

Gene replacement by homologous recombination in the multicellular green alga *Volvox carteri*

ARMIN HALLMANN*, ANNETTE RAPPEL, AND MANFRED SUMPER

Lehrstuhl Biochemie I, Universität Regensburg, D-93053 Regensburg, Germany

Communicated by Adrienne Clarke, University of Melbourne, Parkville, Victoria, Australia, May 9, 1997 (received for review December 20, 1996)

ABSTRACT With only two different cell types, the haploid green alga *Volvox* represents the simplest multicellular model system. To facilitate genetic investigations in this organism, the occurrence of homologous recombination events was investigated with the intent of developing methods for gene replacement and gene disruption. First, homologous recombination between two plasmids was demonstrated by using overlapping nonfunctional fragments of a recombinant arylsulfatase gene (tubulin promoter/arylsulfatase gene). After bombardment of *Volvox* reproductive cells with DNA-coated gold microprojectiles, transformants expressing arylsulfatase constitutively were recovered, indicating the presence of the machinery for homologous recombination in *Volvox*. Second, a well characterized loss-of-function mutation in the nuclear nitrate reductase gene (*nitA*) with a single G → A nucleotide exchange in a 5'-splice site was chosen as a target for gene replacement. Gene replacement by homologous recombination was observed with a reasonably high frequency only if the replacement vector containing parts of the functional nitrate reductase gene contained only a few nucleotide exchanges. The ratio of homologous to random integration events ranged between 1:10 and 1:50, i.e., homologous recombination occurs frequently enough in *Volvox* to apply the powerful tool of gene disruption for functional studies of novel genes.

Green algae of the order Volvocales range in complexity from unicellular *Chlamydomonas* through colonial genera to multicellular organism in the genus *Volvox*. The unicellular members of this order have proven to be excellent model systems for the biochemical and genetic analysis of cellular processes like photosynthesis, phototaxis and motility, and cell wall biogenesis. The more advanced members of this group, like *Volvox* have developed a multicellular form of organization with a complete division of labor between somatic and reproductive cells; in *Volvox carteri*, about 2,000 cells are somatic and only 16 cells are reproductive. Therefore, the Volvocales represent an ideal model system to study the prerequisite for the transition from unicellularity to multicellularity. Recently, the development of nuclear transformation (1, 2) and the introduction of reporter genes (3, 4) for the unicellular as well as the multicellular members of the Volvocales have made it possible to apply the powerful techniques of molecular genetics. However, the important genetic technique of gene replacement and gene disruption by homologous recombination has not yet been established in *Volvox*. Only a few successful reports on homologous recombination events exist for *Chlamydomonas reinhardtii* (5–7).

Gene targeting by homologous recombination is a genetic tool that permits modification of cellular genes in a precise and predetermined fashion. This technique was initially used as a tool by yeast genetics because site-specific rather than random

integration of homologous DNA is the preferred mode of integration in this organism (8, 9). Although homologous integration of transfected DNA into the genome was considered to be an extremely rare event in other organisms, techniques for this process have been established for several model systems (5, 10–16).

As found for higher eukaryotic genomes, nuclear transformation of *Volvox* has, thus far, only been characterized by illegitimate recombination events, resulting in ectopic integration of the introduced DNA. Any strategy for the detection of homologous recombination events in *Volvox* must take into account the technical impossibility of treating high numbers of *Volvox* algae at one time as well as the low level of transformation efficiency. To overcome these difficulties, experimental conditions should increase the frequency of homologous-to-random integration events. To that end, we drew on experience gained with unicellular *Chlamydomonas*, in which it was observed that bombardment with DNA-coated microprojectiles resulted in a much higher frequency of homologous recombination events than did another method of DNA delivery (5). In addition, we made only a very few changes in the nucleotide sequence of the targeting vector to avoid any possible hindrance to the process of homologous recombination.

In this paper, we present evidence for efficient homologous recombination between two introduced DNA fragments as well as for gene replacement events by homologous recombination at the nitrate reductase locus in the nuclear genome of *V. carteri*.

MATERIALS AND METHODS

Recipient Strain. The *Volvox* strain 153–48, obtained from D. L. Kirk (Washington University, St. Louis), was used as the DNA recipient. Strain 153–48 is an F₁-female progeny of HB11A, a female strain of *V. carteri* f. *nagariensis* that has been described (17). This strain with wild-type morphology inherited from HB11A an allele that carries a loss-of-function mutation of *nitA*, the structural gene encoding nitrate reductase (18, 19). The spontaneous reversion rate of this allele is less than 3×10^{-9} per reproductive cell (D. L. Kirk, personal communication).

Culture Conditions. Synchronous *Volvox* recipients were grown in *Volvox* medium (20) at 28°C in an 8-h dark/16-h light (10,000 lx) cycle (21). The nonselective medium used was *Volvox* medium, supplemented with 1 mM NH₄Cl; the selective medium was *Volvox* medium lacking NH₄Cl and containing only nitrate as a nitrogen source.

Construction of the Chimeric β -Tubulin/Arylsulfatase Gene. The *Volvox* arylsulfatase gene was placed under the control of the *Volvox* β -tubulin promoter in *Volvox* by using genomic clones of *Volvox* β -tubulin (22) and *Volvox* arylsulfatase (23). Additional restriction sites were introduced by PCR to facilitate ligation of the parent DNAs. An *EcoRV* site was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1997 by The National Academy of Sciences 0027-8424/97/947469-6\$2.00/0 PNAS is available online at <http://www.pnas.org>.

Abbreviation: RT, reverse transcription.

*To whom reprint requests should be addressed.

generated directly in front of the start codons both of the *Volvox* arylsulfatase gene and the *Volvox* β -tubulin gene. A *KpnI* site was introduced into the β -tubulin promoter region 493 bp upstream of the start codon. Then, a *KpnI/EcoRV* fragment bearing the 493-bp *Volvox* β -tubulin promoter region was ligated to a 10-kb *EcoRV/SalI* fragment containing the *Volvox* arylsulfatase gene with its 15 introns. The complete construct was confirmed by sequencing.

Construction of a Modified Nitrate Reductase Gene Fragment. To introduce conservative nucleotide exchanges into the nitrate reductase gene as a marker, the gene splicing by overlap extension technique, or the recombinant PCR technique, was performed as described by Horton *et al.* (24). To construct the modified *nitA* fragment, the first PCR was performed on *nitA* plasmid pVcNR1 (2) with an 18-mer 5' sense primer (5'-GTTCCGGATCCCGGATCAA) and a 43-mer 3' antisense primer (5'-ACAAACCTGTGATTTTGTGGTTAGGCAATAATGACGTCAGAGG) with only half of the primer completely matching; the other half contains mismatches as a marker. The second PCR was performed on the same template with a 42-mer 5' sense primer (5'-TTGCCTACAAA-CAAAATCACAGGTTTGTCTCCTGTGCTATGTGT), in which the underlined nucleotides are complementary to the corresponding region of the antisense primer of the first PCR), and an 18-mer 3' antisense primer (5'-GGTTGTCCATGAAGTGGT). The third PCR was carried out with both of these PCR products and only the flanking primers. PCRs were performed as described (4). The final construction of the *nitA* fragment was performed by standard techniques (25). All products produced by PCR and cloning were sequenced.

Stable Transformation. Small gold beads (1 μ m in diameter, Bio-Rad) were coated with the required targeting plasmids as described (2, 4, 26) and used as microprojectiles. Stable transformation of reproductive *Volvox* cells of strain 153-48 (carrying a loss-of-function mutation in the nitrate reductase gene) was performed by using a particle gun with flowing helium (BioListic PDS-1000/He System, Bio-Rad) to bombard the cells with the DNA-coated microprojectiles (2, 4, 26). To select for nitrate reductase activity in transgenic *Volvox*, the bombarded cells were resuspended in *Volvox* medium with nitrate as the only nitrogen source (lacking NH_4^+). From the sixth day after transformation onward, bombarded cultures were observed stereomicroscopically, and each green alga was transferred into a microtiter well with fresh selective *Volvox* medium. Transformants were tested for restoration of chlorate sensitivity by cultivation in 8 mM potassium chlorate in *Volvox* medium (2).

Reverse Transcription (RT)-PCR. RT-PCR was performed as described (4, 26). For transformants expressing the artificial tubulin/arylsulfatase gene, the antisense primer 5'-CAGTGGAATAGAAGCCG (arylsulfatase) and the sense primer 5'-ATAACAAGCGACCACTAC (tubulin) were used. For transformants with homologous recombination events at the nitrate reductase gene locus (*nitA*), the antisense primer 5'-GGTTGTCCATGAAGTGGT and the sense primer 5'-CCTGCACTCTCGTATGCG were used. Products of PCR amplification were ligated into the *SmaI* site of pUC18 and sequenced. Amplification, cloning, and sequencing of each RT-PCR fragment were repeated twice.

Genomic PCR. Twenty *Volvox* spheroids were selected under the stereomicroscope and transferred into 10 μ l of sterile lysis buffer (0.1 M NaOH/2.0 M NaCl/0.5% SDS). After 5 min at 95°C, 200 μ l of 50 mM Tris-HCl (pH 7.5) was added immediately. The resulting lysate (2 μ l) was used for PCR (in a total volume of 100 μ l). PCR was performed by standard protocol. Products of PCR amplification were cloned and sequenced. Amplification, cloning, and sequencing of each PCR fragment was repeated twice.

Southern Blot Analysis. Total genomic DNA was prepared from *V. carteri* by using the RapidPrep genomic DNA isolation

kit from Pharmacia. Lysis of *Volvox* cells in extraction buffer was supported by adding glass beads (0.45–0.50 μ m) and vortexing five times at high speed for 1 min. The tube was frozen and thawed between pulses. Restriction digests, gel electrophoresis, and Southern blot procedures followed standard techniques (25). Blots were probed with an \approx 0.3-kb *EcoRI/ApaI* nitrate reductase (18) fragment (nucleotides 1175–1478 of *nitA* sequence as deposited in the GenBank database, accession no. X64136).

RESULTS

Homologous Recombination in *Volvox*. The aim of our initial experiments was to search for an indication that the machinery for homologous recombination exists in reproductive cells of *Volvox*. A plasmid-based test system was used to detect the occurrence of homologous recombination between a pair of plasmids encoding supplementing parts of a reporter gene. The reporter gene used was the *Volvox* arylsulfatase gene (4, 23). To distinguish the plasmid-encoded arylsulfatase from the endogenous arylsulfatase, which is only expressed under conditions of sulfur starvation, the plasmid-encoded arylsulfatase gene was placed under the control of the constitutive β -tubulin-promoter (22) of *Volvox* (Fig. 1A; plasmid 1). First, *Volvox* was transformed with this intact artificial gene construct to check its function; as expected, transformants showed arylsulfatase activity in sulfur-sufficient medium and the presence and the function of the chimeric gene was proven by genomic PCR and RT-PCR.

Then, two plasmids were constructed, each carrying only a fragment of the chimeric tubulin/arylsulfatase gene (Fig. 1B; plasmids 2 and 3). Transformation of *Volvox* with either of these plasmids alone (plus selectable marker plasmid) did not yield any transformant constitutively expressing the arylsulfatase gene, confirming that the deletions had inactivated the artificial tubulin/arylsulfatase gene. Then, *Volvox* was transformed with both (circular) plasmids, that share a 776-bp region of identity but harbor a deletion at the 5' or 3' end. In addition, a selectable transformation marker (the complete *nitA* gene) was supplied by a third plasmid because cotransformation frequency in *V. carteri* is high (40–80%) (2). Transformation with both arylsulfatase plasmids (plus selectable marker plasmid) resulted in a significant number of transformants expressing the arylsulfatase gene in sulfur-sufficient medium. Six out of 20 (or 30%) transformants exhibited this phenotype. These results indicated that a functional chimeric tubulin/arylsulfatase gene had resulted from homologous recombination between the introduced nonfunctional gene fragments.

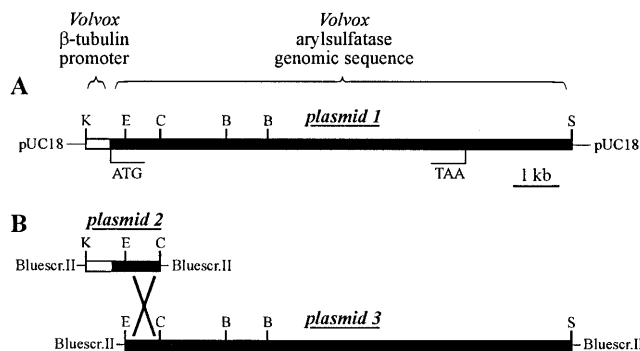


FIG. 1. (A) Structure of the tubulin/arylsulfatase chimeric gene, harbored in plasmid 1, containing the *Volvox* β -tubulin promoter region and the *Volvox* arylsulfatase coding region. (B) Plasmids 2 and 3 carry parts of the original plasmid 1. Plasmids 2 and 3 share a 776-bp overlap of arylsulfatase genomic sequence. B, *BamHI*; C, *ClaI*; E, *EcoRI*; K, *KpnI*; S, *SalI*.

The RT-PCR technique was used to verify the existence of recombinant mRNA in transgenic *Volvox* clones constitutively expressing the arylsulfatase gene. For this purpose, RNA was extracted from 20 *Volvox* spheroids, reverse transcribed, and subsequently amplified by PCR. Oligonucleotide primers that matched sequences up- or downstream from the overlap region of the plasmids and, in particular, that allowed the amplification of the fusion region of the chimeric β -tubulin/aryl-sulfatase gene construct were selected. The sense primer anneals to a sequence on the tubulin part present on the original plasmid 2, and the antisense primer anneals to a sequence on the arylsulfatase part present on the original plasmid 3 (Fig. 1B). A correct RT-PCR product could be expected only if a homologous recombination event, i.e., a single crossover, took place. In this case, a 663-bp cDNA was predicted from the sequence data and the known intron-exon boundaries of the parent genes. As documented in Fig. 2A, the RT-PCR yielded the expected chimeric cDNA fragment in all six *Volvox* clones (named HRA-1 to HRA-6) constitutively expressing the arylsulfatase gene. RT-PCRs with wild-type algae or the parent *nitA*⁻ strain 153-48 failed to produce this product. The corresponding DNA sequence analysis of these hybrid cDNA fragments (663 bp) is shown in Fig. 2B. The five introns originally present within this stretch of chimeric DNA were excised by the splicing machinery of *Volvox* exactly as the parent arylsulfatase gene was spliced. Thus, homologous recombination, i.e., a single crossover, between identical regions of both plasmids took place, resulting in the functional reconstitution of the interrupted artificial tubulin/aryl-sulfatase gene. Homologous recombination between introduced circular DNA molecules was observed to occur with a high frequency (about 1:3) during transformation of *Volvox*.

Chimeric cDNA was detectable in transgenic *Volvox* clones even after 6 months, i.e., after about 90 generations. Because nonreplicating plasmids were used, the product of homologous recombination between both plasmids must have been integrated into the *Volvox* genome, most probably by illegitimate recombination.

Gene Replacement by Homologous Recombination. The above experiments indicated that the machinery for homologous recombination exists in reproductive cells of *Volvox*. Subsequent experiments investigated the possibility of a targeted gene replacement in the *Volvox* genome.

Our first attempts to achieve and detect homologous recombination in the *Volvox* genome followed standard procedures, i.e., a replacement vector in which the coding region of the target gene (*Volvox* arylsulfatase gene; refs. 4 and 23) had been interrupted by inserting a marker gene (neomycin phosphotransferase gene; ref. 27) or by deleting a part (0.3 kb) of the arylsulfatase gene was constructed. All transformants obtained with these constructs turned out to be the result of illegitimate recombination events (data not shown).

Therefore, a different approach was initiated. A nuclear single copy gene was selected as a target in which minor changes, such as exchange of a few nucleotide positions, would lead to a selectable phenotype. The defective nitrate reductase gene in a well characterized *nitA*⁻ mutant of *Volvox* was chosen as an ideal target. The stable *nitA* loss-of-function mutation in the *nitA*⁻ *Volvox* strain 153-81 has been shown to reside in a G \rightarrow A transition of the first nucleotide within the 5'-splice site of the *nitA* intron 2, and, consequently, all splicing products are only nonfunctional mRNA variants (19). Because strain 153-81 does not show wild-type morphology because of additional mutations (*regA* and *gls*), the *nitA*⁻ strain 153-48 with wild-type morphology was selected for all experiments. In strain 153-48, the spontaneous reversion rate of the defective *nitA* allele is less than 3×10^{-9} per reproductive cell (D. L. Kirk, personal communication). Because both strains, 153-81 and 153-48, have a common ancestor, the presence of the same G \rightarrow A transition in the *nitA* gene of strain 153-48 was verified

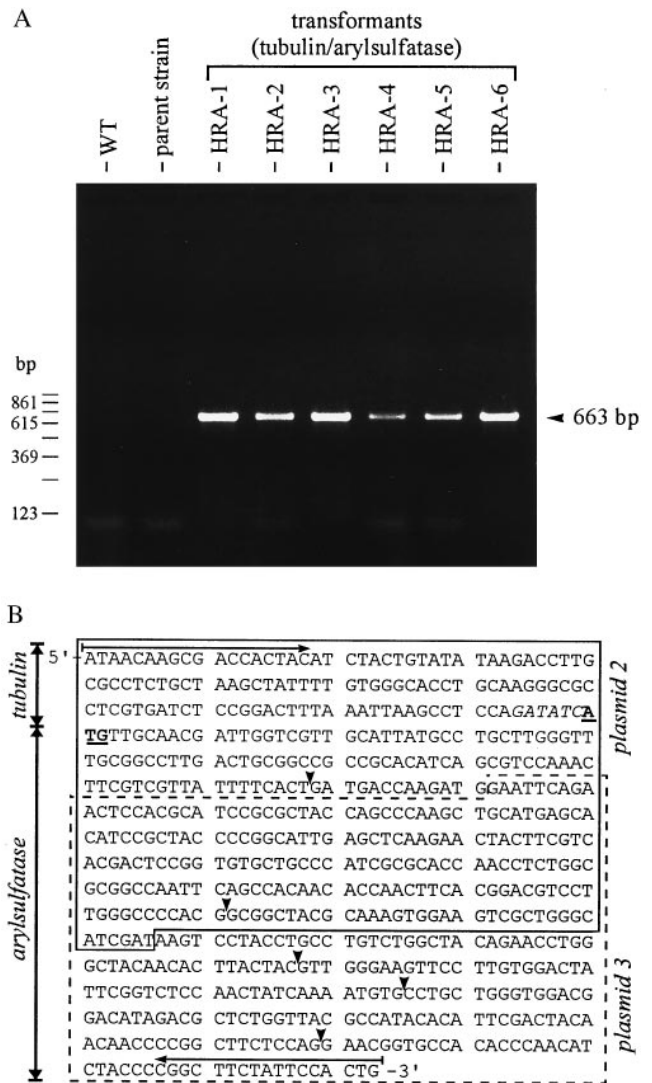


FIG. 2. (A) RT and subsequent PCR amplification of the chimeric mRNA. RNA was extracted from 20 *Volvox* spheroids of parent strain 153-48, wild-type algae (WT), or the six transformants constitutively expressing the arylsulfatase gene (HRA-1 through HRA-6). Sizes of PCR products (663 bp) were determined by using a 123-bp ladder as size marker and by DNA sequencing. (B) Sequence of cDNA (663 bp) obtained from RT and subsequent PCR amplification of the overlap region of the tubulin/aryl-sulfatase gene from *Volvox* clones HRA-1 through HRA-6. All clones resulted from transformation experiments using a mixture of plasmids 2 and 3 (and *nitA* as a selection marker). The sequence stretch contributed by plasmid 2 is boxed with a solid line; the contribution of plasmid 3 is boxed with a dashed line. The translation initiation site (ATG) is highlighted (shown underlined and in boldface type). The artificial *EcoRV* restriction site in front of the translation initiation site, shown in italic type, separates the β -tubulin sequence from the arylsulfatase sequence. Introns were spliced correctly. The positions of the introns within this cDNA-fragment are indicated by solid arrowheads. PCR primers used are indicated by horizontal arrows.

by genomic PCR, followed by DNA sequencing. Next, we tried to repair this defective gene by homologous recombination. The targeting vector carried a G nucleotide at the corresponding position, and another four nucleotides were exchanged within the coding region (exon 2) of the targeting plasmid. These four exchanges did not affect the amino acid sequence, but they allowed the distinction between a homologous recombination event and a spontaneous point mutation. In addition, the 5' region and the first exon of the *nitA* gene were removed on the targeting vector to prevent vector integrations

by illegitimate recombination from producing the *nitA*⁺ phenotype. The *nitA* fragment on the targeting plasmid starts within intron 1 (≈0.5 kb upstream of the five nucleotide exchanges) and ends ≈0.4 kb downstream of the polyadenylation signal (5.4 kb downstream of the five nucleotide exchanges). The complete targeting vector is shown in Fig. 3A (plasmid 4). The differences in sequence between parent strain 153–48 and the targeting plasmid are shown in Fig. 3B.

Volvox strain 153–48 was transformed by bombardment with gold microparticles coated with the targeting plasmid in its circular form. Microparticles were accelerated by the use of a particle gun (28). Seven *nitA*⁺ transformants were recovered (HRN-1 through HRN-7). The number of transformations performed to collect these seven clones usually results in 60–300 transformants (with illegitimate recombinations) if a complete *nitA* gene is used. Therefore, in contrast to illegitimate recombination, which occurs with a frequency of ≈2.5 × 10⁻⁵ per reproductive cell (2), frequency of homologous recombination ranges between ≈2.5 × 10⁻⁶ and ≈5 × 10⁻⁷ per reproductive cell.

The seven *nitA*⁺ transformants were examined by genomic PCR. One of the oligonucleotide primers (Fig. 3A, primer P1) used for PCR anneals to a *nitA* sequence upstream of the 5' end of the *nitA* fragment used for transformation. This strategy prevents the amplification of illegitimately integrated plasmids and allows only the amplification of *nitA* DNA from the *nitA* locus. A 867-bp PCR fragment was predicted from the *nitA* locus with or without the event of homologous recombination. For all seven transformants, genomic PCR yielded this expected 867-bp PCR fragment. Each of the PCR products were cloned into pUC18 vector and sequenced. In all seven transformants, the sequence of the 5' half of the PCR fragment, which cannot be derived from the targeting vector, was the same as the sequence from the intact *nitA* gene. Furthermore, in all seven transformants, the mutation of the 5'-splice site of *nitA* intron 2 was corrected, i.e., it contained a G nucleotide instead of the A nucleotide present in the parent strain. Most importantly, in two (clones HRN-1 and HRN-2) of these seven transformants, the four marker mutations were found in the *nitA* gene exactly where they were introduced into the replace-

ment vector (Fig. 3B, transformants). Another two transformants exhibited again the A → G transition in the splice site as well as one (clone HRN-3) or two (clone HRN-7) marker mutations next to this site. The remaining, more distantly located marker mutations did not show up. The remaining three transformants exhibited only the essential A → G transition within the splice site.

The RT-PCR technique was used to verify the existence of the recombinant mRNA and to prove the correct splicing of intron 2. Again, one of the oligonucleotide primers used for RT-PCR anneals to a sequence upstream of the 5' end of the *nitA* fragment in the replacement vector to prevent the amplification of products resulting from illegitimately integrated plasmids. A 438-bp cDNA fragment was predicted for RT-PCR if homologous recombination took place and if intron 2 of the *nitA* gene was spliced correctly. RT-PCR yielded the expected 438-bp PCR fragment in all of the transformants (Fig. 4A). The DNA sequence analysis of the cDNA fragment of transformants HRN-1 and HRN-2 is shown in Fig. 4B. Again, the four marker mutations were found in the *nitA* cDNA of transformants HRN-1 and HRN-2, and the introns (introns 1 and 2) originally present within this stretch of DNA were excised by the splicing machinery of *Volvox* exactly as the wild-type *nitA* gene was spliced. Thus, gene replacement by homologous recombination restored a functional *nitA* gene containing four marker mutations.

The mode of integration of the targeting vector at the *nitA* locus was investigated in Southern blot experiments. Integration by a single crossover would result in the integration of one copy of the replacement vector at the *nitA* locus and thus would result in a duplication of the *nitA* sequences. As a consequence, the surroundings of the original *nitA* gene would be changed. A double crossover or, alternatively, a recombination event between the duplicated regions generated by a single crossover would restore the original surrounding of the *nitA* gene. A probe (≈0.3 kb) covering the *nitA* gene region upstream of the 5' end of the *nitA* sequence present in the replacement vector was used for the Southern blot experiments. This probe excludes signals resulting from sites of illegitimate plasmid integration. Both a *SalI* and a *EcoRI* digest (see the physical

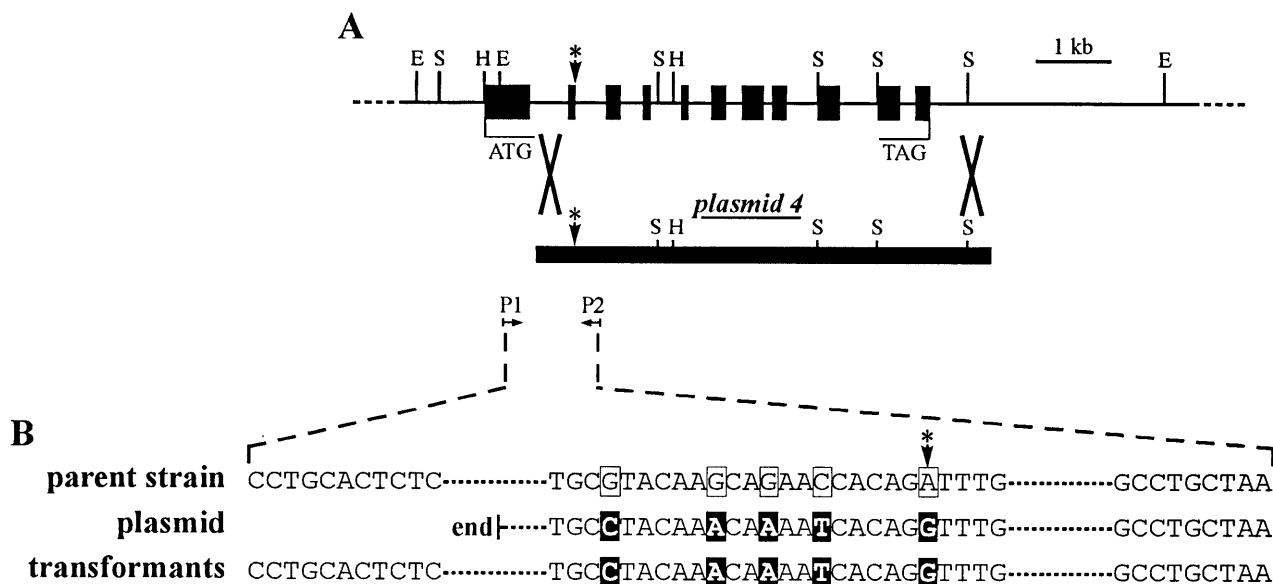


FIG. 3. (A) Physical map and intron–exon structure of the *Volvox* nitrate reductase gene (18) (GenBank accession no. X64136). Plasmid 4 carries a 5'-deleted part of the original nitrate reductase gene with five base pair exchanges with respect to the parent gene, one of them repairing the ruined intron–exon splice site (which is marked by an asterisk). The primers used in genomic PCR are indicated (P1 and P2). P1 matches only the genomic sequence and not the plasmid sequence. For clarity, only the insert of the circular plasmid 4 is shown. (B) Part of the genomic sequence of the nitrate reductase gene in the original *Volvox* strain 153–48 (parent strain), part of the sequence of the 5'-deleted nitrate reductase plasmid 4 (plasmid), and part of the genomic sequence in the recombinant *Volvox* strains HRN-1 and HRN-2 (transformants), showing a homologous recombination event. E, *EcoRI*; H, *HindIII*; S, *SalI*.

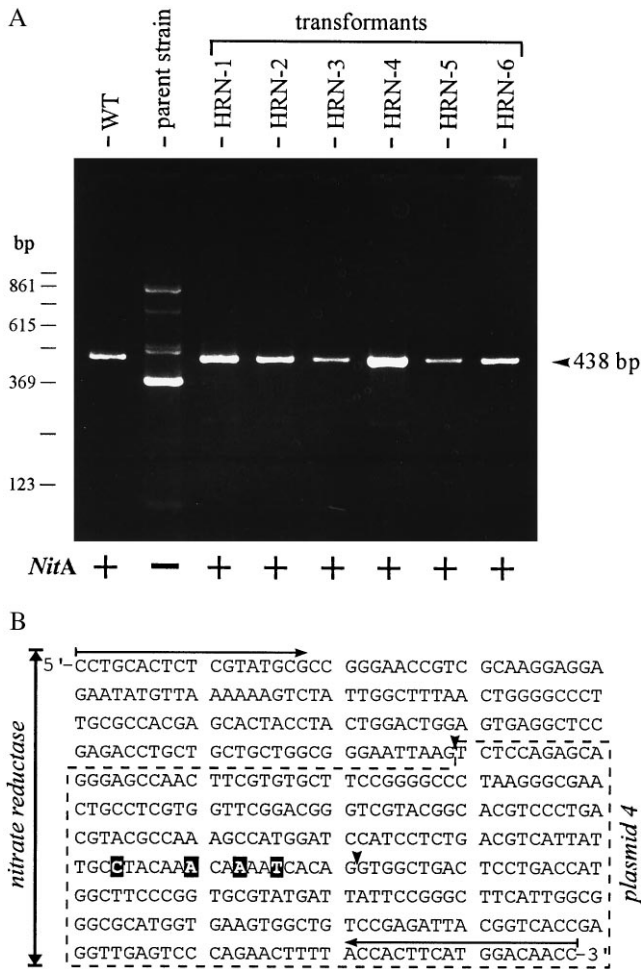


FIG. 4. (A) RT and subsequent PCR amplification of the (modified) nitrate reductase mRNA. RNA was extracted from 20 *Volvox* spheroids of parent strain 153–48, wild-type algae (WT), or transformants HRN-1 through HRN-6 (RT-PCR of clone HRN-7 is not shown). As expected, RT-PCR with parent strain 153–48 yielded no correct product (19). Expression of nitrate reductase (*NitA*) is indicated by a plus or a minus. Sizes of PCR products (438 bp) were determined by using a 123-bp ladder as a size marker and by DNA sequencing. (B) Sequence of cDNA (438 bp), corresponding to nucleotides 1400–1837 of *nitA* sequence (18), obtained from RT and subsequent PCR amplification of the modified nitrate reductase gene harbored in *Volvox* clones HRN-1 and HRN-2, showing a homologous recombination event. Clones HRN-1 and HRN-2 resulted from transformation with plasmid 4. The sequence harbored in plasmid 4 is boxed (dashed line). The artificial base exchanges, with regard to the parent strain, are highlighted. Both introns (solid arrowheads) within this part of the cDNA were spliced correctly; in parent strain 153–48, splicing of the second intron is affected by mutation (G → A transition). The PCR primers used are indicated by horizontal arrows.

map in Fig. 3) of genomic DNA extracted from transformants HRN-1, HRN-2, and HRN-3 were probed, and the results are shown in Fig. 5. All transformants exhibited the same hybridization patterns as those found for the parent *Volvox* strain, 153–48 (Fig. 5), i.e., the homologous recombination event did not affect the immediate surroundings at either the 5' end or the 3' end of the *nitA* gene.

DISCUSSION

The targeted introduction of *in vitro* manipulated DNA to homologous sites in the genome provides a powerful tool for the study of physiological processes. Gene knockout or targeted gene disruption techniques provide important informa-

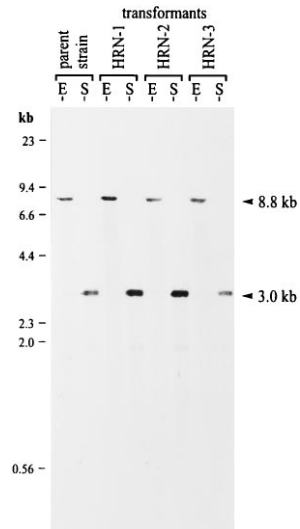


FIG. 5. Southern blots of genomic DNA from the parent *Volvox* strain, 153–48, and from transformants HRN-1, HRN-2, and HRN-3. The sizes of DNA standards are indicated on the left, and hybridizing bands (arrowheads) are indicated on the right. DNA was digested with *EcoRI* or *SalI*, and the blot was probed with an ≈ 0.3 -kb *EcoRI/ApaI* nitrate reductase fragment. The probe covers the *nitA* gene region upstream of the 5' end of the *nitA* sequence present in the replacement vector used for the Southern blot experiments and, therefore, excludes signals resulting from sites of illegitimate plasmid integration. An 8.8-kb (*EcoRI*) fragment and a 3.0-kb (*SalI*) fragment were predicted if the homologous recombination event did not affect the immediate surroundings at the 5' end or at the 3' end of the *nitA* gene. E, *EcoRI*; S, *SalI*.

tion about the biological function of unknown genes. Any organism allowing the application of these techniques fulfills an important prerequisite for serving as a model system. The green algae of the order Volvocales offer useful candidates for model organisms because this order includes organisms that demonstrate as clearly as a diagram the different stages of the evolutionary transition from unicellularity to multicellularity. Targeted gene replacement has recently been shown to be applicable to the unicellular member of this order, *Chlamydomonas* (5). The detection of targeted gene replacement in the most advanced member of the Volvocales makes this group of organisms an attractive model for molecular genetic and biochemical studies.

With a few exceptions, such as yeast, targeted gene replacement by homologous recombination is a rare event and requires highly selective screening procedures for detection; these procedures are available for a number of animal and plant cell systems (5, 10–16). The ratio of homologous to nonhomologous recombination events ranges between 10^{-2} and 10^{-5} in animal cells and between 10^{-4} and 10^{-5} in plant cells (10–16). In the unicellular green alga *Chlamydomonas*, the frequency ranged between 1:24 and 1:1000 (5). As a consequence, large numbers of transformants are required for the detection of homologous recombination events. This is not a serious problem if one is dealing with small unicellular algal cells such as *Chlamydomonas*. In this case, more than 10^7 cells can be handled per shot by using the particle gun for transformation. In contrast, a reproductive cell of *Volvox* with a mean diameter of 80 μm is a huge cell compared with *Chlamydomonas*, and at best 10^4 cells can be handled per shot. With a transformation rate of about 2.5×10^{-5} per reproductive cell, the detection of homologous recombination events becomes a serious problem. Indeed, we were unable to detect homologous recombination events using standard selection techniques such as the insertion of a selectable marker gene within the target gene sequence. Minimizing the extent of

sequence modification to only a few nucleotide exchanges (a total of five in our experiments) within the targeting sequence increased the ratio of homologous to nonhomologous recombination events up to 1:10, thus greatly facilitating the detection of gene replacement events.

Genetic recombination involves the exchange of DNA strands between two duplex DNA partners. The Holliday junction, the branch point between two duplex DNAs that have exchanged a pair of strands, is thought to be an important intermediate in homologous recombination (29). Movement of the Holliday junction by branch migration can increase the amount of genetic information exchanged between homologues. But a single base mismatch, insertion, or deletion is sufficient to pose *in vitro* a substantial barrier to spontaneous branch migration (30). In our experiments, three of our gain-of-function transformants exhibited only the essential A → G nucleotide exchange, two transformants exhibited in addition either only one or two out of the four nucleotide exchanges serving as a marker, and two transformants exhibited all five nucleotide exchanges. Because of the low number of processed cells, it is highly unlikely that the first group of transformants arose by a spontaneous mutation event (spontaneous reversion rate $< 3 \times 10^{-9}$). Therefore, our data indicate that, even *in vivo*, single mismatches may pose a barrier to the movement of the Holliday junction.

This paper has shown that gene replacement by homologous recombination occurs frequently enough in *Volvox* to apply the powerful tool of gene disruption. A general and routine system for knocking out nuclear genes of the haploid multicellular *Volvox* by homologous recombination is an important goal in the further development of this organism as a model system for molecular genetic analyses.

We wish to thank C. Friederich for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 521).

1. Kindle, K. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1228–1232.
2. Schiedlmeier, B., Schmitt, R., Müller, W., Kirk, M. M., Gruber, H., Mages, W. & Kirk, D. L. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 5080–5084.
3. Davies, J. P., Weeks, D. P. & Grossman, A. R. (1992) *Nucleic Acids Res.* **20**, 2959–2965.
4. Hallmann, A. & Sumper, M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 11562–11566.
5. Sodeinde, O. A. & Kindle, K. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9199–9203.
6. Gumpel, N. J., Rochaix, J. D. & Purton, S. (1994) *Curr. Genet.* **26**, 438–442.
7. Nelson, J. A. & Lefebvre, P. A. (1995) *Mol. Cell. Biol.* **15**, 5762–5769.
8. Hinnen, A., Hicks, J. B. & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1929–1933.
9. Orr-Weaver, T. L., Szostak, J. W. & Rothstein, R. S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6354–6358.
10. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A. & Kucherlapati, R. S. (1985) *Nature (London)* **317**, 230–234.
11. Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S. & Smithies, O. (1987) *Nature (London)* **330**, 576–578.
12. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51**, 503–512.
13. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336**, 348–352.
14. Eid, J. & Sollner-Webb, B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2118–2121.
15. Gaertig, J., Thatcher, T. H., Gu, L. & Gorovsky, M. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4549–4553.
16. Lee, K. Y., Lund, P., Lowe, K. & Dunsmuir, P. (1990) *Plant Cell* **2**, 415–425.
17. Adams, C. R., Stamer, K. A., Miller, J. K., McNally, J. G., Kirk, M. M. & Kirk, D. L. (1990) *Curr. Genet.* **18**, 141–153.
18. Gruber, H., Goetinck, S. D., Kirk, D. L. & Schmitt, R. (1992) *Gene* **120**, 75–83.
19. Gruber, H., Kirzinger, S. H. & Schmitt, R. (1996) *Plant Mol. Biol.* **31**, 1–12.
20. Provasoli, L. & Pintner, I. J. (1959) in *The Ecology of Algae*, Special Publication No. 2, Pymatuning Laboratory of Field Biology, eds. Tyron, C. A. & Hartman, R. T. (Univ. Pittsburgh Press, Pittsburgh), pp. 84–96.
21. Starr, R. C. (1969) *Arch. Protistenk.* **111**, 204–222.
22. Harper, J. F. & Mages, W. (1988) *Mol. Gen. Genet.* **213**, 315–324.
23. Hallmann, A. & Sumper, M. (1994) *Eur. J. Biochem.* **221**, 143–150.
24. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77**, 61–68.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
26. Hallmann, A. & Sumper, M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 669–673.
27. Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., Bittner, M. L., Brand, L. A., Fink, C. L., Fry, J. S., Galluppi, G. R., Goldberg, S. B., Hoffmann, N. L. & Woo, S. C. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4803–4806.
28. Takeuchi, Y., Dotson, M. & Keen, N. T. (1992) *Plant Mol. Biol.* **18**, 835–839.
29. Holliday, R. (1964) *Genet. Res.* **5**, 282–304.
30. Panyutin, I. G. & Hsieh, P. (1993) *J. Mol. Biol.* **230**, 413–424.