossover would result in the integration of one copy of p5′Δ at the *nit1* locus, as shown in Fig. 3*c*. The formation of p5′Δ multimers prior to integration or additional single crossovers into the locus would result in integration of multiple copies of the plasmid (Fig. 3*d*). Either of these alternatives would incorporate at least one copy of the sequence tag. A double crossover could also repair the mutation, and, depending on where the crossovers occurred, either would or would not incorporate the tag (Fig. 3 *b* and *a*, respectively). A structure identical to that resulting from a double crossover could also arise from recombination between the duplicated regions generated by a single crossover event (at either site 2 or site 3 in Fig. 3*c*). Gene conversion events could also give rise to structures shown in Fig. 3 *a* and *b*, but their detection would require the analysis of both products of the recombination event. This was not possible in these experiments because the vectors currently available cannot replicate autonomously in the nucleus of *Chlamydomonas*.

DNA blot analysis, described below, demonstrates that the tagged *nit1* gene was integrated at the *nit1* locus, providing

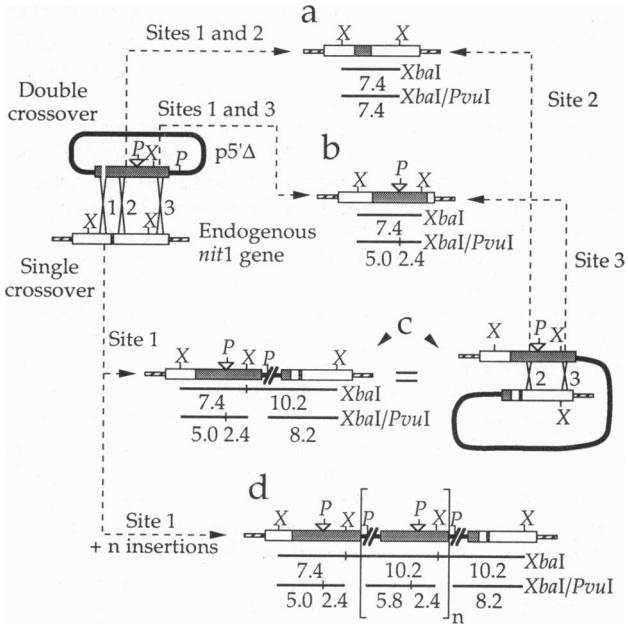

FIG. 3. Homologous recombination between p5′Δ and the endogenous *nit1* gene. The expected products are shown from a single crossover at the double-strand break in p5′Δ (blank region at site 1) or a double crossover between site 1 and either site 2 or site 3. Also shown are the sizes of fragments expected to hybridize with the *nit1* probe after digestion with *Xba* I or *Xba* I/*Pvu* I. The narrow black bar in the endogenous *nit1* gene represents the hypothetical location of the *nit1-305* mutation. The inverted arrow in p5′Δ represents the inserted sequence tag. P, *Pvu* I; X, *Xba* I.

conclusive evidence for gene-targeted homologous recombination. Genomic DNA from nit$^+$ transformants was digested with *Xba* I or *Xba* I/*Pvu* I and was hybridized with a *nit1* probe that spans the site of the tag insertion. Fig. 4 shows that in transformants a, b and c, the 7.4-kb *Xba* I fragment, which contains the endogenous *nit1* gene, is digested by *Pvu* I, giving rise to fragments of 5.0 and 2.4 kb. This is consistent with integration of *nit1* DNA from p5′Δ at the *nit1* locus or with a homologous replacement event. In transformant c, only the 5.0- and 2.4-kb *Xba* I/*Pvu* I fragments hybridize with the *nit1* probe, the expected result from a double crossover or gene conversion event (Fig. 3*b*). Transformants a and b contain an additional 8.2-kb *Xba* I/*Pvu* I fragment, which is consistent with integrated plasmid copies at the *nit1* locus (Fig. 3 *c* and *d*). Transformant b contains multiple copies of the 5.8- and 8.2-kb *Xba* I/*Pvu* I fragments as would be

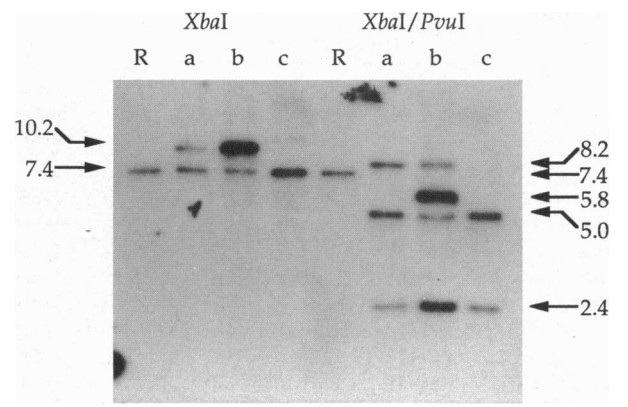

FIG. 4. DNA blot analysis of Nit$^+$ colonies resulting from glass-bead transformation of the *nit1-305 cw15* strain with p5′Δ. The blot was hybridized with the *nit1* probe described in Fig. 2. Lanes: R, recipient; a–c, transformants.

expected of tandemly integrated copies of p5′Δ at the *nit1* locus (Fig. 3*d*). Revertants and products of homologous recombination events that exclude the tag are indistinguishable from the parental strain.

Glass-bead-mediated transformation of the *nit1-305 cw15* strain with p5′Δ gave rise to 41 Nit$^+$ colonies from 143 plates in five independent experiments. Of these, 30 had not incorporated the tag and were thus indistinguishable from the recipient strain. These Nit$^+$ colonies arose either from reversion or from transformation via homologous recombination events that excluded the tag. Since few revertants were recovered from transformations performed in the absence of DNA (2 colonies from 28 plates), two-thirds of these Nit$^+$ colonies were probably products of a homologous recombination event. The remaining 11 transformants incorporated the tag at the *nit1* locus. Of these 11, 3 contained a single tagged copy of the *nit1* gene, consistent with either a double crossover (Fig. 3*b*) or recombination between the duplicated copies arising from a single crossover (site 3 in Fig. 3*c*). Eight transformants arose from single crossovers, having integrated either one copy (3 transformants) or multiple copies (5 transformants) of p5′Δ at the *nit1* locus. A comparison of the number of transformants obtained with p5′Δ (homologous recombination events) with the number obtained with *Eco*RI-digested pnit1 (random integration events) indicates that homologous recombination events occur at frequencies ranging from 1:200 to 1:1000 (Table 1).

Chlamydomonas cells were bombarded with DNA-coated microprojectiles to determine whether this would increase the recovery of transformants due to homologous recombination. Transformants obtained by this method generally incorporate multiple copies of the transforming plasmid DNA into the nuclear genome (17, 18). Analysis of genomic DNA isolated from nine Nit$^+$ colonies that arose following bombardment with p5′Δ revealed that four (44%) had not incorporated the tag (Fig. 5, lanes d and g). However, five (56%) were clearly products of homologous recombination events—i.e., they contained a 5.0-kb *Xba* I/*Pvu* I fragment that hybridized with the *nit1* probe (Fig. 5, lanes a–c, e, and f). Two of the transformants exhibited hybridization patterns consistent with a double-crossover event—i.e., the 7.4-kb *Xba* I fragment was digested by *Pvu* I, and the 5.8- and 8.2-kb *Xba* I/*Pvu* I fragments indicative of tandem insertions were not present (Fig. 5, lanes e and f). Two transformants exhibited patterns consistent with single-crossover events, containing either a few or multiple insertions (Fig. 5, lanes c and b, respectively). One transformant (Fig. 5, lane a) exhibited an unusual hybridization pattern with the *nit1* probe: a single 7.4-kb *Xba* I fragment but five *Xba* I/*Pvu* I fragments (including three at 7.4, 5.0, and 2.4 kb). This transformant may have arisen by gene conversion of the 5′ deletion in p5′Δ by the endogenous *nit1* gene, followed by integration into an ectopic location. We have not analyzed this transformant further.

Table 1. Frequency of homologous recombination at the *nit1* locus

Exp.	Transformants: p5′Δ, total no. (no. of plates)	Transformants: pnit1, no. per plate	Targeting frequency*
	Glass-bead transformation		
1	4 (17)	160	1/680
2	16 (51)	300	1/960
3	2 (15)	28	1/210
4	2 (18)	72	1/650
5	17 (42)	175	1/430
	Particle-bombardment transformation		
1	13 (12)	63	1/58
2	92 (44)	50	1/24

*Targeting frequency is expressed as the ratio of Nit$^+$ colonies obtained from transformation with p5′Δ to the number obtained with pnit1 linearized with *Eco*RI, assuming that all Nit$^+$ colonies arising from transformation with p5′Δ are homologous recombinants.

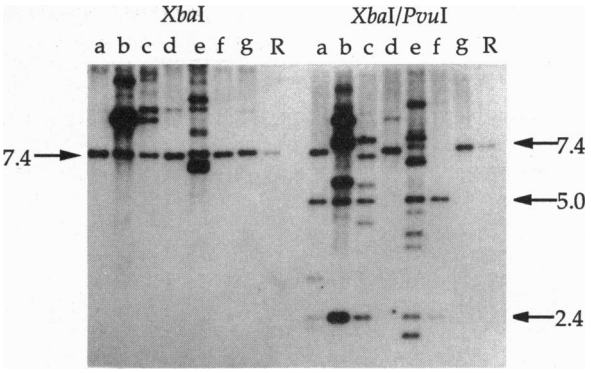

FIG. 5. DNA blot analysis of Nit$^+$ colonies resulting from transformation of the *nit1-305* strain via particle bombardment with p5′Δ. The blot was hybridized with the *nit1* probe described in Fig. 2. Lanes: R, recipient; a–g, transformants.

As is evident from the autoradiograph in Fig. 5, most of the transformants obtained by particle bombardment contained additional hybridizing fragments, which presumably represent random integrations. However, one transformant, which exhibited a hybridization pattern consistent with a double crossover event, did not harbor additional random integrations (Fig. 5, lane f). The frequency of homologous to nonhomologous integrations in bombardment experiments ranged from 1:24 to 1:58, an increase compared with the frequency obtained by glass-bead-mediated transformation (Table 1).

DISCUSSION

The ability to target introduced DNA sequences to homologous sites in the genome provides a powerful tool for molecular genetic analyses. In this report, we present evidence for efficient homologous recombination machinery that is capable of targeting recombination events to the *nit1* locus in the nuclear genome of *C. reinhardtii*. Transformation by both the glass-bead and particle-bombardment methods yielded homologous recombination events; each approach has advantages and shortcomings. Transformation by particle bombardment resulted in a high frequency of homologous to nonhomologous recombination (1:24–1:58) but requires specialized equipment and usually results in additional random integration events (Fig. 5). The glass-bead method, on the other hand, is a fast, simple procedure that does not require expensive equipment and rarely gives rise to additional random integration events. However, the latter method is less efficient for homologous recombination (1:200–1:1000) and requires the use of strains lacking cell walls or enzymatic removal of cell walls.

The detection of homologous recombination events in *Chlamydomonas* was greatly facilitated by the use of a plasmid vector that conferred a selectable phenotype. Although much less efficient than in bacteria and yeast, the frequency of homologous to nonhomologous events in *Chlamydomonas* is comparable to that obtained in mammalian cells (7–9, 16) and at least 2 orders of magnitude higher than has been reported in plant cells (10–12). At this point, it is not clear why bombardment yields higher targeting frequencies than glass-bead-mediated transformation. This may be a consequence of introducing a larger number of DNA molecules into each cell, although observations in mamma-

lian cells have indicated that the concentration of the substrates involved in recombination has a negligible effect on the targeting frequency (14, 16). Alternatively, particle bombardment may introduce DNA directly into the nucleus; experiments in mammalian cells suggest that nuclear microinjection is much more efficient than $Ca_3(PO_4)_2$ transformation for gene targeting. Finally, the tungsten particles used for bombardment may induce DNA damage and, as a result, stimulate homologous recombination.

The experiments in this report do not directly address the mechanism(s) involved in nuclear homologous recombination. In yeast, integrative recombination is enhanced by introducing a double-stranded break in a region of homology shared between the transforming vector and the targeted gene (33). Similarly, our experiments (data not shown) indicate that the presence of contiguous homologous sequences at the double-stranded-break junction is important, since transformations with a plasmid containing double-stranded breaks flanking the region of homology (p5′Δ digested with *Sal* I/*Eco*RI) did not give rise to transformants. This suggests that the majority of homologous recombination events are likely to be integrations and that even the apparent gene conversion or double-crossover events may arise by recombination between duplicated regions that result from a single integration event. Indeed, we selected a chlorate-resistant (Nit^-) colony from a transformant containing a single insertion and demonstrated that the tandem duplication had been eliminated (data not shown).

It is not clear why Nit^+ colonies containing the tag are recovered less frequently than those that lack it (3 vs. 30 for glass-bead transformants; 2 vs. 4 for particle-gun transformants). This bias toward non-tag-containing DNA could be a result of appropriately positioned hotspots for recombination or a function of the distance between the site of the unmapped *nit1-305* mutation and the tag. Alternatively, the p5′Δ deletion and tag on the introduced plasmid could be corrected by using the endogenous *nit1* sequence as template, followed by nonhomologous integration of this corrected version into the genome. As mentioned above, a similar event, in which the tag was not corrected, may have given rise to the transformant shown in Fig. 5, lane a.

High-frequency intermolecular recombination has been observed previously in studies with mammalian cells and may have practical applications (34, 35). For instance, limitations on the size of DNA that can be introduced into the cell as a single fragment may be overcome by cotransformation with smaller overlapping subclones which, once within the cell, may recombine to generate an intact larger fragment (36, 37).

One important consideration that may influence the frequency of gene targeting is the transcriptional activity of the target locus. It should be noted that, in all of the experiments described here, the cells were grown under conditions that are known to induce *nit1* gene expression.

This paper has shown that gene-targeted homologous recombination occurs frequently enough to make targeted-gene disruption feasible. With the possibility of nuclear gene-targeted disruption, it may soon be possible to alter both nuclear and chloroplast genes in *Chlamydomonas*. The application of these technologies in *Chlamydomonas* promises to yield important insight into many processes unique to photosynthetic eukaryotes.

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