Isolation of the Chlamydomonas Regulatory Gene *NIT2* by Transposon Tagging

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ABSTRACT

Genetic evidence suggests that the *NIT2* gene of *Chlamydomonas reinhardtii* encodes a positive regulator of the nitrate-assimilation pathway. To learn more about the function of the *NIT2* gene product, we isolated the gene using a transposon-tagging strategy. A *nit2* mutation caused by the insertion of a transposon was identified by testing spontaneous *nit2* mutants for the presence of new copies of *Gulliver* or *TOC1*, transposable elements that have been identified in Chlamydomonas. In 2 of the 14 different mutants that were analyzed, a *Gulliver* element was found to be genetically and phenotypically associated with the *nit2* mutation. Using the *Gulliver* element as a probe, one of the transposon-induced *nit2* alleles was isolated, and a sequence adjoining the transposon was used to isolate the corresponding wild-type locus. The *NIT2* gene was delimited by mapping DNA rearrangements associated with *nit2* mutations and mutant rescue by genetic transformation. The *NIT2* gene encodes a 6-kb transcript that was not detected in cells grown in the presence of ammonium. Likewise, *NIT2*-dependent genes are repressed in ammonium-grown cells. These results suggest that repression of the *NIT2* gene may mediate metabolite repression of the nitrate assimilation pathway in Chlamydomonas.

THE assimilatory reduction of nitrate to ammonium by plants, algae and some fungi and bacteria is the predominant point of entry of inorganic nitrogen into the biological realm (GUERRERO, VEGA and LOSADA 1981). In this process, nitrate is transported into the cell and is reduced to nitrite and to ammonium by the consecutive action of nitrate and nitrite reductases. In the unicellular green alga *Chlamydomonas reinhardtii*, mutations in seven genes (*NIT1–NIT7*) have been described that block the assimilation of nitrate (AGUILAR *et al.* 1992; FERNÁNDEZ and MATAGNE 1984; NICHOLS and SYRETT 1978; SOSA, ORTEGA and BAREA 1978). These mutations were isolated because they confer resistance to chlorate, an analog of nitrate. It has been suggested that chlorate toxicity in wild-type cells is mediated by nitrate reductase, which converts chlorate to a toxic compound (ÅBERG 1947), although other mechanisms of chlorate toxicity have been proposed (BAEUERLE and HUTTNER 1986; COVE 1976; PRIETO and FERNÁNDEZ 1993). The *NIT1* gene encodes the nitrate reductase apoenzyme and *NIT3* through *NIT7* are required for synthesis of a molybdopterin cofactor that is essential for nitrate reductase activity (AGUILAR *et al.* 1992; FERNÁNDEZ and AGUILAR 1987; FERNÁNDEZ and MATAGNE 1984; FERNÁNDEZ *et al.* 1989).

The *NIT2* gene is proposed to encode a positive regulator of the nitrate assimilation pathway (FERNÁNDEZ and MATAGNE 1986; FERNÁNDEZ *et al.* 1989). In *nit2* mutants, nitrate reductase, nitrite reductase, nitrate permease, and nitrite uptake activities are undetectable or greatly reduced compared to their wild-type levels (FERNÁNDEZ and MATAGNE 1984; GALVÁN, CÁRDENAS and FERNÁNDEZ 1992; NICHOLS, SHEHATA and SYRETT 1978; E. FERNÁNDEZ, personal communication). Comparison of *NIT1* transcript levels in wild-type and *nit2* mutant strains confirmed that the wild-type *NIT2* gene is required for accumulation of the *NIT1* transcript (FERNÁNDEZ *et al.* 1989). To our knowledge, *NIT2* is the first regulatory gene involved in nitrate assimilation to be identified in a photosynthetic eukaryote. Progress toward identifying the product of the *NIT2* gene and understanding its regulatory role is contingent upon isolation of the gene.

Transposable elements have provided an invaluable means for isolating genes whose products are unknown (BINGHAM, LEVIS and RUBIN 1981; MOERMAN, BENIAN and WATERSTON 1986; OKKEMA and KIMBLE 1991; SCHMIDT, BURR and BURR 1987; TOMLINSON, KIMMEL and RUBIN 1988). Cloning a gene by transposon tagging requires an active transposable element for which a molecular probe is available, and a readily identifiable mutation caused by insertion of the transposable element into or near the gene of interest. In Chlamydomonas, two transposable elements have been identified. The *TOC1* element has an unusual long terminal direct repeat structure at its ends and resembles RNA-mediated transposons (DAY and ROCHAIX 1991a,b; DAY *et al.* 1988). The *Gulliver* element has a short inverted repeat at its ends and is

flanked by an 8-bp target site duplication, hallmarks of DNA-mediated transposons (FERRIS 1989).

If members of the transposable element families described in Chlamydomonas transpose during mitotic growth, and if integration occurs randomly throughout the genome, then it should be possible to recover mutations caused by insertion of a transposon into a particular gene. The *NIT2* gene is well-suited for testing the feasibility of transposon tagging in Chlamydomonas because loss-of-function *nit2* mutations can be selected on medium containing chlorate and wild-type revertants can be selected on medium containing nitrate as the sole nitrogen source.

In this study a transposon-induced *nit2* mutation was identified by analyzing spontaneous *nit2* mutant strains for the presence of additional or rearranged copies of transposable elements. A *Gulliver* element associated with a *nit2* mutation was isolated, and sequences contiguous to the transposon were used to clone the wild-type *NIT2* gene. Analysis of transcript abundance revealed that expression of the *NIT2* gene was repressed in medium containing ammonium. In light of these observations, working models for the role of *NIT2* in the regulation of nitrate assimilation are discussed.

MATERIALS AND METHODS

Strains: Chlamydomonas strains used in this study are listed in Table 1. Mutant alleles *nit1-305* and *nit2-203* were obtained from strains 305 and 203, which were provided by E. FERNÁNDEZ (University of Cordoba, Cordoba, Spain; SOSA, ORTEGA and BAREA 1978). Mutant alleles *nit1-137* and *nit2-137* arose spontaneously in the Ebersold-Levine line 137c (FERNÁNDEZ and MATAGNE 1984). Strains A54 and A55 were progeny from a cross between strain 305 (*nit1-305*, *mt*$^-$) and strain C6$^+$ (*ac17*, *sr1*, *mt*$^+$). The *supcs1-2* mutation confers a cold-sensitive growth phenotype and was originally isolated as an extragenic suppressor of *ory1-1* (Rcs2) (JAMES *et al.* 1989). Strains C6$^+$ and T897-B2 (*supcs1-2 mt*$^-$) were provided by S. JAMES (Gettysburg College, Gettysburg, Pennsylvania). Strains D66 and D67 were obtained from the fifth consecutive cross in which meiotic progeny containing the cell wall mutation *cw15* (DAVIES and PLASKITT 1971; HYAMS and DAVIES 1972), were successively backcrossed to strains A33, A53, 305, B27, B26 and A35.

Culture conditions, media and genetic analysis: TAP medium (GORMAN and LEVINE 1965) contained 20 mM Tris (Sigma7-9), 7.5 mM NH_4Cl, 0.01% $Mg_2SO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$, 0.62 mM K_2HPO_4, 0.41 mM KH_2PO_4, 1 ml Hutner trace elements (SURZYCKI 1971), and 1 ml glacial acetic acid per liter. TAP-N was identical to TAP except that NH_4Cl was omitted. M medium (minimal medium I) (SAGER and GRANICK 1953) was prepared according to HARRIS (1989) except that the trace metal concentration was reduced fourfold and the phosphate composition was changed to 0.53 mM K_2HPO_4 and 0.51 mM KH_2PO_4. R medium was the same as M except that it also contained 13 mM sodium acetate and the phosphate concentration was increased to 1.5 mM K_2HPO_4 and 1.5 mM KH_2PO_4. SGII medium (SAGER and GRANICK 1953) was the same as M except that it also contained 14.7 mM sodium acetate and the phosphate was changed to 6.6 mM K_2HPO_4 and 26.6 mM NaH_2PO_4.

Where appropriate, NH_4NO_3 (5 mM) was replaced by KNO_3 (4 mM), KNO_2 (2 mM), or urea (2 mM). Filter-sterilized solutions of KNO_2, urea and potassium chlorate were added to media after autoclaving. Solid media contained 1% agar that had been washed extensively with distilled water. Chlamydomonas strains were grown at 25° under continuous light, except that strains containing *supcs1-2* were grown at 33° and maintained at 25°.

Standard methods were used for tetrad analysis (LEVINE and EBERSOLD 1960). For complementation testing, Nit$^-$ mutant strains were mated with *nit2* and *nit1* tester strains (B20, B21, B14, B15) and stable diploids were selected using complementing mutations (EBERSOLD 1967). For this selection, cold-sensitive *supcs1-2* strains were mated with acetate-requiring *ac17* strains and the mating mixtures were plated onto solid medium without acetate (M) and incubated at the restrictive temperature for *supcs1-2* (15°) for 10 days. To assay complementation, at least three colony-purified diploid isolates were spotted onto medium containing nitrate as the sole nitrogen source. Growth was scored after 3 days at 25°. Noncomplementing mutations should result in diploid strains that are unable to grow on nitrate.

Chlamydomonas cells were transformed with plasmid DNA using a previously described method (KINDLE 1990). Briefly, cells containing *nit2-203* and *cw15* were grown in SGII medium and cells ($2–5 \times 10^7$) were vigorously mixed with supercoiled plasmid DNA (5–10 μg), glass beads (1-mm diameter), and polyethylene glycol (M_r 8,000) using a Vortex mixer (Scientific Products) at top speed for 45 sec. Plasmids used for transformation were obtained by subcloning restriction fragments from λ47-7 (Fig. 5) into pUC118 or pUC119 (VIERA and MESSING 1987): pMN60 contains the 6.2-kb *Hin*dIII fragment, pMN68 contains the 12-kb *Sst*I fragment and pMN72 contains the 10.5-kb *Sal*I fragment.

Spontaneous, chlorate-resistant mutants were obtained by plating cells from 16 independent cultures on solid R medium containing urea as the sole nitrogen source and either 2.5 or 5 mM potassium chlorate. Chlorate-resistant colonies arose at frequencies of 0.8×10^{-5} to 5.6×10^{-5} per cell. Mutants were named according to the strain and culture from which they were isolated. For example, mutant A55-c9 is the ninth mutant obtained from culture "c" of strain A55.

Revertibility of Nit$^-$ mutations was assayed qualitatively by plating approximately 10^6 cells (100 μl of a 200-μl culture), grown nonselectively from a single colony, onto medium containing nitrate as the sole nitrogen source. Reversion frequencies from 10^{-3} to 10^{-6} revertants per cell were detected by this method. The rates of reversion of *nit2-1* and *nit2-2* were measured using the method of the median (JAMES *et al.* 1989; LEA and COULSON 1948). For this assay, cultures were grown from individual whole colonies in TAP medium at 25° under constant light. In strain A55-c9 (*nit2-1*) and A55-a5 (*nit2-2*), Nit$^+$ revertants arose at a rate of 4.4×10^{-7} and 5.0×10^{-5} reversions per cell division, respectively.

To determine whether *nit2* mutants contained *TOC1* dimorphisms, genomic DNA (3.5 μg) from each mutant was digested with *Sst*I or with *Pvu*II, fractionated through 0.8% agarose, transferred to a nylon membrane, and hybridized with the radiolabeled TOC1.1R1 plasmid (DAY *et al.* 1988). To test mutants for *Gulliver* dimorphisms, genomic DNA from each mutant was digested with *Hin*dIII, transferred to a nylon membrane and hybridized with the λB3 clone (FERRIS 1989).

Isolation of nucleic acids from Chlamydomonas: Chlamydomonas DNA was purified using a procedure adapted from WEEKS, BEERMAN and GRIFFITH (1986). Sta-

tionary phase cells grown in 250 ml of TAP medium bubbled with filtered air, were pelleted by centrifugation and resuspended in water to a volume of 2 ml. Cells were lysed by the addition of 0.35 ml 20% SDS, followed immediately by the addition of 0.5 ml Extraction Buffer (0.138 M EDTA, 1.38 M NaCl, and 0.345 M Tris-HCl, pH 8.0), and incubated for 30–60 min at 42°. The cell lysate was mixed with solid CsCl (3.1 g), loaded into a quick-seal tube (Beckman No. 358980) containing 0.15 ml ethidium bromide (10 mg/ml), and centrifuged at 90,000 rpm for at least 4 hr (Beckman TLN100 rotor). The banded DNA was collected using a syringe adapted with an 18-gauge needle and ethidium bromide removed by extraction using isopropanol saturated with water and sodium chloride. The DNA was precipitated by addition of one volume of water and one volume of isopropanol. The precipitated DNA was washed once with 70% ethanol, dried and resuspended in TE for at least 2 days at 4°.

Chlamydomonas RNA was isolated from cells grown to a density of 1.5–3.5 × 10^6 cells per ml in 1.5 liters TAP medium. Cultures were bubbled continuously with air supplemented with 2–5% CO_2. For derepression, cells were washed three times with TAP-N (without ammonium), resuspended in 1 liter TAP-N and bubbled with air for 45 min. Approximately 90 min elapsed between the first wash with TAP-N and the harvesting of the cells following derepression. Total RNA was isolated by phenol-chloroform extraction and centrifugation through cesium chloride as described previously (SCHLOSS, SILFLOW and ROSENBAUM 1984). Poly(A)$^+$ RNA was selected by batchwise adsorption to oligo(dT) cellulose (Collaborative Research) and size-fractionated through agarose in the presence of formaldehyde (MANIATIS, FRITSCH and SAMBROOK 1982).

Nucleic acid hybridizations: DNA fragments to be used as hybridization probes were purified from low-melting-temperature agarose (SeaPlaque, FMC) by phenol extraction and ethanol precipitation (MANIATIS, FRITSCH and SAMBROOK 1982). Probes were radiolabeled by nick-translation (RIGBY *et al.* 1977). Conditions for transfer of nucleic acids from agarose gels to Nytran membranes and for hybridization were as described by the manufacturer (Schleicher & Schuell, 1987).

Library construction and screening: To generate a wild-type Chlamydomonas DNA library, DNA from strain 21gr *mt*$^+$ was isolated as described above except that, following gradient centrifugation, CsCl was removed by dialysis against TEN (5 mM Tris-HCl, 2.5 mM EDTA, 20 mM NaCl, pH 7.6) rather than by precipitation in ethanol. The resulting DNA was partially digested with *Mbo*I, and the vector DNA (λFIXII; Stratagene) was digested with *Xho*I, and both were partially filled in using the Klenow fragment of *Escherichia coli* DNA polymerase I to generate compatible overhangs and to prevent the formation of multiple inserts during ligation. The ligated mixture was packaged *in vitro* (GigapakIIXL; Stratagene) and plated on *E. coli* strain P2392. A library containing 0.5 × 10^6 recombinant phage was amplified by plating on *E. coli* strain LE392. Using a single-copy sequence as a probe (probe 2, Figure 4) positively hybridizing phage were detected at a frequency of 2 × 10^{-4} per phage and the insert lengths averaged 18 kb (Figure 5). Standard methods were used for plating phage and for screening by hybridization (MANIATIS, FRITSCH and SAMBROOK 1982) and for isolation of λ DNA (CHISHOLM 1989).

To isolate the *Gulliver* element associated with the *nit2-1* mutation, genomic DNA from strain A55-c9 was digested with *Hin*dIII and size-fractionated by electrophoresis through a low-melting-temperature agarose gel. DNA from the fraction containing the desired fragment was ligated into the plasmid vector pUC119 and transformed into DH5α (Bethesda Research Laboratories). Plasmids containing *Gulliver* sequences were identified by colony hybridization (MANIATIS, FRITSCH and SAMBROOK 1982).

TABLE 1

***Chlamydomonas reinhardtii* strains**

Strain	Genotype	Alias[a]
A35	*nit2-203 mt*$^+$	
A54	*ac17 sr1 mt*$^+$	
A55	*ac17 sr1 mt*$^-$	
A55-c9	*nit2-1 ac17 sr1 mt*$^-$	(CC-2841)
A55-a5	*nit2-2 ac17 sr1 mt*$^-$	(CC-2842)
A55-d8	*nit2-3 ac17 sr1 mt*$^-$	
A54-a2	*nit2-4 ac17 sr1 mt*$^+$	
B14	*supcs1-2 nit1-137 mt*$^+$	
B15	*supcs1-2 nit1-137 mt*$^-$	
B20	*supcs1-2 nit2-137 mt*$^+$	
B21	*supcs1-2 nit2-137 mt*$^-$	
B26	*sr1 mt*$^+$	
B27	*sr1 mt*$^-$	
B52	*nit2-2 sr1 mt*$^+$	
D66	*nit2-203 cw15 mt*$^-$	
D67	*nit2-203 cw15 mt*$^+$	
S1-D2	*mt*$^-$	(CC-2290)

[a] Strains available from the Chlamydomonas Genetics Center.

RESULTS

Isolation of mutants: Spontaneous, chlorate-resistant mutants were selected from Nit$^+$ strains A54 and A55 (Table 1) and assayed for their ability to grow on nitrate as a sole nitrogen source. Most of the chlorate-resistant mutants (335 out of 384) were unable to utilize nitrate (Nit$^-$). To identify putative *nit2* alleles, 122 Nit$^-$ mutants were analyzed for their ability to complement a known *nit2* mutation in diploids (see MATERIALS AND METHODS). When mated with a *nit2* strain (B20 or B21), approximately one-third of the mutants (42 out of 122) gave rise to Nit$^-$diploids. When mated with a *nit1* strain (B14 or B15), all but two of these 42 mutants gave rise to Nit$^+$ diploids. Genetic mapping confirmed that one of the mutants that failed to complement both a *nit1* and a *nit2* mutation (A54-f24) contained mutations in both genes (data not shown). Only mutations that failed to complement the *nit2* mutation, and fully complemented the *nit1* mutation were retained for further analysis. FERNÁNDEZ *et al.* (1989) observed that a strain containing a *nit2* mutation grew poorly on nitrite. To determine the generality of this phenotype among *nit2* alleles, we tested the ability of each of the Nit$^-$ mutants to grow on medium containing nitrite as the sole nitrogen source. Each of the 40 mutants assigned to the *nit2* complementation group grew slowly on nitrite compared to wild-type strains. Thus, the "nitrite minus" phenotype would be a useful test for identifying potential *nit2* mutants among large numbers of chlorate-resistant strains.

Mutability, or phenotypic instability has been observed in association with transposable element-in-

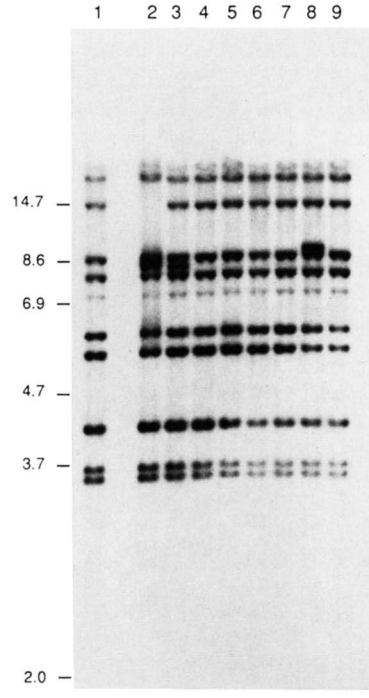

FIGURE 1.—Hybridization analysis of *Gulliver* elements in *nit2* mutant strains. DNA from wild type (lane 1) and isogenic *nit2* mutant strains (lanes 2–9) was digested with *Hin*dIII, fractionated through 0.8% agarose, transferred to a nylon membrane and hybridized to a fragment corresponding to one end of the *Gulliver* transposon (Figure 4A, probe 1). Lanes contain DNA (3.5 μg) from strain A55 (lane 1), A55-c9 (lane 2), A55-a5 (lane 3), A55-c8 (lane 4), A55-e12 (lane 5), A55-h1 (lane 6), A55-h9 (lane 7), A55-d8 (lane 8) and A55-a14 (lane 9). DNA size markers are indicated in kilobase pairs (kb) throughout.

duced mutations in a number of organisms (SHAPIRO 1983). To determine whether the *nit2* mutations isolated in this study were unstable, multiple cultures of each mutant strain were grown from single cells without selection and spotted onto nitrate medium. More than half of the *nit2* mutant strains (24 out of 40) gave rise to Nit^+ colonies. Thirteen of these unstable mutants and one nonreverting mutant (A55-d8) were selected for further analysis.

***Gulliver* elements associated with *nit2* mutations:** To determine whether a *Gulliver* element was associated with any of the *nit2* mutations, DNA from 14 different or independently isolated *nit2* mutant strains was analyzed for the presence of a *Gulliver* dimorphism. For this experiment, genomic DNA was cleaved with *Hin*dIII, fractionated through agarose, blotted, and hybridized to a radiolabeled DNA fragment containing one end of a *Gulliver* element. Each fragment detected by the *Gulliver* end-specific probe corresponds to a single *Gulliver* element (FERRIS 1989). Compared with the isogenic wild-type strain (A55), three of the *nit2* mutant strains contained a new *Gulliver* band (A55-c9, A55-a5 and A55-d8) and in one of these strains (A55-c9) a band was also lost (Figure 1, lanes 2, 3 and 8). The appearance of a new *Gulliver* band in these three strains might reflect transposition of a *Gulliver* element into the *NIT2* locus. Alternatively, the new band might have arisen by a transposition or recombination event independent of the *nit2* mutation. To distinguish between these alternatives, we tested whether the new *Gulliver* element in each of these strains was genetically linked with the *nit2* mutation. Each of the mutant strains (A55-c9, A55-a5 and A55-d8) was mated with a wild-type strain (B26) and meiotic progeny were isolated. In more than 20 tetrads from each cross, the Nit^- phenotype segregated as a single nuclear mutation and was linked to the *ac17* locus on linkage group III (less than 15 cM), as predicted for mutations at the *NIT2* locus (HARRIS 1989). In all three tetrads analyzed by hybridization from the first cross, the new *Gulliver* band in A55-c9 cosegregated with the Nit^- phenotype (Figure 2A). For the second cross, a *nit2-2* mt^+ strain (B52), obtained by crossing A55-a5 and a wild-type strain (B26), was mated with a wild-type field isolate (S1-D2) which is devoid of *Gulliver* elements. (FERRIS 1989; GROSS, RANUM and LEFEBVRE 1988). In all four tetrads analyzed from this cross, the new *Gulliver* element cosegregated with the Nit^- phenotype (Figure 2B). In contrast, in the third cross, between mutant A55-d8 and a wild-type strain (B26), the new *Gulliver* band segregated independently from the *nit2* mutation (1 PD and 2 NPD tetrads; data not shown). These results indicated that the Gulliver dimorphisms in the first two strains (A55-c9 and A55-a5) were linked to the *nit2* mutations in those strains and that the dimorphism in the third strain (A55-d8) was not associated with the *nit2* mutation in that strain.

To determine whether the *nit2* mutations in strains A55-a5 and A55-c9 were caused by the insertion of *Gulliver* into the *NIT2* locus, or whether the new *Gulliver* elements in these strains were simply linked to the *nit2* mutations, Nit^+ revertants were isolated. For each of the *nit2* mutants, genomic DNA from each of three independent revertant strains was examined for the presence of the *Gulliver* dimorphism (Figure 3). In each case, loss of the dimorphic *Gulliver* band accompanied reversion to the wild-type phenotype. These results confirmed that the *nit2* alleles in strains A55-c9 and A55-a5 were caused by the insertion of a *Gulliver* element into or near the *NIT2* gene.

Cloning of a *nit2*-associated transposon and the wild-type *NIT2* locus: To isolate sequences flanking a *nit2*-associated transposable element, genomic DNA from mutant strain A55-c9 was digested with *Hin*dIII and fractionated on an agarose gel. DNA fragments from the region of the gel containing the 8-kb dimorphic *Gulliver* fragment were purified, ligated into a plasmid vector, and transformed into *E. coli*. Among 6,000 colonies that were screened, nine hybridized to the *Gulliver* probe. To identify plasmids that contained inserts of the appropriate size, plasmid DNA from each of the positive bacterial clones and genomic

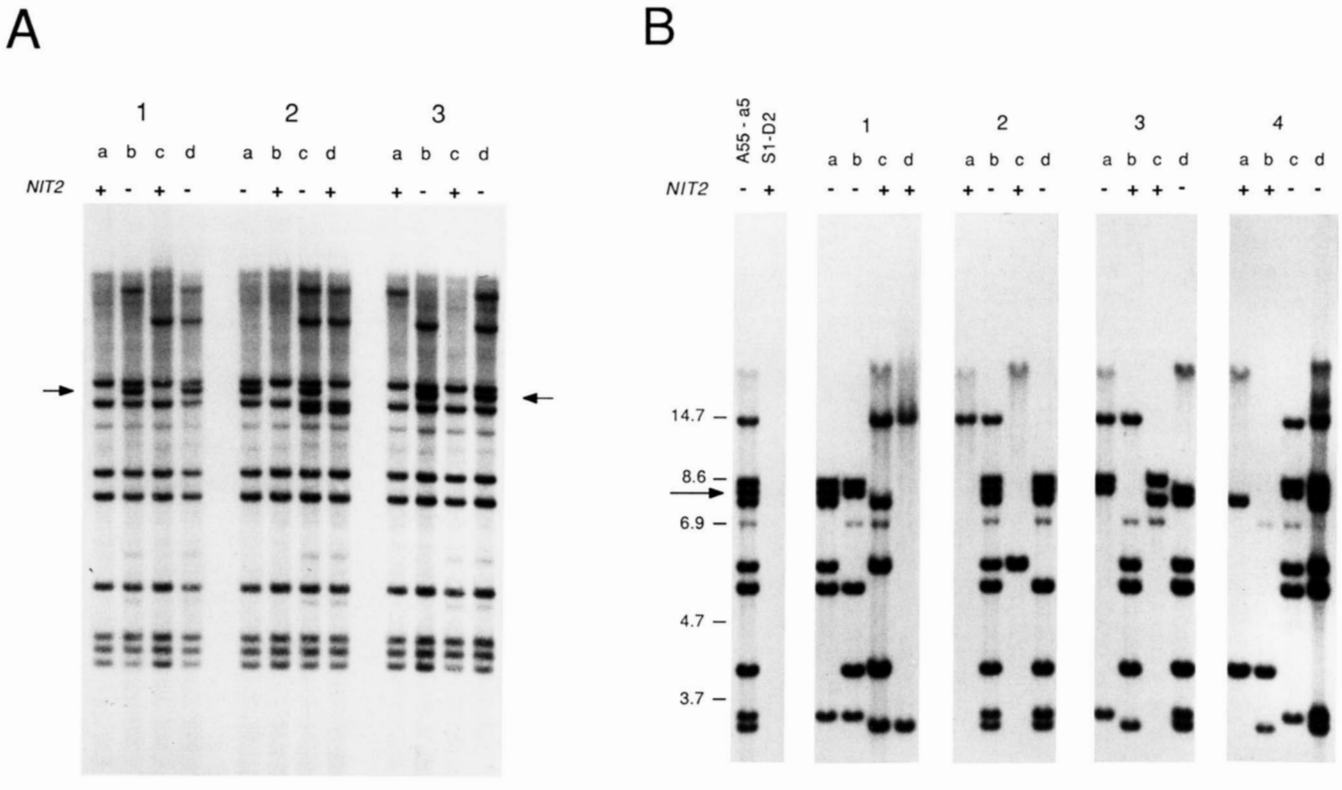

FIGURE 2.—Hybridization analysis of tetrad progeny. (A) Cosegregation of a *Gulliver* element with *nit2-1*. Progeny from tetrads (labeled 1–3) were obtained by crossing a *nit2-1 mt*$^-$ strain (A55-c9) with a wild-type *mt*$^+$ strain (B26). DNA samples (3.5 μg) were digested with *Hin*dIII, fractionated through 0.8% agarose, and hybridized with λB3, a clone containing one end of a *Gulliver* element (FERRIS 1989). The *NIT2* phenotype, listed above each lane, was determined by testing growth on medium containing nitrate as the sole nitrogen source. The arrows show the position of the relevant *Gulliver* band. (B) Cosegregation of a *Gulliver* element with *nit2-2*. Tetrads were obtained by crossing a *nit2-2 mt*$^+$ strain (B52) with a wild-type *mt*$^-$strain (S1-D2). The progeny were analyzed as in panel A except that a purified fragment was used as the *Gulliver* probe (probe 1, Figure 4).

DNA from strain A55-c9 were digested with *Hin*dIII, size-fractionated and hybridized with the *Gulliver* probe. Inserts from six of the plasmids comigrated with the 8-kb dimorphic band in A55-c9. A representative plasmid from this set, designated pMN47, was characterized further (Figure 4).

To determine the relative position of *Gulliver* and flanking sequences within the pMN47 insert, the cloned region was subdivided into smaller fragments which were used as probes for genomic hybridization analysis. The rightward probe (probe 1, Figure 4) hybridized exclusively to *Gulliver* elements (Figure 1), whereas the leftward probe (probe 2, Figure 4) hybridized to a 6.2-kb *Hin*dIII fragment in the wild-type strain (A55) and an 8-kb *Hin*dIII fragment in the *nit2–1* strain. In tetrads from a cross between the two strains, the 6.2-kb *Hin*dIII fragment cosegregated with the Nit$^+$ phenotype (Figure 4B). These results suggested that the leftward fragment from pMN47 hybridized to the *NIT2* locus. To isolate the complete *NIT2* gene, the leftward fragment was used to screen a phage λ library containing DNA from a wild-type Chlamydomonas strain. Restriction analysis of DNA from eight of the positively hybridizing phage indicated that the inserts were overlapping and spanned a 27-kb region (Figure 5).

The cloned sequence encoded a functional *NIT2* gene product: To test whether the cloned fragments were able to complement a *nit2* mutation, restriction fragments from the phage inserts were subcloned into a plasmid vector and introduced into Chlamydomonas cells by transformation. For this experiment, three plasmids were introduced into a *nit2–203, cw15* strain (D66 or D67) by vortexing cells and plasmid DNA in the presence of glass beads and polyethylene glycol (KINDLE 1990). The treated cells were spread onto medium containing nitrate as the sole nitrogen source and the number of Nit$^+$ colonies was scored after 10 days (Figure 6A). In four control transformations, in which plasmid DNA was omitted, only one Nit$^+$ colony was observed. When plasmid pMN60 or pMN68 was added, the total number of colonies increased to 37 and 105, respectively. Addition of plasmid pMN72 resulted in five colonies.

Since nuclear transformation in Chlamydomonas occurs almost exclusively by integration of the transforming DNA into the genome by nonhomologous recombination (DIENER *et al.* 1990; KINDLE *et al.* 1989; MITCHELL and KANG 1991), a transformant should contain at least one extra copy of the introduced gene. Putative transformants were tested for

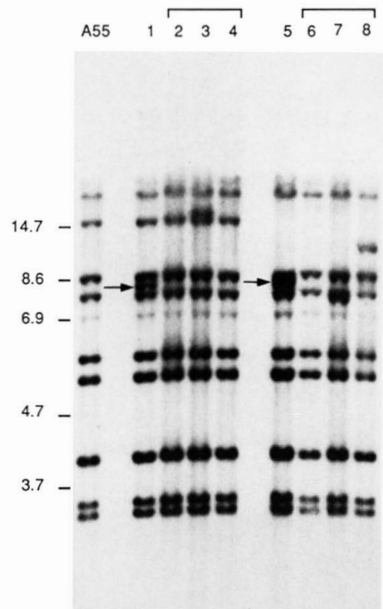

FIGURE 3.—Hybridization analysis of Nit$^+$ revertant strains. Genomic DNA from *nit2* mutants and revertants was digested with *Hin*dIII and hybridized to a *Gulliver* probe as described in the legend for Figure 1. Lanes contain DNA from *nit2* strains A55-a5 (lane 1) and A55-c9 (lane 5) and from independently isolated Nit$^+$ revertants obtained from A55-a5 (lanes 2–4) and from A55-c9 (lanes 6–8). The arrows indicate the positions of the polymorphic *Hin*dIII fragments associated with the Nit$^-$ phenotype of the *nit2* mutant strains.

the presence of extra gene copies by digesting genomic DNA with *Sst*I and using a fragment from within the transforming DNA as a probe. Transformants obtained with pMN68 or pMN60 contained one or more new bands compared with the untransformed parent strain (Figure 6B, lanes 1–12). In contrast, putative transformants obtained using pMN72 contained a single band and were indistinguishable from the untransformed strain, suggesting that these colonies arose by reversion of the *nit2-203* mutation rather than by transformation with pMN72 (Figure 6B, lanes 13–15).

If the introduced gene rescues the *nit2* mutation, then the Nit$^+$ phenotype should segregate with the extra gene copy during meiosis. To test this prediction, a pMN68-transformed strain (Figure 6B, lane 2) was mated with a *nit2* mutant strain (A35). In all four tetrads examined, the extra gene copy segregated with the Nit$^+$ phenotype (Figure 6C). Together, these results indicate that plasmids pMN68 and pMN60, but not pMN72, were able to rescue the *nit2-203* mutation.

Physical mapping of *nit2* mutations: Transformation experiments showed that a 6.2-kb *Hin*dIII fragment was sufficient to complement a *nit2* mutation. A reciprocal approach, to identify functionally important sequences, is to map mutations that disrupt a gene's function. For this experiment, DNA from 14 spontaneous *nit2* mutant strains was digested with *Hin*dIII and hybridized with a fragment from the

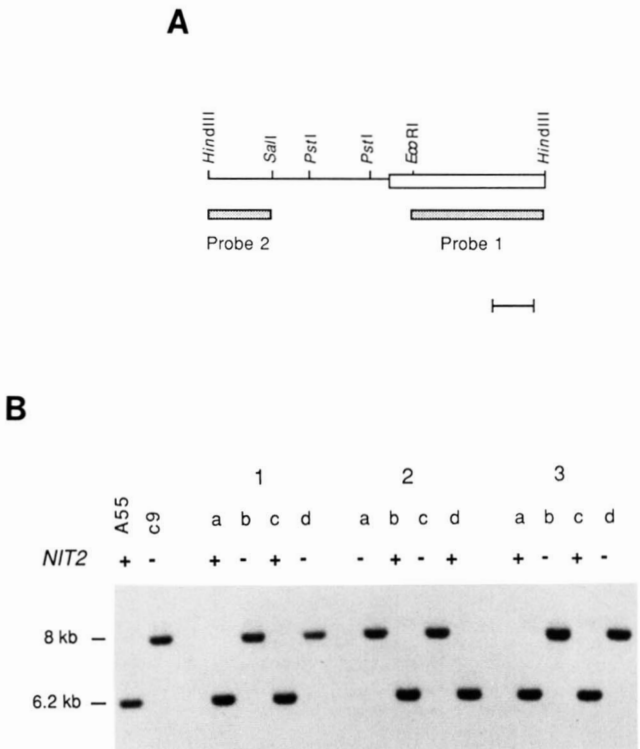

FIGURE 4.—The 8-kb transposon-tagged *Hin*dIII fragment from A55-c9. (A) Restriction map of the pMN47 insert. The shaded areas indicate fragments that were used for hybridization probes (probe 1 and 2). The unshaded rectangle represents the portion of the insert corresponding to the transposon. (B) The filter shown in Figure 2A was stripped and rehybridized with the 1.5-kb *Hin*dIII-*Sal*I fragment from pMN47 (panel A, probe 2).

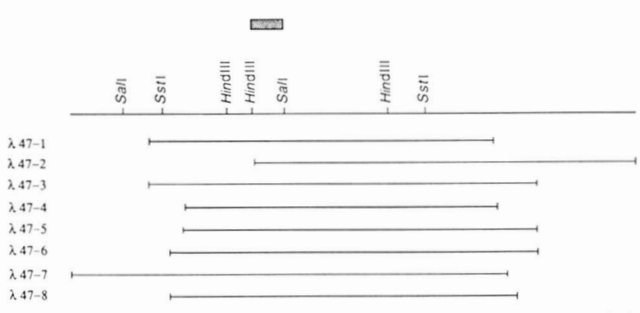

FIGURE 5.—Restriction map of wild-type *NIT2* genomic clones. Lines beneath the map represent lambda inserts. The rectangle corresponds to probe 2 in Figure 4. The scale bar represents 1 kb.

cloned *NIT2* gene (probe B, Figure 7A). In the wild-type strain and in four of the mutant strains, the probe hybridized to a 6.2-kb fragment. However, in 10 of the mutant strains, the probe hybridized to fragments of various lengths (Figure 7B and data not shown). Therefore, 10 of the 14 mutant strains contained a restriction fragment length polymorphism within the 6.2-kb *Hin*dIII region. The location of each of the 10 polymorphisms was determined more precisely by digesting genomic DNA with both *Hin*dIII and *Sal*I, or with *Hin*dIII and *Kpn*I, and hybridizing filters with subfragments from the cloned region (probes A through E were used to detect polymorphisms within regions a through e, respectively; Figure 7A). A

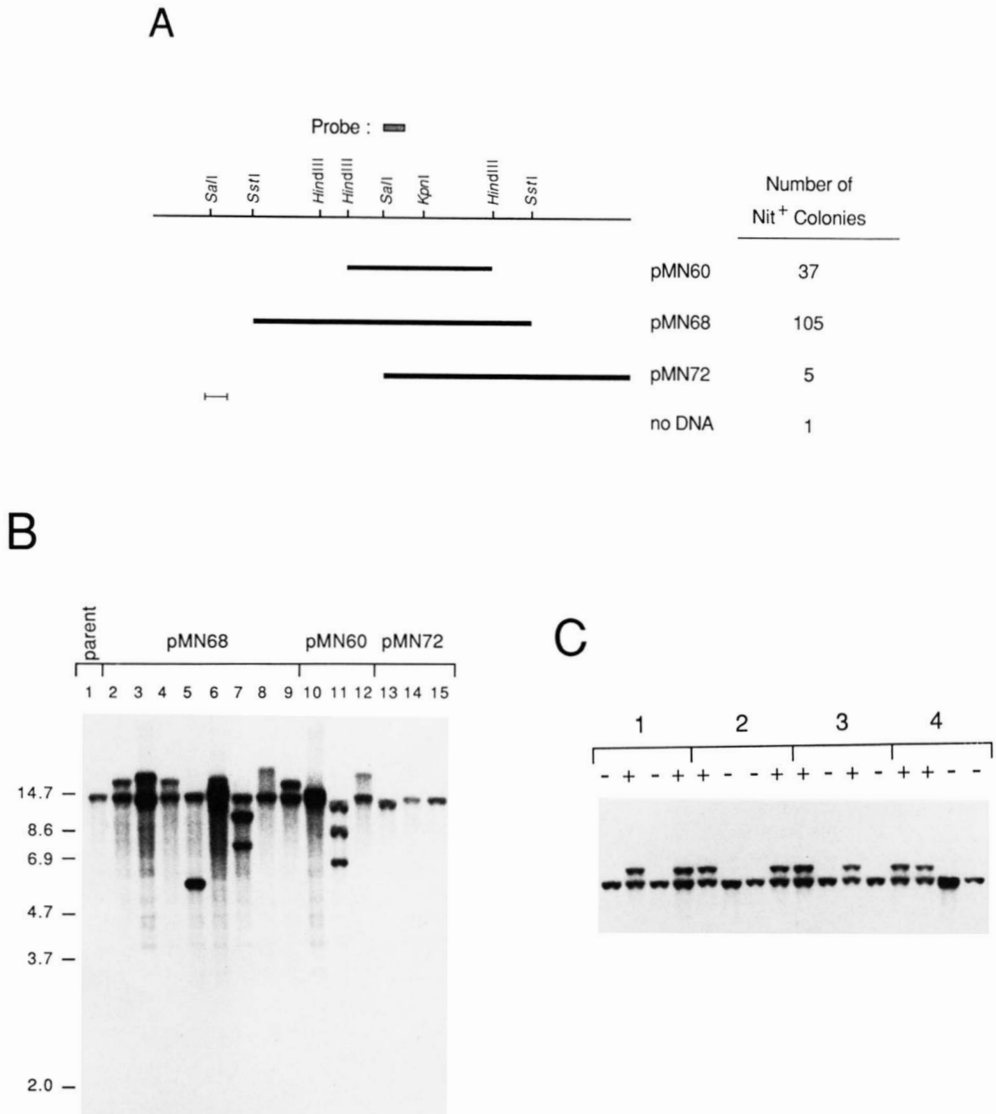

FIGURE 6.—The cloned wild-type *NIT2* sequence rescues a *nit2* mutation. (A) Mutant rescue by genetic transformation. Regions contained in plasmid subclones are indicated by lines beneath the restriction map. The scale bar represents 1 kb. The number of Nit$^+$ colonies refers to the total number obtained in four trials. The line above the restriction map shows the 0.9-kb *Sal*I-*Pst*I fragment used as a probe in panels B and C. (B) Hybridization analysis of transformants. Genomic DNA (2 μg) from the untransformed strain D66 (lane 1) or from putative transformants obtained with plasmid pMN68 (lanes 2–9), pMN60 (lanes 10–12) or pMN72 (lanes 13–15), was digested with *Sst*I, fractionated through 0.8% agarose, transferred to a nylon membrane and hybridized with the probe indicated in panel A. (C) Cosegregation of a transgene and the Nit$^+$ phenotype. Tetrads were obtained by crossing the pMN68-transformant shown in panel B, lane 2, and a *nit2-203* strain (A35). The progeny from four tetrads were analyzed as described for panel B. Segregation of the *NIT2* phenotype is indicated above each lane.

change in the hybridization pattern relative to the wild-type control indicated a mutation within the region detected by that probe. The results of this analysis are summarized in Table 2. For example, DNA insertions associated with *nit2–4*, *nit2–2* and *nit2–1* resided within region b, c and d, respectively. No restriction fragment length polymorphisms were observed within regions a or e in any of the mutants. These results are consistent with the suggestion from transformation experiments that the *NIT2* gene resides within the 6.2-kb *Hin*dIII fragment, although sequences important for the regulation of the *NIT2* gene may extend beyond this region.

Identification of the *NIT2* transcript and its regulation by ammonium: When ammonium, the product of nitrate assimilation, is available, each component of the nitrate assimilation pathway is repressed (FLORENCIO and VEGA 1983; GALVAN *et al.* 1991; HERRERA *et al.* 1972). In the case of *NIT1*, the only component of the pathway whose expression has been examined at the RNA level, ammonium stringently represses accumulation of the transcript (FERNÁNDEZ *et al.* 1989). In addition, transcripts from at least four genes that are tightly linked to *NIT1*, but whose functions are unknown, are repressed by ammonium (QUESADA 1992). To ask whether ammonium also affects expression of the *NIT2* gene, we compared the abundance of the *NIT2* transcript in cells grown in

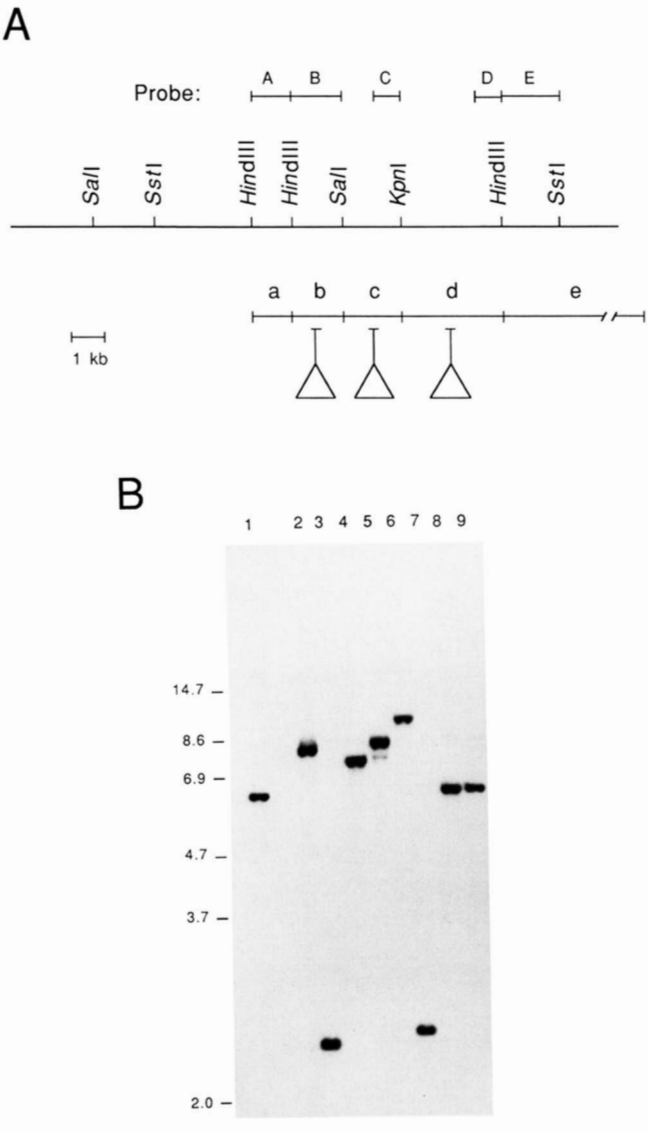

FIGURE 7.—Localization of *nit2* mutations. (A) Genomic restriction map of the wild-type *NIT2* locus. DNA regions that were tested for the presence of restriction length polymorphisms are designated by small letters below the restriction map (a through e). Fragments used as probes are designated by capital letters above the restriction map (A through E). Polymorphisms in four mutants were located within region b (A54-a2, A54-g24, A55-c8 and A55-e12); four were in region c (A55-a5, A54-f10, A55-h1 and A55-h9); and two resided in region d (A55-c9 and A54-g18). (B) Hybridization analysis of *Hin*dIII-digested DNA from strain A55 (lane 1) and from *nit2* mutant strains A55-c9 (lane 2), A55-a5 (lane 3), A55-c8 (lane 4), A55-e12 (lane 5), A55-h1 (lane 6), A55-h9 (lane 7), A55-d8 (lane 8) and A55-a14 (lane 9). For this experiment, the filter described in the legend to Figure 1 was rehybridized with probe B, shown in panel A of this figure.

the presence of ammonium and in cells transferred to nitrogen-free medium for one hour prior to harvesting. To test the effectiveness of the repression and derepression regimens, a fragment that encompasses the *NIT1* locus was used as a probe for RNA hybridization analysis. In derepressed wild-type cells, the control probe detected the *NIT1* transcript (4 kb) plus two shorter transcripts from genes that flank *NIT1* (Figure 8A, lane 2). Whereas in ammonium-grown wild-type cells, and in derepressed *nit2-1* mutant cells, all three transcripts were undetectable (Figure 8A, lanes 1 and 3). As a control for equal loading, the filter was rehybridized with an α-tubulin probe (Figure 8B). These results confirm that ammonium repressed the expression of *NIT1* and of two neighboring genes, that transcripts from all three genes were detectable within 1 hr after removal of ammonium, and that accumulation of these transcripts required the wild-type *NIT2* gene.

To determine whether the *NIT2* gene was regulated by ammonium, the filter was rehybridized using a fragment from the cloned *NIT2* gene as a probe (probe C, Figure 7A). This probe detected a 6-kb RNA in derepressed wild-type cells and a shorter, 2.45-kb RNA in *nit2-1* mutant cells (Figure 8C, lanes 2 and 3). The altered length of the RNA in the *nit2* mutant confirmed that the 6-kb RNA detected in wild-type cells is the *NIT2* transcript. In the presence of ammonium, the level of the *NIT2* transcript was dramatically reduced (Figure 8B, lane 1). Therefore, accumulation of the *NIT2* transcript was repressed by ammonium.

TABLE 2

Summary of new *nit2* mutations

Allele	Isolate[a]	Type of mutation[b]	Location[c]
nit2-4	A54-a2	Insertion (T1)	b
nit2-12	A54-f2	ND	
nit2-6	A54-f10	Insertion (T1)	c
nit2-7	A54-g18	Insertion (T2)	d
nit2-13	A54-g21	ND	
nit2-11	A54-g24	Rearrangement	b
nit2-2	A55-a5	Insertion (G)	c
nit2-14	A55-a14	ND	
nit2-10	A55-c8	Rearrangement	b
nit2-1	A55-c9	Insertion (G)	d
nit2-3	A55-d8	ND	
nit2-5	A55-e12	Insertion (T1)	b
nit2-8	A55-h1	Insertion (T2)	c
nit2-9	A55-h9	Insertion (T3)	c

[a] These 14 different *nit2* mutants were isolated from eight independent cultures, designated by the parental strain name (A54 or A55) and the culture name (a, b, etc.).

[b] Polymorphisms caused by DNA insertions are classified according to the identity of the inserted sequence: G denotes a *Gulliver* element and T1, T2, and T3 denote three unrelated sequences. The abbreviation ND indicates that no DNA polymorphisms were detected.

[c] Regions that contain DNA polymorphisms (b, c, and d) are defined in Figure 7A.

DISCUSSION

Isolation of the *NIT2* gene from *C. reinhardtii* demonstrates the first use of transposon tagging in this organism. In principle, transposon tagging provides a means for isolating any locus for which a transposon insertion allele can be identified. However, if *Gulliver* integrates preferentially into particular sequences or if the rate of transposition is influenced by environ-

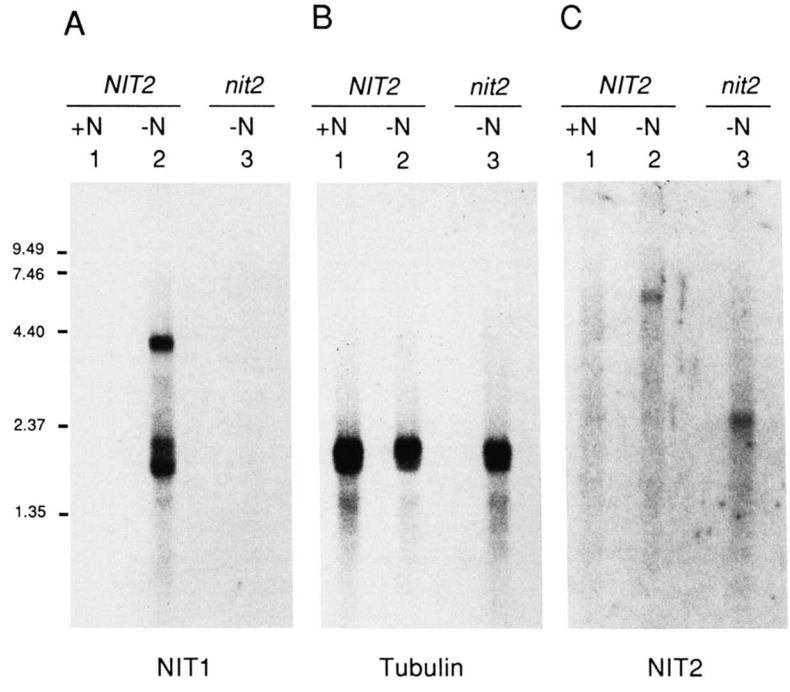

FIGURE 8.—RNA hybridization analysis of the *NIT2* transcript. RNA from wild-type *NIT2* strain A55 (lanes 1 and 2) or the isogenic *nit2-1* strain A55-c9 (lane 3), was isolated from cells grown in the presence of ammonium (+N) or from cells transferred to ammonium-free medium for one hour before harvesting (−N). In each lane, 10 μg poly(A)$^+$ RNA were fractionated through a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane and hybridized with (A) the 13.5-kb *Sph*I fragment that contains the *NIT1* gene from plasmid pMN24 (KINDLE *et al.* 1989); (B) an α_1-tubulin cDNA (α10-2) (SILFLOW *et al.* 1985); (C) the 1-kb *Pst*I-*Kpn*I fragment corresponding to probe C in Figure 7A.

mental or genetic factors, then this transposon may not be useful for tagging certain genes. One approach for overcoming this limitation would be to isolate probes for additional transposable element families, thereby increasing the number of transposon-induced mutations that can be detected. Strikingly, most of the spontaneous *nit2* mutations isolated in this study contained insertions or rearrangements within the *NIT2* gene. Six of the mutations were caused by the insertion of sequences that share no homology with *Gulliver* or *TOC1*. Characterization of these sequences has so far led to the identification of two new transposable element familes, *Tcr1* and *Tcr2* (our unpublished results). Future studies will resolve whether transposon tagging can be used routinely for isolating genes in Chlamydomonas.

Most significantly, isolation of the *NIT2* gene provides a means of determining the product of this gene. The properties of *nit2* mutants suggest that the *NIT2* gene encodes a positive regulator of the nitrate assimilation pathway. Specifically, *nit2* mutations are recessive and prevent the utilization of both nitrate and nitrite. The role of *NIT2* appears to be specific for the nitrate assimilation pathway, since *nit2* mutants grew as well as wild-type strains in the presence of other nitrogen sources, including acetamide, adenine, ammonium, arginine, asparagine, glutamine, guanine, ornithine and urea (data not shown). Consistent with their inability to utilize either nitrate or nitrite, *nit2* mutants contain little if any nitrate reductase or nitrate permease activity and significantly reduced nitrite reductase and nitrite uptake levels (GALVÁN, CÁRDENAS and FERNÁNDEZ 1992; NICHOLS, SHEHATA and SYRETT 1978; E. FERNÁNDEZ, personal communication). In the case of nitrate reductase, regulation by *NIT2* occurs at the RNA level, since accumulation of the nitrate reductase transcript requires the presence of a wild-type *NIT2* gene. Whether *NIT2* activates other components of the pathway at the RNA level is not known since those genes have not been identified. However, at least four genes of unknown function, noted by their proximity to the *NIT1* gene, require *NIT2* for their expression (QUESADA 1992).

The 6-kb *NIT2* transcript could be unequivocally identified because in a mutant strain that contained a transposon inserted within the *NIT2* locus, this transcript was replaced by a shorter RNA. Furthermore, the accumulation of this mutant *nit2* transcript suggests that the *NIT2* gene does not require the product of its own gene to be expressed. Comparison of the abundance of the *NIT2* transcript in cells under ammonium-repressed and derepressed conditions revealed that the *NIT2* gene was repressed by ammonium.

Previous studies have shown that every gene or biochemical activity that has been found to be activated by *NIT2* is also repressed by ammonium. The observation that the *NIT2* gene itself is subject to metabolite repression suggests that ammonium might repress the expression of *NIT1* and other *NIT2*-dependent genes by controlling the amount of the *NIT2* gene product. Assuming that regulation occurs at the

level of RNA synthesis rather than RNA stability, it should be possible to test this model by introducing a *NIT2* gene placed under the control of a constitutive promoter. The model predicts that expression of the *NIT2* gene in the presence of ammonium would lead to constitutive expression of *NIT2*-dependent genes, including *NIT1*. Alternatively, if ammonium represses both *NIT2* and *NIT2*-dependent genes directly, then constitutive expression of the *NIT2* gene would not influence the ability of ammonium to repress *NIT1* and other *NIT2*-dependent genes.

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