Stable Nuclear Transformation of *Chlamydomonas* Using the *Chlamydomonas* Gene for Nitrate Reductase

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Abstract. We have developed a nuclear transformation system for *Chlamydomonas reinhardtii*, using microprojectile bombardment to introduce the gene encoding nitrate reductase into a *nit1* mutant strain which lacks nitrate reductase activity. By using either supercoiled or linear plasmid DNA, transformants were recovered consistently at a low efficiency, on the order of 15 transformants per microgram of plasmid DNA. In all cases the transforming DNA was integrated into the nuclear genome, usually in multiple copies. Most of the introduced copies were genetically linked to each other, and they were unlinked to the original *nit1* locus. The transforming DNA and nit⁺ phenotype were stable through mitosis and meiosis, even in the absence of selection. *nit1* transcripts of various sizes were expressed at levels equal to or greater than those

in wild-type nit⁺ strains. In most transformants, nitrate reductase enzyme activity was expressed at approximately wild-type levels. In all transformants, *nit1* mRNA and nitrate reductase enzyme activity were repressed in cells grown on ammonium medium, showing that expression of the integrated *nit1* genes was regulated normally. When a second plasmid with a nonselectable gene was bombarded into the cells along with the *nit1* gene, transformants carrying DNA from both plasmids were recovered. In some cases, expression of the unselected gene could be detected. With the advent of nuclear transformation in *Chlamydomonas*, it becomes the first photosynthetic organism in which both the nuclear and chloroplast compartments can be transformed.

THE unicellular green alga *Chlamydomonas reinhardtii* has long been a favorite experimental organism for studying the genetics and molecular biology of a number of cellular processes, including photosynthesis and chloroplast development (Levine and Goodenough, 1970; Rochaix and Erickson, 1988), flagellar assembly and motility (Luck, 1984; Lefebvre and Rosenbaum, 1986), and cell–cell interactions during mating (Snell, 1985). The attractiveness of *Chlamydomonas* for these studies has been hampered by the lack of a reproducible, generally applicable technique for stably transforming the nuclear genome.

Previous authors who have reported nuclear transformation in *Chlamydomonas* have used genes from other organisms as selectable markers. For example, the yeast *ARG4* gene was used to complement a *Chlamydomonas arg7* mutation (Rochaix and van Dillewijn, 1982; Rochaix et al., 1984) and a bacterial neomycin phosphotransferase gene under the control of the SV-40 early promoter was used to confer resistance to the aminoglycoside antibiotic G418 (Hasnain et al., 1985). In neither case was it clearly demonstrated that the foreign DNA was responsible for the selected phenotype. The reversion of *arg7* to prototrophy and the frequency of spontaneous G418 resistant colonies are significant problems with these systems. To date, no transformation system for nuclear genes has proven to be experimentally useful.

Genes from other organisms may make poor selectable markers for nuclear transformation in *Chlamydomonas* because of inefficient expression in *Chlamydomonas*. Codon usage is highly biased in many *Chlamydomonas* genes, especially those encoding abundant proteins (Silflow and Youngblom, 1986; Goldschmidt-Clermont and Rahire, 1986). In addition, the putative polyadenylation signal for *Chlamydomonas* genes is unusual and is not found in the genes that have been used as selectable markers (Silflow and Youngblom, 1986). The use of a *Chlamydomonas* gene as the selectable marker in nuclear transformation experiments should circumvent these problems. Moreover, it is possible that the frequency of stable integration into the *Chlamydomonas* genome could be increased by using homologous DNA.

It has recently been demonstrated that DNA can be introduced into a variety of plant cells using DNA-coated tungsten microprojectiles accelerated by a particle gun (Klein et al., 1987, 1988a, b). This technique has been used to transform the organelles of lower eukaryotes, e.g., the chloroplasts of *Chlamydomonas* (Boynton et al., 1988; Blowers et al., 1989) and the mitochondria of yeast (Johnston et al., 1988; Fox et al., 1988). In *Chlamydomonas*, the nucleus is actually a smaller target for particle bombardment than is the single large chloroplast that surrounds it. However, our preliminary experiments showed that the particle gun could be used

to generate unstable nuclear transformants using foreign genes (Kindle, K. L., T. Klein, and J. C. Sanford, unpublished data).

As a selectable marker for nuclear transformation in *Chlamydomonas*, we have used the *Chlamydomonas* gene encoding nitrate reductase. Nitrate reductase (NR)[1] is the first enzyme in the pathway for nitrate utilization, and is required for growth when nitrate is the sole nitrogen source. The structural gene for NR (*nit1*) has recently been cloned from wild-type *Chlamydomonas* using a partial cDNA clone for barley NR as a heterologous probe (Fernández et al., 1989). We report here that the cloned *nit1* gene can be used to complement a *nit1* mutation using microprojectile bombardment to introduce the cloned DNA into the mutant cells. In all cases, the transforming DNA integrated stably into the genome. Although nit⁺ transformants arose at a relatively low frequency, they usually contained multiple copies of the transforming DNA. In addition, when cells were bombarded with both the NR plasmid and a second plasmid containing a nonselectable gene, as many as half of the nit⁺ transformants carried DNA from both plasmids. In at least one case the unselected DNA was expressed at the level of mRNA. Therefore, microprojectile bombardment using the *nit1* gene as a selectable marker should be a reproducible and generally useful system for the introduction of DNA into the nuclear genome of *Chlamydomonas*.

Materials and Methods

Chlamydomonas Strains and Culture Media

Chlamydomonas strain *nit1-305* was derived from 6145c (Sosa et al., 1978). Strains 21gr and 6145c were obtained from Dr. Ruth Sager (Sidney Farber Cancer Center, Boston, MA). The *nit1* mutation in *nit1-305* is very stable; revertants were rarely found when as many as 10^8 cells were plated on nitrate-containing medium. *pf13* was obtained from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC.

In most experiments, cells were grown in Sager-Granick medium II containing NH_4NO_3 (SGII; Sager and Granick, 1953), a high phosphate, acetate-containing medium. After transformation, nit⁺ cells were selected on SGII medium in which KNO_3 was substituted for NH_4NO_3. For solid media, Difco or Gibco agar was washed extensively with distilled water to remove contaminating NH_4^+ (Difco Laboratories Inc., Detroit, MI; Gibco Laboratories, Grand Island, NY). Because the cells tended to clump in SGII medium, in some experiments SGII was modified by reducing the phosphate concentration 60% and the acetate concentration 50% ("1/2 R" medium). For synchronous growth of cells, cultures were exposed to a 12-h light, 12-h dark illumination regime in HS medium (Sueoka, 1960).

For induction of NR, cells were first grown to mid-exponential phase in 10 mM NH_4Cl minimal medium (Sueoka, 1960) with bubbling (2-5% CO_2 in air) under continuous light. Cells were harvested by centrifugation at 10,000 g for 5 min, washed twice with nitrogen-free minimal medium, and transferred immediately to minimal medium containing 4 mM KNO_3.

Enzymatic Assays

Enzymatic assays were performed in situ after permeabilizing cells by shaking 1 ml of cells vigorously with 500 U of nystatin for 1 min (Fernández and Matagne, 1986). NADPH-nitrate reductase activity (overall-NR) was assayed in 1.35 ml of a mixture containing 1 ml of permeabilized cells, 100 μmol Tris-HCl (pH 8.0), 10 μmol KNO_3, and 0.4 μmol NADPH. The reaction was stopped at acidification with reagents for nitrite determination (Barea and Cardenas, 1975). Terminal nitrate reductase activity was assayed in 1.35 ml of a mixture containing: 1 ml of permeabilized cells, 100 μmol Tris-HCl (pH 8.0), 10 μmol KNO_3, 2 μmol benzyl viologen, and 2 mg so-

dium dithionite. The reaction was stopped by vigorous shaking to oxidize reduced benzyl viologen. One unit of activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol nitrate to nitrite per minute at 30°C. Nitrite reductase was assayed as described by Barea and Cardenas (1975).

Plasmid DNAs

pMN24 (Fernández et al., 1989; see Fig. 2 a) carries the entire *nit1* gene on a 14.5 kb Sal I-Bgl II fragment cloned into pUC119. The unique Bam HI site in the insert was digested to produce linear DNA for transformation in some experiments. The plasmid DNA used for cotransformation experiments consisted of a *Chlamydomonas* gene encoding a chlorophyll a/b binding protein into which an insertion had been made to allow unambiguous detection of the transforming DNA. A λ clone containing genomic DNA encoding one of the *Chlamydomonas* chlorophyll a/b binding protein genes was isolated using a cDNA clone as probe (Shepherd et al., 1983). The genomic DNA library was constructed in λEMBL4 from a Sau 3A partial digestion of DNA from *Chlamydomonas* strain CC400G. A 4.0-kb Sma I-Eco RI fragment which contains the entire coding region of the gene plus 2.0 kb of upstream sequence was subcloned into pIBI76 (International Biotechnologies, Inc., New Haven, CT). The *cabII-1* coding region contains a single Nco I site in the 3'-nontranslated region of the gene (Imbault et al., 1988). Two different insertions were made by ligating Hae III fragments of bacteriophage λ DNA into this site; *cab*λ3 contains a ∿500-bp insertion of λ DNA while *cab*λ9 has a ∿150-bp insertion (see Fig. 7 a).

Bombardment and Selection of Transformants

nit1-305 cells were grown in ammonium-containing liquid medium in continuous light. Cells from mid-logarithmic to early stationary phase (5 × 10^5-2 × 10^6 cells/ml) were concentrated by centrifugation and resuspended at a density of ∿2 × 10^8 cells/ml in nitrate-containing SGII medium. 0.4 ml of concentrated cells was used for each bombardment. In initial experiments, cells were spread either on selective plates in soft agar as described by Boynton et al. (1988) or as a thin liquid layer in an otherwise empty 60-mm tissue culture dish (Falcon Labware, Oxnard, CA) (Table I). Cells bombarded in liquid were diluted into ammonium-containing SGII medium and grown for 18 h in the light before being harvested and spread on selective SGII agar plates. In subsequent experiments 0.4 ml of cells were spread in the center of a 90-mm selective SGII agar plate and allowed to dry uncovered until bombardment. After bombardment plates were sealed with parafilm.

DNA-coated tungsten spheres were prepared as described by Klein et al. (1987). Approximately 800 ng of plasmid DNA and 0.5 mg of 1.2 μm tungsten spheres were used in each bombardment with a No. 1 nail gun powder charge. Where indicated, cells were exposed to UV light from an 8 W Sylvania germicidal G8T5 bulb, calibrated with a Spectroline DM-254 N shortwave UV light meter (Spectronics Corp., Westbury, NY).

1-3 wk after bombardment, transformed colonies were visible and could be streaked onto fresh selective SGII plates.

Genetic Analysis

Tetrad analysis was performed at 24°C as described by James et al. (1988). Meiotic products were separated and allowed to grow in constant light for 1 wk on the same plates used for zygote germination. The colonies were then transferred by toothpick into 400 μl of medium I (Sager and Granick, 1953) in the wells of a 96-well culture dish. After 3-5 d of growth in constant light, motility of each of the tetrad progeny was scored by examining each well at 80× using a stereomicroscope (Zeiss DR-C). Approximately 50 μl of each culture was then spotted onto an agar plate containing nitrate as the sole nitrogen source to score nit⁺ and nit⁻ phenotypes.

Preparation of Nucleic Acids

Transformed cells were grown in 50-100 ml of selective (nitrate-containing) or nonselective (ammonium-containing) SGII or 1/2R media. DNA was either CsCl purified (Weeks et al., 1986) or prepared using a miniprep procedure adapted from Rochaix (1980). For the miniprep, cells were harvested by low speed centrifugation and resuspended in 4 ml 10 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8. SDS was added to 0.1%, proteinase K to 200 μg/ml and the mixture was incubated at 55°C for 2 h. DNA was phenol extracted, and precipitated with 0.5 vol isopropyl alcohol in the presence of 0.3 M NaOAc, pH 4.5. The DNA was further purified by precipitation

Table I. Recovery of nit⁺ Colonies Using Different Transformation Conditions

Experiment number	Cells	pMN24 DNA	UV	nit⁺ colonies	Number of plates bombarded
			J/m^2		
1	Log	sc	–	19	4
2	Late	sc	–	19	8
3	Late	Linear	–	4	6
4	Late	None	–	0	2
5	Late	sc	–	4	6
6	Late	Linear	–	4	6
7	Late	None	–	1	1
8	Mixed	sc	–	3	1
9	Mixed	sc	2.5	14	2
10	Mixed	sc	5	2	2
11	Mixed	sc	10	4	2
12	Mixed	sc	20	7	2
13	Mixed	sc	40	14	2
14	Mixed	sc	80	14	2
15	Mixed	sc	160	14	2

nit1-305 cells were grown to a density of ~5 × 10⁵ (early) or 2 × 10⁶ (late) cells/ml and suspended at ~2 × 10⁸ cells/ml. In cases labeled mixed, equal numbers of early and late cells were mixed before bombardment. *sc*, supercoiled; *linear*, linearized at the unique Bam HI site. Experiments 1–4, 8–18, cells were bombarded on solid media containing nitrate as the sole nitrogen source. Experiments 5–7, cells were bombarded in liquid media containing ammonium; they were then diluted and allowed to grow without selection for 18 h before being plated on selective media.

with 10% polyethylene glycol/1 M NaCl, ethanol precipitation in the presence of 2.5 M NH₄OAc, and digestion with 20 µg/ml ribonuclease A at 37°C for 45 min. After phenol extraction and ethanol precipitation, DNA prepared in this way can be digested by most restriction enzymes. Southern blots from genomic DNA digests were prepared using standard methods.

RNA was prepared from 11 cultures of cells as described (Shepherd et al., 1983) and poly(A)⁺ RNA was selected by oligo(dT) cellulose chromatography as described (Maniatis et al., 1982). RNA blots were prepared by transferring RNA that had been size-fractionated on 1.2% agarose formaldehyde gels to Schleicher and Schuell NYTRAN (Schleicher & Schuell, Inc., Keene, NH) using standard methods (Maniatis et al., 1982).

Probes were synthesized using ³²P-dATP by extension from random primers (Feinberg and Vogelstein, 1983). DNA and RNA blots were hybridized according to standard methods. After hybridization, the filters were washed three times (30 min each) at 65°C in 0.2× SSC, 0.5% SDS. Autoradiograms were made by exposing blots to Kodak XAR5 film using intensifying screens at –70°C.

Results

Bombardment of nit1 Cells with the Wild-type nit1 Gene Leads to Correction of the nit1 Deficiency

The *nit1* locus of *Chlamydomonas reinhardtii* is the structural gene for NR (Sosa et al., 1978; Fernández and Matagne, 1984). *nit1* mutants require a reduced nitrogen source (such as ammonium) for growth. By introducing the cloned *nit1* gene into a *nit1* mutant strain it should be possible to recover transformed cells by their ability to grow on nitrate as the sole nitrogen source.

The *nit1-305* mutant strain (Fernández and Matagne, 1984) was bombarded with plasmid-coated tungsten particles. The plasmid used in these experiments, pMN24, contained the complete wild-type *nit1* gene (Fernández et al., 1989). After 1–3 wk on selective media, colonies were visible in a ring surrounding an area of high cell mortality at the center of the

blast (Fig. 1). It appears that there is only a narrow zone in which the microprojectiles penetrate the cells at an appropriate frequency and velocity to cause transformation. Hybridization analysis indicated that nearly all of the nit⁺ colonies contained DNA from the transforming plasmid (see below).

The different experimental conditions used for transformation are outlined in Table I. nit⁺ transformants were obtained from cells grown in ammonium-containing medium before transformation, indicating that the cells need not be incubated under inducing conditions for NR before transformation and selection. Transformants were also recovered when cells were bombarded directly on selective (nitrate-containing) plates, indicating that there is no need for a period of nonselective growth before selection. Furthermore, the addition of carrier DNA or an osmoticum, such as mannitol, did not improve the recovery of nit⁺ colonies (data not shown). Both linear and supercoiled plasmid DNA produced transformation events, with no dramatic difference in efficiency. We compared the success of transformation using cells grown to logarithmic phase or to early stationary phase and found little difference. We pretreated the cells with UV light before transformation in order to stimulate DNA repair systems which might promote recombination and saw no pronounced effect on the efficiency of transformation. Most importantly, transformation by bombardment was reproducible; >40 plates were bombarded and the majority of these plates had 2–10 transformants growing on them (Table I). In subsequent experiments, we have seen up to 25 nit⁺ colonies resulting from a single bombardment (data not shown).

nit⁺ Colonies Contain Extra DNA Fragments that Hybridize to pMN24

To determine whether the nit⁺ colonies obtained by bombardment contained DNA sequences derived from pMN24, the DNA from each of 13 nit⁺ isolates was analyzed by hybridization to the insert from pMN24. Undigested genomic DNA from transformants showed only a single band hybridizing to pMN24 after size fractionation on agarose gels, and this comigrated with undigested genomic DNA (data not shown). This observation and genetic evidence discussed below suggested that the pMN24 DNA had integrated into the genome. When genomic DNA was digested with Pvu II and hybridized to the pMN24 insert, DNA from the untransformed recipient strain (*nit1-305*), showed four fragments that hybridized to the probe (Fig. 2 *b*, fragments *A*, *B*, *C*, and *E*). The transformants contained these same four fragments plus additional fragments. Each transformant displayed a unique pattern of new fragments, varying in number from 1 (number 12) to 16 (numbers 1 and 5). These new fragments may represent junction fragments between plasmid and genomic DNA or they may reflect rearrangements within the transforming plasmid before integration. Besides these extra fragments, multiple copies of the *nit1* fragments of pMN24 were found in the transformants. Fig. 2 *a* shows that four Pvu II fragments of pMN24 should hybridize to the Sph I fragment probe; fragments A and C comigrate with *Chlamydomonas* genomic DNA fragments, while fragments C' and F are unique to the plasmid. The stoichiometry of Pvu II fragments which comigrated with fragments A, C, C', and F of pMN24 ranged from 1 to more than 10 copies per cell in the transformed strains. When the same filter was hybridized using the vector

Figure 1. nit⁺ colonies on bombarded plates. Approximately 4×10^7 cells were spread in the central area of a 90-mm petri plate containing SGII agar medium with nitrate as sole nitrogen source. Cells were bombarded with pMN24 DNA-coated tungsten particles, and grown for ~4 wk. Areas where agar has been blown away can be seen in the center, surrounded by six nit⁺ colonies.

pUC119 as probe, similar results were obtained. The transformants contained from 1 to 10 fragments which hybridized to the vector, and those that comigrated with the vector fragments were present in stoichiometries varying from 1 to more than 10 copies per cell (data not shown).

Some of the extra Pvu II fragments which hybridized to the pMN24 insert failed to hybridize to pUC119 DNA (data not shown). That these may represent truncated *nit1* genes is suggested by the following observation. When genomic DNA was digested with Cla I + Eco RI and probed with the *nit1* Cla I + Eco RI fragment, there were fragments that migrated ahead of the intact *nit1* fragment in some cases (data not shown). These must represent truncated *nit1* genes, which are probably not functional. Whether the complex Southern patterns result from multiple independent integration events or from plasmid rearrangements before integration remains to be determined.

Transforming pMN24 DNA Is Mitotically Stable Even in the Absence of Selection

Most of the transformants grew well in selective media, suggesting that the *nit1* gene was expressed at a level high enough to allow relatively rapid growth rates. If the pMN24 DNA was stably integrated into the genome, then one would expect it to be maintained under either selective or nonselective conditions. The stability of the transforming DNA was tested by the experiment shown in Fig. 3. Transformants were grown in either selective (nitrate as sole nitrogen source) or nonselective (ammonium-containing) SGII medium for ~10 generations. DNA was prepared and digested with Pvu II, and the genomic fragments hybridizing to labeled pMN24

DNA were detected by Southern transfer and hybridization. In all cases the same fragments were labeled whether the cells were grown with or without selection. Subsequently, we have routinely grown samples for DNA isolation under nonselective conditions, and have not detected any rearrangements of the additional DNA fragments in transformed strains after >6 mo of growth in the absence of selection.

nit⁺ Phenotype Is Inherited as a Single Mendelian Trait Which Is Unlinked to the Original nit1 Locus

To determine whether the nit⁺ phenotype of the transformants was inherited as a Mendelian trait and whether it was located at the *nit1* locus or elsewhere in the genome, 11 of the transformants were backcrossed to a strain that carried both the *nit1* mutation and the paralyzed-flagella mutation, *pf13*. *pf13* is closely linked (<5 cM) to *nit1* on linkage group IX (Harris, 1989). Progeny from each cross were scored for motility and for the ability to grow using nitrate as the sole nitrogen source. The results of this experiment are shown in Table II.

In all crosses, the nit⁺ phenotype was inherited as a single Mendelian trait: every tetrad contained two nit⁺ and two nit⁻ cells. This result was somewhat surprising in view of the high copy number of pMN24 sequences present in some of the transformants (Fig. 2). However, as shown below for two of the high copy number transformants, most of the extra bands hybridizing to pMN24 were linked to one another, which would explain the Mendelian segregation of the nit⁺ phenotype.

In each of the 11 backcrosses, the nit⁺ phenotype segregated independently of the parental *nit1* locus, as seen by
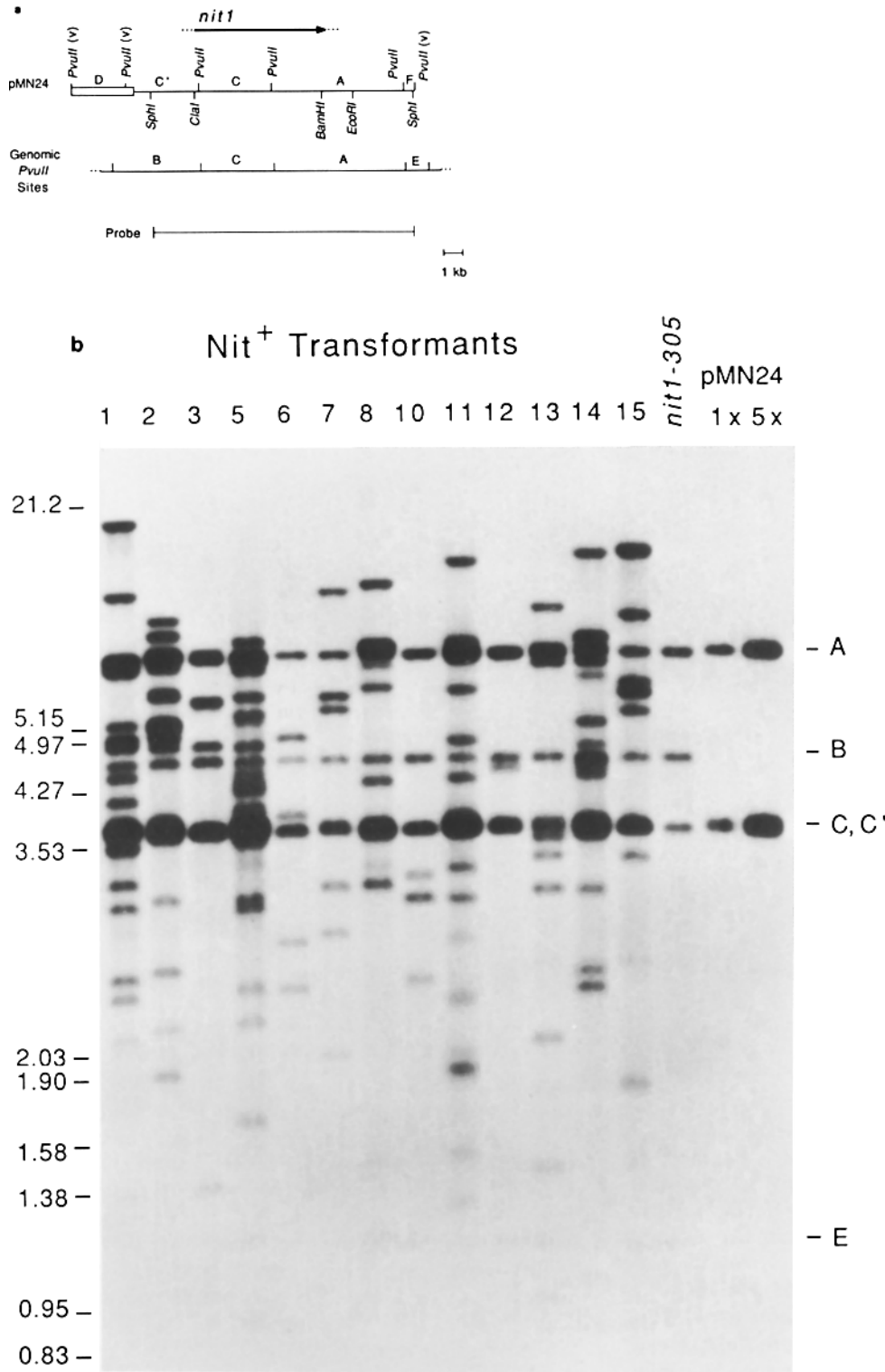
Figure 2. Hybridization analysis of nit⁺ transformants. (*a*) The restriction map of pMN24 is shown linearized at one of the Pvu II sites within the vector (*v*). Vector sequences are depicted by the open rectangle. The approximate endpoints of the *nit1* transcript and its orientation are represented by the arrow. Pvu II sites within the corresponding genomic region are shown on the lower map. Fragments C' and F of pMN24 have one Pvu II site in *Chlamydomonas* DNA and one in pUC119, and so differ in length from the corresponding genomic Pvu II fragments. (*b*) Strains were grown in liquid media without selection and genomic DNA was prepared. DNA from the untransformed parent (*nit1-305*) and 13 independent nit⁺ transformants was digested with Pvu II and fractionated on a 0.8% agarose gel (3.5 μg DNA per lane). Transfer to a Schleicher and Schuell nylon filter and hybridization were performed according to the manufacturer's recommendations using the nick-translated 13.5-kb Sph I fragment from pMN24 as probe (Rigby et al., 1977). Lanes labeled 1× and 5× contain 0.6 and 3.0 ng, respectively, of pMN24 DNA digested with Pvu II. A, B, C, and E refer to fragments present in the untransformed parent. Fragments A and C are also present in pMN24.

the independent inheritance of the *nit*⁺ and *pf13*⁺ markers (Table II). For 10 of the 11 transformants, the nit⁺ marker was completely unlinked to *pf13*; for the other (number 10), close linkage to *pf13* was not seen, but the number of tetrads analyzed was not sufficient to determine whether the *nit*⁺ marker was loosely linked to *pf13*.

Most of the Newly Introduced nit1 DNA Fragments Cosegregated in Tetrad Progeny of Three Transformants

The pattern of inheritance of each of the pMN24-homologous Pvu II fragments introduced during transformation was
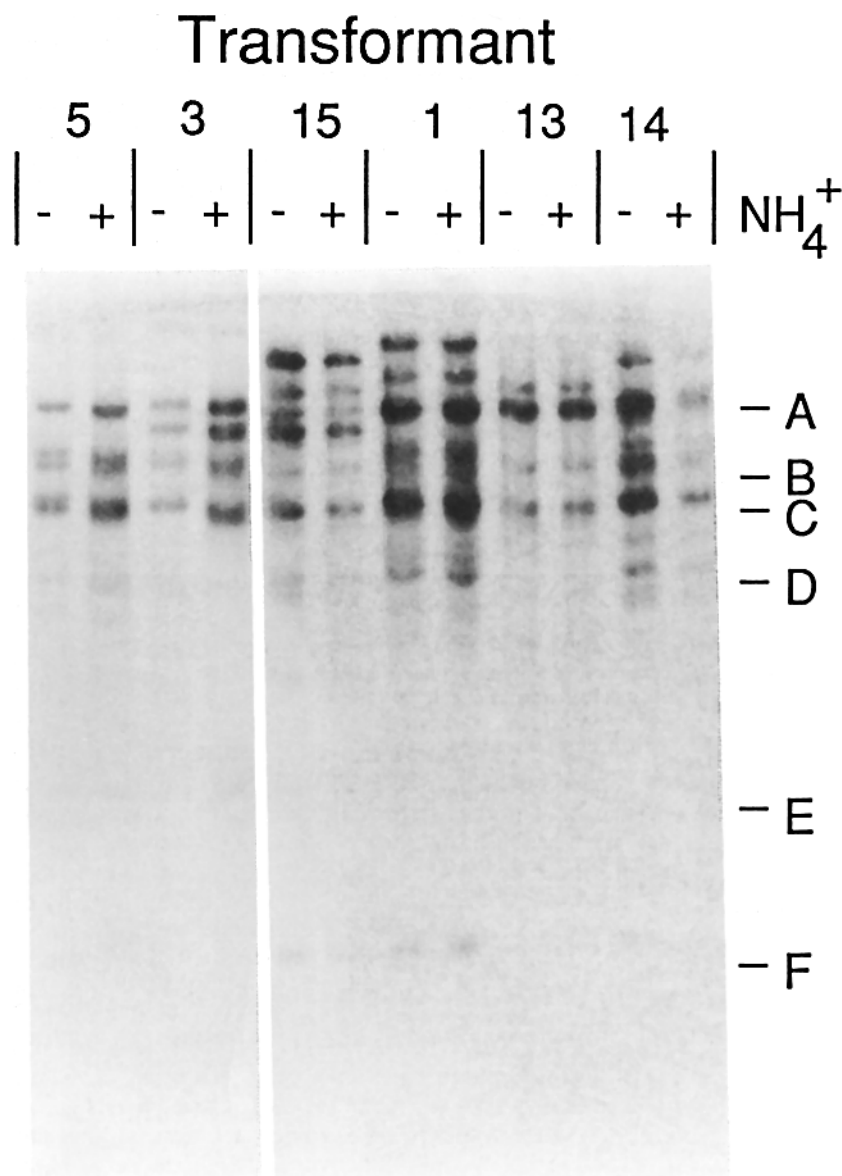
Transformant

Figure 3. Stability of pMN24 DNA under selective and nonselective conditions. Transformants were grown in SGII media with (+) or without (−) NH₄⁺ in the medium for ~10 generations. DNA was prepared, digested with Pvu II, and Southern blots were probed with pMN24 DNA. Fragments A–F are as described in legend to Fig. 2 a.

examined in progeny of backcrosses of three of the transformants (numbers 1, 5, and 10). Transformant 10 produced the simplest pattern of segregation. Three extra Pvu II fragments were seen in the DNA from transformant 10 (Fig. 2 b). In addition, bands B and C, normally present in a single copy per genome, showed increased hybridization intensity in the transformant relative to nitl-305. These three extra fragments cosegregated with the nit⁺ phenotype in three tetrads (Fig. 4 a). We conclude that transformant 10 probably resulted from a single integration event; therefore the extra DNA fragments cosegregated with the nit⁺ phenotype in tetrad progeny as a single Mendelian trait.

Transformant 1 provided a more complex picture. At least 18 additional Pvu II fragments which hybridized to pMN24 were seen in transformant 1 (Fig. 2 b); at least eight of these contain plasmid vector sequence (data not shown). In tetrads 2 and 3 (Fig. 4 b) all of the extra bands cosegregated, and they were found only in the nit⁺ progeny. In tetrad 1, however, one band (marked with an asterisk in Fig. 4 b) segregated in a nonparental ditype pattern relative to the other bands and relative to the nit⁺ marker. Although the number

of tetrads is too small to determine linkage with certainty, this DNA fragment appears to be unlinked to the others. This band did not encode a functional gene for NR, as it was found in two nit⁻ progeny.

Similarly, transformant 5 showed a complex pattern of additional Pvu II fragments hybridizing to pMN24 DNA (Fig. 2 b). In one tetrad and one complete octad, all of the extra bands cosegregated in the nit⁺ progeny, suggesting that they are linked (Fig. 4 c). In tetrad 1, however, at least four Pvu II fragments (fragment D and the three fragments marked with arrows) segregated in a tetratype pattern relative to the nit⁺ marker and the other extra bands. In tetrad 1, these four bands are present in one nit⁻ progeny (Fig. 4 c, lane 1 b), and they are missing from one nit⁺ progeny (Fig. 4 c, lane 1 c). These four Pvu II fragments are thus either unlinked to the other supernumerary bands, or they are distantly enough linked that a crossover event occurred between the two groups of DNA fragments. The DNA in these four bands, however, was not expressed as functional NR, as they were present in DNA from a nit⁻ segregant.

The results from transformant 5 also illustrate an intrigu-

Table II. Backcross Data: Transformants Backcrossed to nit1 pf13 Double Mutants

Transformant	Tetrad type		
	PD	NPD	T
1	4	8	10
2	12	6	7
3	2	2	6
5	4	1	8
6	6	6	10
7	4	3	10
8	0	3	10
10	3	0	9
11	4	5	7
14	3	3	16
15	5	4	7

Each of the transformants was backcrossed to a double mutant strain that contained both the *nit1* and *pf13* mutations. Each of the tetrad progeny was scored for growth on nitrate as sole nitrogen source, and for swimming phenotype. *PD*, 2 nit⁺ pf⁺ : 2 nit⁻ pf⁻. *NPD*, 2 nit⁺ pf⁻ : 2 nit⁻ pf⁺. *T*, 1 nit⁺ pf⁺ : 1 nit⁻ pf⁻ : 1 nit⁺ pf⁻ : 1 nit⁻ pf⁺.

ing example of non-Mendelian inheritance which was occasionally observed in the backcross experiments. A gene conversion event (5:3 nit⁺/nit⁻) was scored in octad 2. Surprisingly, all of the supernumerary bands hybridizing to pMN24 were seen in all five of the nit⁺ progeny. Mendelian segregation was seen for the other phenotypic marker in this cross, *pf13*, for which four swimming and four paralyzed progeny were seen. The "co-conversion" of each of the extra bands is especially surprising in view of the fact that four of the bands are distant enough from the others that a recombination event occurred in one of the analyzed tetrads.

nit1 mRNA Expression Is Regulated by Ammonium in Transformants

In nit⁺ strains, a high molecular weight NR transcript (~3.5 kb) is induced when cells are switched from ammonium- to nitrate-containing medium; this transcript is undetectable in noninduced cells, presumably because NR gene expression is repressed by ammonium (Fernández et al., 1989). To examine *nit1* gene expression in transformants, a *nit1–305* revertant and two transformants with multiple copies of pMN24 DNA were grown in the presence or absence of ammonium, and poly(A)⁺ RNA was prepared. To detect *nit1* transcripts, a Hind III fragment internal to the coding region was used as a hybridization probe to RNA blots (shown in Fig. 5 *a*). A ~3.6-kb transcript was present in the *nit1–305* revertant, only when the cells were grown in the absence of ammonium. One to three transcripts (2.9–3.6 kb) hybridized to the *nit1* probe in these transformants (Fig. 5 *b*). The minimum transcript length that could encode a mature NR (105 kD) is 2.9 kb. Since ammonium repressed the expression of all *nit1* transcripts in these transformants, it appears that the DNA sequences that are necessary and sufficient for regulation of *nit1* gene expression by ammonium are present in pMN24 and that they function in chromosomal locations outside the original *nit1* locus.

The *nit1–305* mutant retains one of the two partial enzyme activities of NR (Sosa et al., 1978; Fernández and Matagne, 1984; see below) and so must retain some form of *nit1*

mRNA. The *nit1* transcripts in the *nit1–305* mutant and in the 13 transformants that had been characterized in detail were analyzed as follows. Cells that had been grown in ammonium-containing medium were transferred to nitrate medium, and after 2 h, total RNA was isolated. RNA blots were prepared and hybridized to the *nit1* probe described above. Fig. 5 *c* shows that approximately equal amounts of *nit1* mRNA were present in *nit1–305* and the *nit1–305* revertant. Some of the transformants showed additional *nit1* mRNA species, at 2.9 kb (numbers 2, 5, and 6) and 4–4.5 kb (numbers 3 and 7). In most transformants, *nit1* mRNA abundance was higher than in the untransformed strains, although in the cases of transformants 3, 8, and 14, *nit1* mRNA abundance was low. There was no correlation of high *nit1* mRNA abundance with high copy number of pMN24 DNA fragments.

Transformants Express Varying Levels of Nitrate Reductase Activity

NR catalyzes a two-step electron transfer reaction whose components can be assayed separately. Thus, we measured two enzyme activities: overall NR activity, and the so-called "terminal NR" activity observed using benzyl viologen as substrate (Fernández and Matagne, 1986). These activities were analyzed in 12 transformants, and were compared to the activities in the wild-type strains (21gr and 6145c), in mutant *nit1–305*, and in a spontaneous revertant of *nit1–305* (*rev9*).

In each of the transformed strains, as in the wild-type strains, NR activities were absent in cells grown in ammonium medium (data not shown). In most cases, the time course of accumulation of the enzyme activities upon transfer to nitrate medium and the levels of enzyme activity that accumulated were similar in the transformants and in the wild type (data not shown). However, transformants 8, 11, and 14 had very low overall NR activity, though the terminal NR activity was comparable to wild-type strains. Overall NR activity could not be detected in *nit1–305* and was extremely low in transformant 13, which grew very poorly in nitrate-containing medium. All of the other transformants grew as well as the wild-type strains in nitrate medium, indicating that the low overall NR activity in transformants 8, 11, and 14 was not limiting for growth.

The ratio of the terminal NR activity to overall activity for the different transformed strains is shown in Table III. It was lowest in the wild-type and revertant strains (2.1–2.7), while transformants 1, 2, 3, 5, 6, 7, 10, and 12 showed a somewhat higher ratio (3.3–5.3). The much higher ratio in transformants 8, 11, and 14 (7.4–18.6) and 13 (184.5) may reflect inefficient expression of the introduced genes or the synthesis of a defective gene product, perhaps resulting from the expression of a truncated *nit1* gene.

Nitrite reductase activity was also repressed in ammonium medium and derepressed in nitrate with a very similar time course in all the above strains. Maximum activity levels appeared after 2–4 h and ranged between 340 and 820 mU/mg chl (results not shown).
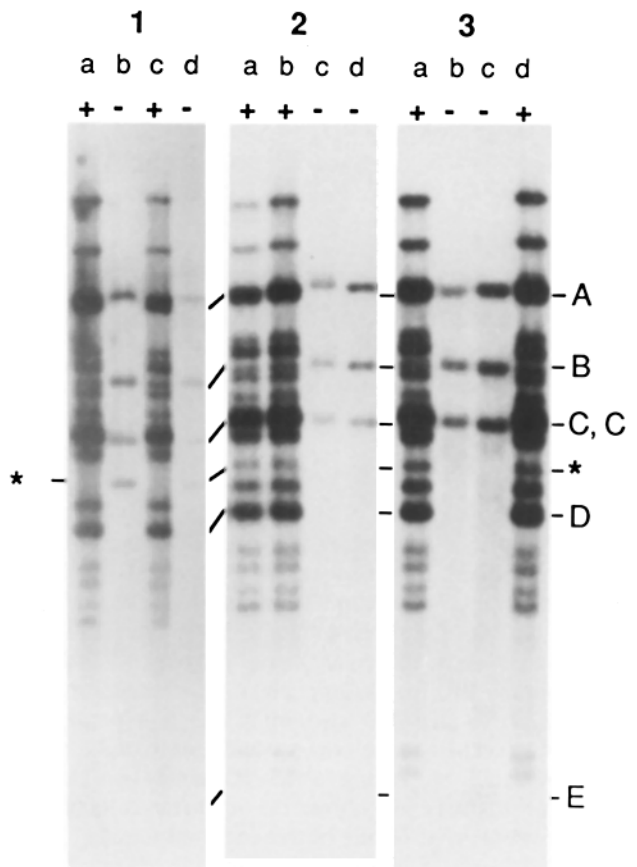
Cobombardment with pMN24 and a Second Nonselected Plasmid Results in Integration of Both DNAs

The fact that many of the transformants contained multiple copies of *nit1* DNA after transformation suggested that

a Tft 10 x *nit1⁻*
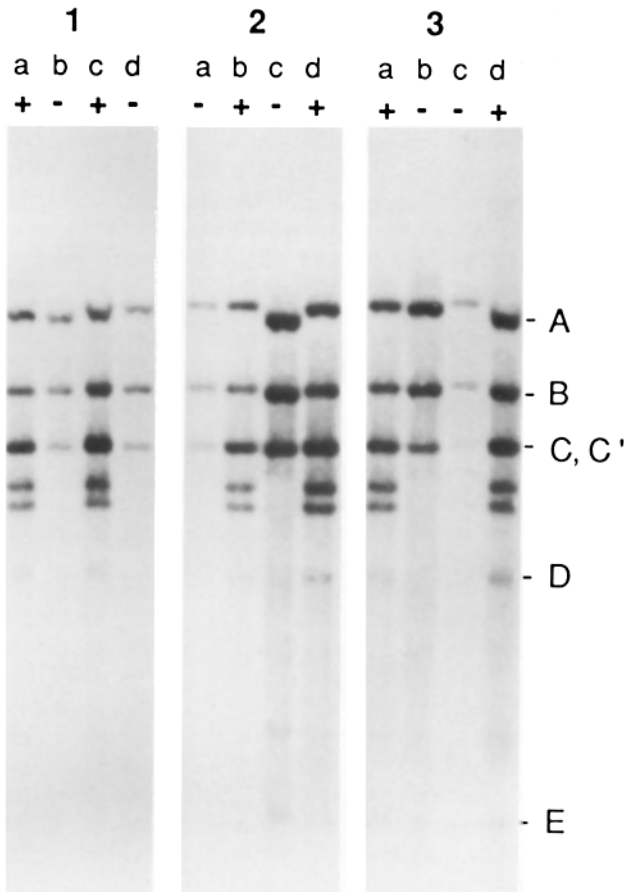
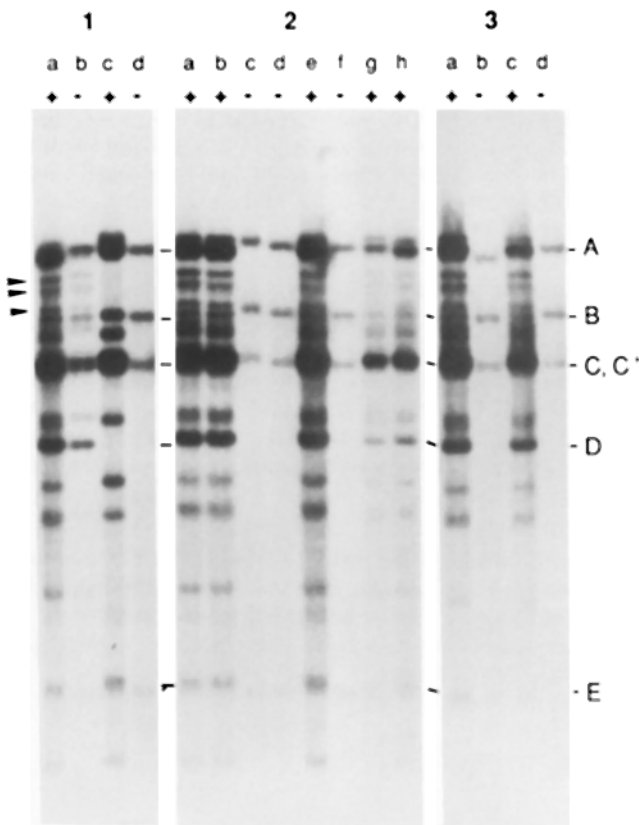**b** Tft 1 x *nit1⁻*

c Tft 5 x *nit1⁻*

Figure 4. Inheritance of pMN24 DNA in genetic backcrosses. DNA was prepared from meiotic progeny of three tetrads or octads from backcrosses of three transformants to a *nit1⁻ pf13⁻* strain. Pvu II fragments hybridizing to pMN24 DNA were analyzed as described in the legend to Fig. 2. Each set of lanes corresponds to one tetrad or octad; + and − refer to the ability to grow on nitrate as sole nitrogen source. Tetrads from backcrosses with: (*a*) transformant 10; (*b*) transformant 1; and (*c*) transformant 5.
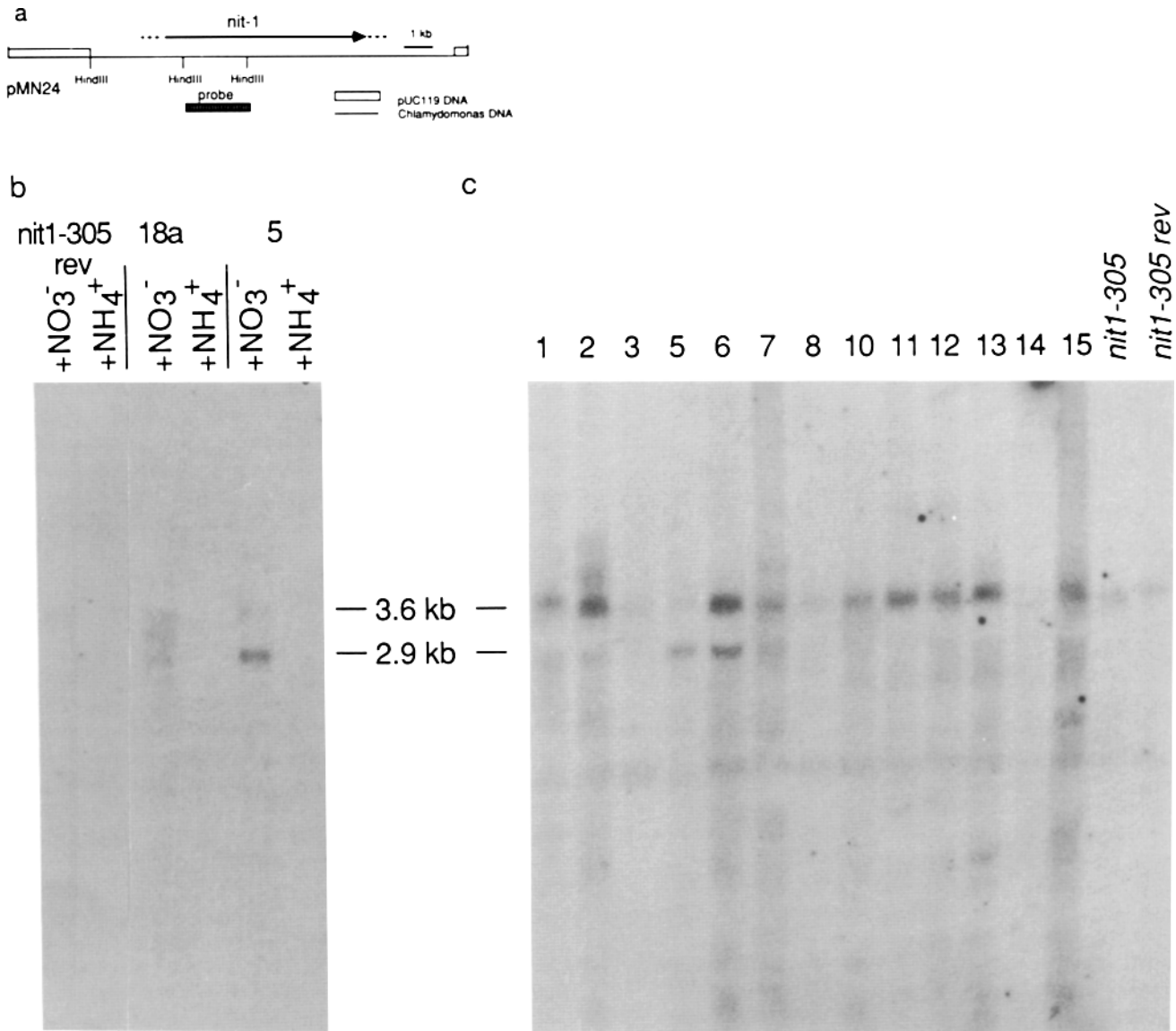
Figure 5. Regulation of *nit1* mRNA in transformed strains. (*a*) Map of pMN24 DNA showing location of Hind III fragment used as hybridization probe. (*b*) Cells were grown in 1/2R medium lacking (−) or containing (+) NH_4^+, and poly(A)$^+$ RNA was prepared as described in Materials and Methods. Approximately 1 µg of poly(A)$^+$ RNA was run on each lane of an agarose/formaldehyde gel, transferred to a nylon filter and hybridized to the gene-specific Hind III fragment. (*c*) Cells were grown in 1/2R medium containing NH_4^+ and switched to nitrate medium for 2 h. Total RNA was prepared, and 20 µg from each sample was size fractionated on an agarose/formaldehyde gel, and hybridized to the gene-specific Hind III fragment. That each lane contained the same amount of RNA was verified by ethidium bromide staining of the gel before transfer (not shown).

cobombardment with a second nonselectable plasmid might result in transformants carrying both the selected and the nonselected DNA.

To test this possibility, we used as a nonselected DNA a *Chlamydomonas* gene for a chlorophyll a/b binding protein of photosystem II (*cabII-1*, one member of the family of *cabII* genes in *Chlamydomonas*). A piece of bacteriophage λ DNA was inserted into the 3′ noncoding region of this gene, so that integrated copies of the transforming *cabII* gene could be easily distinguished from the resident *cabII* genes. Two different plasmids were used: *cab*λ3, with a 500-bp insert, and *cab*λ9, with a 150-bp insert (see Fig. 6 *a*). In cotransformation experiments, supercoiled pMN24 DNA provided the selected marker, and either supercoiled or linearized *cab*λ3

or *cab*λ9 was used as the nonselected DNA. DNA was prepared from 23 nit$^+$ colonies, digested with Pst I, separated on agarose gels, and transferred to nylon membranes for hybridization to a *cab*λ DNA probe. Both untransformed and transformed strains showed hybridization to the resident *cabII-1* gene (fragments labeled A, B, and C of the *cabII-1* gene; see Fig. 6). In addition, numerous fragments that hybridized less intensely were present in all strains; these represent the other members of the *cabII* gene family. Transformants 16b and 16c, which had been bombarded with linearized *cab*λ3, showed enhanced hybridization to fragments A and B and also showed hybridization to a new Pst I fragment that comigrated with the *cab*λ3 fragment labeled C(*cab*λ3). This fragment, which contains the λ insert in

Table III. Ratios of Terminal to Overall Nitrate Reductase Activities

Strain	Mean ratio
Untransformed	
21 gr	2.1
6145c	2.7
rev9	2.3
nit1-305	NA
Transformed	
1	5.3
2	4.6
3	3.9
5	3.3
6	4.6
7	4.2
10	4.1
12	5.1
8	18.6
11	13.3
14	7.4
13	184.5

Mean values for the ratio of terminal to overall NR activity were calculated from in situ activity values in ammonium grown cells that were derepressed by transfer to nitrate medium for 2, 4, 6, and 24 h. NA, not applicable, since overall NR activity was undetectable (0).

cabλ3, is not present in untransformed cells or in cells cotransformed with cabλ9. As shown in Fig. 6 c, fragment C(cabλ3) in transformants 16b and 16c hybridized to labeled λ DNA. Similarly, transformants 17a, 17d, and 18a, which had been cobombarded with linearized cabλ9 DNA, contained DNA fragments homologous to cabλ9, which carries a smaller insert of λ DNA. We have detected integration of the nonselected DNA in at least 50% of nit⁺ transformants, using either linear or supercoiled cabλ3 or cabλ9 DNA.

It appears that the integrated copies of the cabλ3 and cabλ9 genes contain the complete, unrearranged coding region of the cabII gene. Extra copies of all three Pst I fragments that contain the cabII coding region were present in the cotransformants, and almost all of the hybridization of the probe to new cabII fragments was to these fragments. If there had been significant rearrangement of the coding regions of cabλ3 and cabλ9 genes, numerous fragments of altered mobility should have been evident.

We were interested in determining whether the unselected cabλ3 and cabλ9 genes were transcribed. The high level of expression of the resident cabII-1 gene made detection of these transcripts difficult, despite their larger size. Cotransformants were grown in HS minimal medium under a 12-h light/12-h dark illumination schedule, and RNA was prepared at the end of the dark period or after exposure to 6 h of white light to induce cabII gene expression. RNA blots were hybridized to a Pst I-Fsp I DNA fragment, which contains the λ insert and a small amount of flanking cab sequence (shown in Fig. 7 a). The abundant 1.2-kb transcript from the resident cabII-1 gene was seen in all untransformed and transformed strains, and its abundance increased dramatically in cells exposed to light (Fig. 7 b and data not shown). In addition, a transcript of ~1.7 kb was seen in transformant 16c; this transcript was not present in 16b and has never been detected in untransformed cells (Kindle, 1987;

unpublished data; Shepherd et al., 1983). Although this transcript was much less abundant than the cabII-1 transcript, it was present only in the cells that had been exposed to light.

In summary, by adding a second nonselected DNA to the nit1 transformation, we have demonstrated that integration of the second DNA occurs frequently, and that the nonselected gene can be expressed at the transcriptional level.

Discussion

Using the Chlamydomonas gene for nitrate reductase as a selectable marker, we have demonstrated that a nit1 mutant can be transformed to nit⁺ using microprojectile bombardment. Although the frequency of transformation is low, the process is reproducible. Moreover, the transformants grow well under selective and nonselective conditions, and both the nit⁺ phenotype and the extra nit1 DNA are stable in the absence of selection both during mitotic growth and through meiosis. S. P. Mayfield and K. L. Kindle (manuscript submitted for publication) have also reported stable nuclear transformation of a nonphotosynthetic mutant, FUD44 (defective in OEE1, the oxygen evolving enhancer protein) using the Chlamydomonas psbI gene, and Day et al. (1989) have demonstrated nuclear transformation using the Chlamydomonas arg7 gene to complement an arg7 mutation. Thus, nuclear transformation of Chlamydomonas using Chlamydomonas genes as selectable markers is feasible.

It is difficult to estimate the efficiency of transformation by the particle gun on a per cell basis because many of the cells are outside of the target zone. In these experiments, using ~10⁷ cells and 800 ng of DNA, we recovered 1-25 transformants per bombardment. The particle gun has the advantage of introducing a large amount of DNA into those cells which are successfully penetrated, which results in the integration of multiple copies of the transforming DNA.

In the 12 transformants analyzed, the transforming DNA was not linked to the nit1 locus, indicating that integration was due to nonhomologous recombination. Many of the transformants carried multiple copies of the transforming DNA, but the large number of new fragments that hybridized to pMN24 DNA argues against a simple tandem repetition. For the three transformants for which the DNA of tetrad progeny was isolated, most of the new fragments were linked. If the fragments were produced by independent integration events, these events must be limited to specific chromosomal loci for reasons that are not understood. Alternatively, pMN24 DNA may undergo complex rearrangement and concatemer formation before integration, resulting in a complex tandem array of pMN24 DNA which is stable after integration into the genome. It will be interesting to see whether the cabλ and pMN24 DNAs are genetically linked in cotransformants.

The presence of many integrated copies of transforming DNA increases the likelihood of interrupting genes to produce observable phenotypic effects. When two of the transformants were backcrossed to a nit1 mutant, new phenotypic markers for colony morphology were observed; these segregated as Mendelian markers unlinked to the nit⁺ phenotype. We have not yet determined whether these new phenotypic markers are linked to new DNA restriction fragments introduced by transformation.
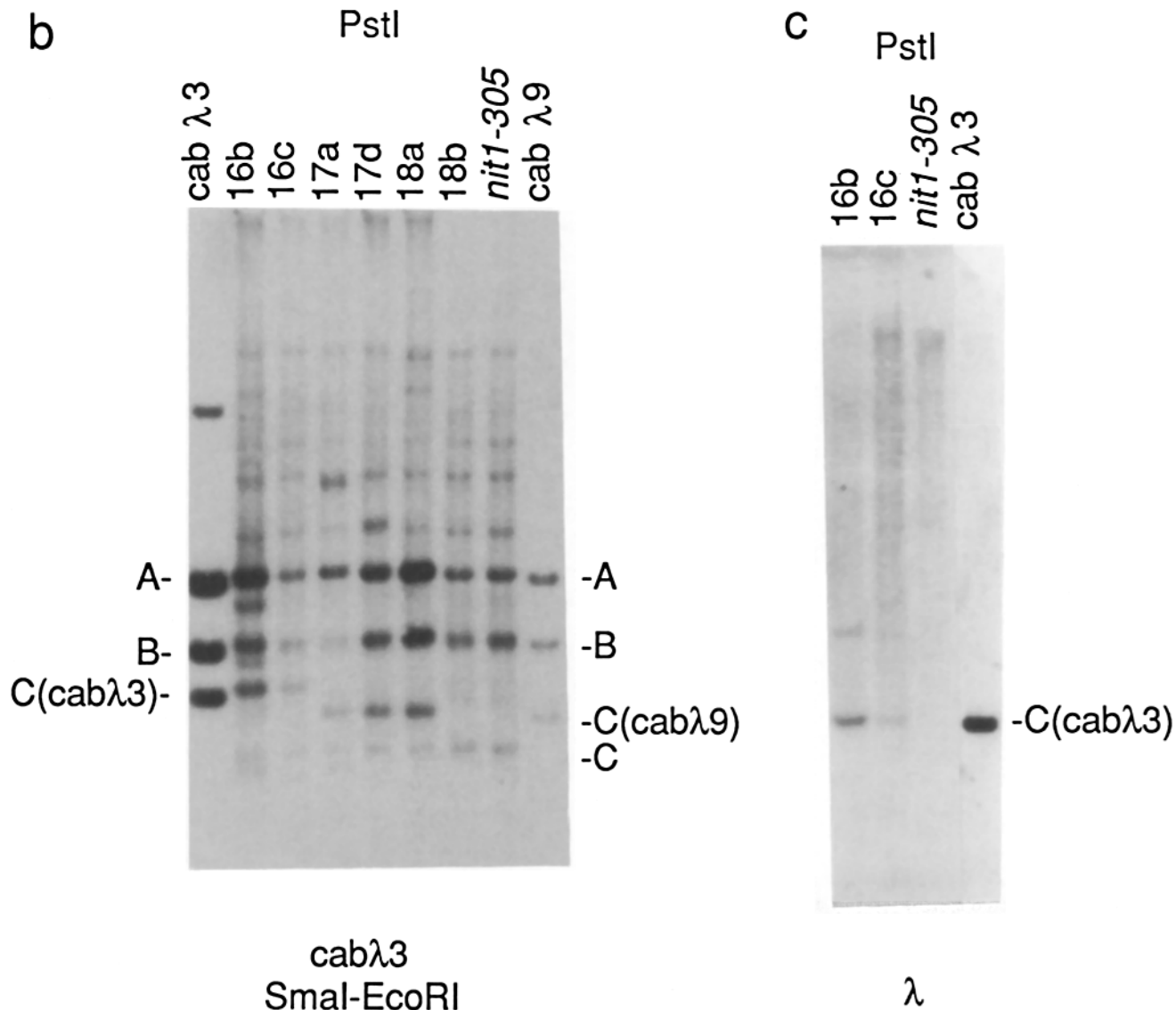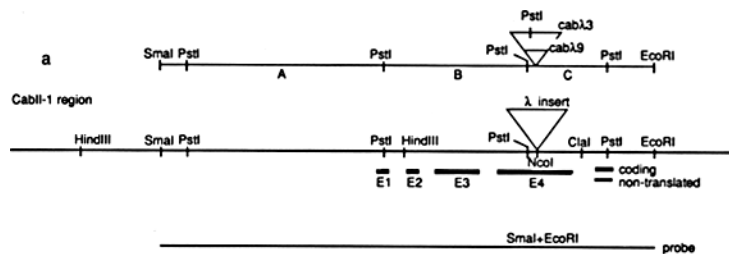
The newly introduced nit1 DNA was expressed at levels

Figure 6. Presence of extra *cabII* gene copies in cotransformants. (*a*) Map of the *cabII-1* region of *Chlamydomonas* DNA (Kindle, K. L., and W.-K. Lee, unpublished data; Imbault et al., 1988). The site of insertion of λ DNA into the unique Nco I site in the noncoding region of exon 4 is shown. The 500-bp insert of *cab* λ3 contains a Pst I site. Pst I fragments A, B, and C are present in the resident *cabII-1* gene of untransformed strains. DNA was prepared from cotransformants and controls and digested with Pst I. Southern blots were probed with either the Sma I + Eco RI fragment of *cabλ3*, which contains the entire gene plus 5' and 3' flanking regions (*b*) or with total λ DNA (*c*). Cotransformant 18b does not contain *cabλ* DNA.

high enough to allow nearly wild-type growth rates in most cases, and in most transformants *nitl* mRNA was expressed at levels as high as wild type. In transformants producing multiple transcripts, the abundance of all transcripts was regulated by ammonium. NR enzyme activity was not over-produced in the transformants, even when the transcripts were present at higher levels than in the *nitl-305* revertant. Some of the transcripts may be nonfunctional or produce a nonfunctional protein, or alternatively, the NR complex could be subject to autoregulatory control as recently reported
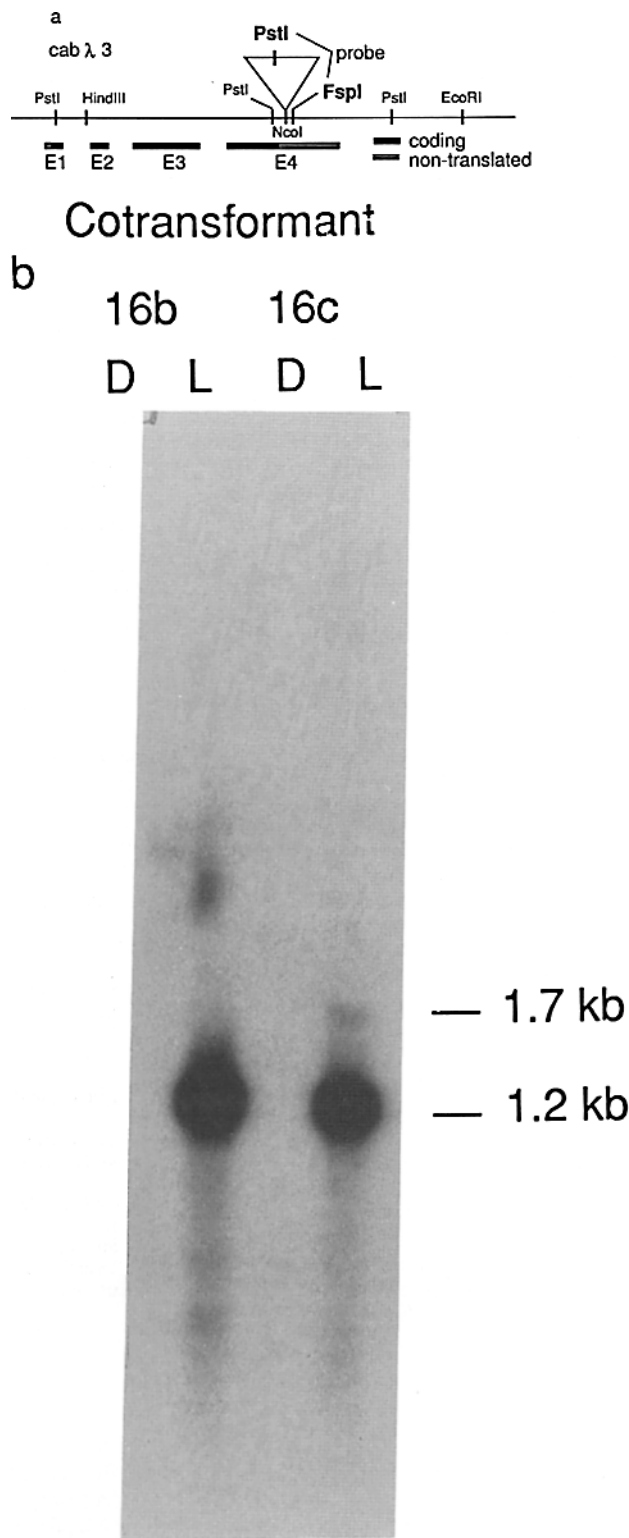
Figure 7. Expression of the *cabλ3* gene in cotransformants. *Chlamydomonas* transformants carrying *cabλ3* DNA were grown in HS minimal medium under a 12-h light/12-h dark light regime. RNA was prepared at the end of the dark period (*D*) or after exposure to 6 h of white light (*L*). Poly(A)+ RNA was isolated, separated on a 1.2% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized to a ~300-bp Pst I–Fsp I fragment that contains both λ and *cabII* sequences. (*a*) Map of *cabλ3*, showing location of probe. (*b*) Hybridization of probe to RNA blot.

(Franco et al., 1987), which would prevent accumulation of NR protein. The elevated ratio of terminal to overall NR activity in the transformants suggests that defective NR subunits with only terminal NR activity continue to be synthesized from the mutant locus. Hybrid NR complexes could be formed by assembly of intact and defective subunits, as was found in in vivo complementation experiments with NR-deficient mutants (Fernández and Matagne, 1986).

Although the introduced *nitl* gene(s) were expressed efficiently, we have so far detected only weak expression of the *cabλ* DNA in a small fraction of cotransformants. Perhaps the efficiency of expression of the integrated DNA depends on the site of insertion; with *nitl* we may only detect integration events in which the DNA is expressed efficiently enough to allow selection of the nit+ phenotype. Alternatively, the *cabλ* clones may not contain all the control elements required for efficient expression, or the λ DNA insert may affect gene expression or mRNA stability. Understanding the factors that determine whether introduced DNA is expressed efficiently could significantly increase the frequency of transformation and the general usefulness of the system.

The *nitl* gene has a number of advantages as a selectable marker for nuclear transformation in *Chlamydomonas*. The *nitl-305* mutation is stable, making the ratio of transformants to revertants high, even though transformation rates are presently low. In addition, the selection for transformation is tight; *nitl-305* cells do not grow on nitrate as sole nitrogen source. The *nitl* gene does not normally synthesize an abundant transcript, and the expression of the *nitl* gene at only 5% of wild-type levels is sufficient to allow growth rates approaching wild-type under selective conditions (Fernández and Matagne, 1986). The need for only low levels of NR expression for growth may explain why the recovery of transformants with *nitl* has been better than with *oeel* using the particle gun (unpublished observations). Finally, a direct selection against the nit+ phenotype is available; nit− cells are resistant to killing by chlorate (Sosa et al., 1978). Selection for expression of one cloned gene and against expression of another has recently been used to develop an elegant selection for integrants occurring via homologous recombination in mice (Mansour et al., 1988). The cloned nitrate reductase gene and the transformation techniques described in this report should be useful in developing such a selection in *Chlamydomonas*.

Recently our laboratories, in collaboration with Joel Rosenbaum's lab (Yale University, New Haven, CT), have been able to complement the *pf14* flagellar mutation in radial spoke protein 3 by cotransforming a *nitl pf14* double mutant with the genes for nitrate reductase and the radial spoke gene 3. Over half of the nit+ transformants generated by bombardment with these two genes also recover motility. This finding dramatically demonstrates the general utility of this contransformation protocol to allow the selection of nit+ colonies likely to be transformed by any unselected gene.

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