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A Forward Genetic Screen Identifies Mutants Deficient for Mitochondrial Complex I Assembly in *Chlamydomonas reinhardtii*

M. Rosario Barbieri,*,† Véronique Larosa,*,‡ Cécile Nouet,* Nitya Subrahmanian,*,† Claire Remacle*,‡,[1] and Patrice P. Hamel*,†,[1]

**Department of Molecular Genetics and Department of Molecular Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210 ‡Genetics of Microorganisms Laboratory, Department of Life Sciences, Université de Liège, B-4000 Liège, Belgium and †Plant Cellular and Molecular Biology graduate program, The Ohio State University, Columbus, Ohio 43210*

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ABSTRACT

Mitochondrial complex I is the largest multimeric enzyme of the respiratory chain. The lack of a model system with facile genetics has limited the molecular dissection of complex I assembly. Using *Chlamydomonas reinhardtii* as an experimental system to screen for complex I defects, we isolated, via forward genetics, *amc1–7* nuclear mutants (for **a**ssembly of **m**itochondrial **c**omplex I) displaying reduced or no complex I activity. Blue native (BN)-PAGE and immunoblot analyses revealed that *amc3* and *amc4* accumulate reduced levels of the complex I holoenzyme (950 kDa) while all other *amc* mutants fail to accumulate a mature complex. In *amc1*, *-2*, *-5–7*, the detection of a 700 kDa subcomplex retaining NADH dehydrogenase activity indicates an arrest in the assembly process. Genetic analyses established that *amc5* and *amc7* are alleles of the same locus while *amc1–4* and *amc6* define distinct complementation groups. The locus defined by the *amc5* and *amc7* alleles corresponds to the *NUOB10* gene, encoding PDSW, a subunit of the membrane arm of complex I. This is the first report of a forward genetic screen yielding the isolation of complex I mutants. This work illustrates the potential of using Chlamydomonas as a genetically tractable organism to decipher complex I manufacture.

MULTIMERIC respiratory complexes I, III, and IV in the mitochondrial inner membrane generate the proton motive force that is critical for ATP production. Complex I, the largest respiratory complex, is a NADH-ubiquinone oxidoreductase with a hydrophilic peripheral arm protruding into the mitochondrial matrix and a membrane arm (Sazanov and Hinchliffe 2006; Efremov *et al.* 2010; Hunte *et al.* 2010). It is known that the assembly of multimeric enzymes is assisted by factors absent from the mature enzyme but nevertheless essential in promoting its assembly into an active form. In humans, complex I dysfunction is the cause of severe diseases (Distelmaier *et al.* 2009). Since only 40% of the nuclear mutations in complex I-linked diseases occur in structural subunits of complex I, it is generally accepted that the other 60% represent defects in factors recruited to assemble or regulate the complex (Loeffen *et al.* 2000; Thorburn 2004).

In recent years, the use of model organisms has contributed to the identification of factors involved in the assembly of respiratory complexes. Most of these studies have been carried out in the model organism *Saccharomyces cerevisiae*, which has a broad spectrum of genetic and molecular tools facilitating research (Barrientos 2003). However, given that *S. cerevisiae* and its related species have lost complex I subunits (Gray *et al.* 2001), they cannot be used as a model for the study of complex I assembly. Instead, the lack of complex I in *S. cerevisiae* and other eukaryotes has been instrumental in revealing candidate complex I assembly factors through subtractive phylogenetic analyses. In conjunction with mitochondrial proteomic data from complex I-bearing organisms, these analyses were recently used to identify a number of candidate assembly factors (Pagliarini *et al.* 2008). Three of these factors, C8ORF38, C20ORF7, and FOXRED1, were found to be mutated in complex I-deficient patients (Pagliarini *et al.* 2008; Sugiana *et al.* 2008; Fassone *et al.* 2010). A limitation of subtractive phylogenetics is that assembly factors are assumed to have been systematically lost from organisms lacking complex I, hence excluding the possibility of finding conserved genes that have acquired dual or novel function. Therefore, there is still a need for a genetic approach to discover loci controlling complex I assembly on the basis of loss-of-function phenotypes.

Fungi, animals, and vascular plants have been used extensively as experimental systems for the study of complex I defects (Remacle *et al.* 2008). Although such

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[1]*Corresponding authors:* 500 Aronoff Laboratory, 318 W. 12th Ave., Columbus, OH 43210. E-mail: hamel.16@osu.edu; and Genetics of Microorganisms Laboratory, Department of Life Sciences, Université de Liège, B-4000 Liège, Belgium. E-mail: c.remacle@ulg.ac.be

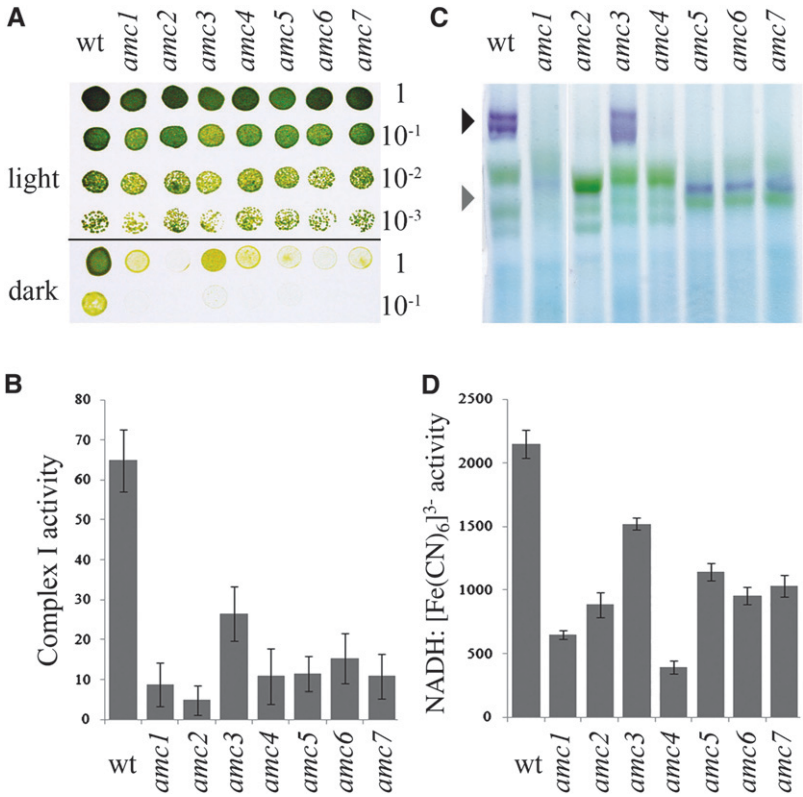

FIGURE 1.—Six novel *sid* mutants display a defect in complex I activity. (A) Tenfold dilution series of *amc1–7* and wild-type (wt) cells were plated on acetate-containing medium and incubated 3 days in the light or 5 days in the dark. (B) Complex I-specific activity (nmol NADH reduced/min/mg of protein) is indicated as averages of 10 independent measurements. (C) BN-PAGE and in-gel NBT staining of NADH dehydrogenase activity. The black and gray arrowheads indicate the position of the mature 950-kDa complex I and the 700-kDa subcomplex, respectively. The purple bands indicate staining for NADH oxidase activity in complex I and subcomplexes. Note that complex I is often resolved into two or more purple bands of high molecular weight. The identity of such bands is unclear but they do not correspond to the supercomplex I + III_2 (CARDOL *et al.* 2008). The green bands correspond to photosystems I and II and their respective light harvesting complexes of the chloroplast (REXROTH *et al.* 2003; CARDOL *et al.* 2006). (D) NADH: ferricyanide oxidoreductase activity (nmol $K_3[Fe(CN)_6]$ reduced/min/mg of protein). The results represent the average of three independent determinations. (B and D) Error bars indicate standard error (SE).

models were invaluable in documenting the impacts of specific mutations in complex I function, they were not developed for the isolation of complex I mutants through forward genetics.

Chlamydomonas reinhardtii, a unicellular green alga, is a very promising experimental system to address the question of complex I assembly (REMACLE *et al.* 2008). It is an ideal organism to study mitochondrial biogenesis because complex I-less mutants are viable due to the operation of alternative NADH dehydrogenases (REMACLE *et al.* 2001a). Moreover, complex I-deficient mutants can be maintained in phototrophic (light) or mixotrophic (light + carbon source) conditions because they are not defective in photosynthesis (CARDOL *et al.* 2003). This provides an advantage to screen for complex I mutants, as they display robust growth under mixotrophic/phototrophic conditions but slow growth under heterotrophic conditions (dark + carbon source) (REMACLE *et al.* 2001a; CARDOL *et al.* 2002). In contrast, complex III or complex IV mutants display arrested growth in the dark (***d***ar***k*** dier or *dk*). That a complex I mutant retains two respiratory complexes (III and IV) out of the three is believed to account for the "slow" growth phenotype. Indeed, in a complex I mutant, complexes III and IV still contribute to the formation of the proton electrochemical potential, and thus to ATP formation. However, in a complex III or complex IV mutant, the contribution of complex I to the proton gradient is assumed to be insufficient to sustain growth in the dark (WIKSTRÖM 1984; GALKIN *et al.* 2006). Several mutations in complex I subunits encoded by the mitochondrial genome and one nuclear mutation (*amc2*, ***a****ssembly of* ***m****itochondrial* ***c****omplex I*, not yet characterized molecularly) have been described in Chlamydomonas (REMACLE *et al.* 2001a). Here, we used a mutagenesis approach to search for additional *amc* mutants of nuclear origin. Six *amc* mutants, defining five loci, were isolated. The *amc* mutants are affected to different extents in complex I activity and most of them result in the accumulation of subcomplexes, an indication that the assembly process

TABLE 1

Complex I-dependent oxygen consumption of *amc* and wild-type strains

Strain	Respiration (μmol O_2 h^{-1} mg^{-1} of chlorophyll)	
	−rotenone	+rotenone
wt	61.0 ± 2.0	42.0 ± 4.0
amc1	44.2 ± 3.2	39.8 ± 5.7
amc2	42.7 ± 0.8	41.0 ± 5.4
amc3	44.6 ± 5.0	40.1 ± 6.8
amc4	43.9 ± 5.3	43.7 ± 7.8
amc5	37.0 ± 4.2	33.4 ± 5.9
amc6	36.2 ± 4.6	34.4 ± 3.1

Wild-type and *amc* strains representative of the six different *AMC* loci were grown mixotrophically (TAP + arginine, light). Oxygen consumption was measured in the dark, with or without the addition of 100 μM rotenone to specifically inhibit complex I. Values represent the average of three independent measurements and ± indicates standard error.

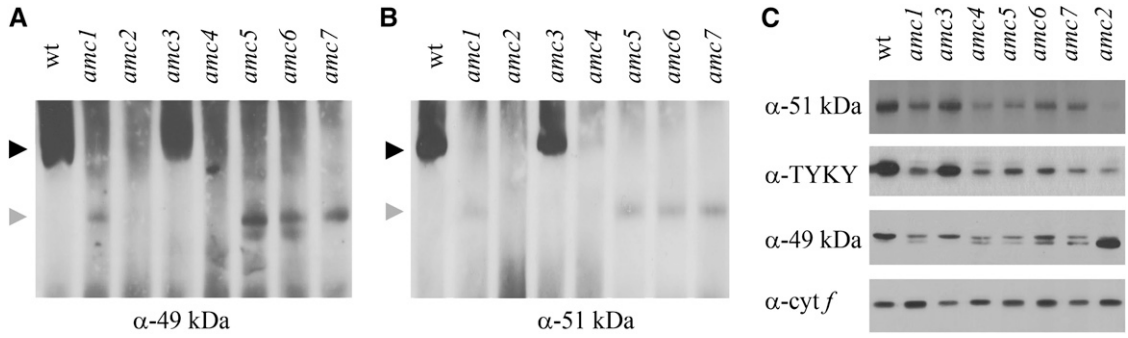

FIGURE 2.—The *amc* mutants are deficient in complex I assembly. (A and B) Immunoblot analyses of membrane fractions, separated by BN-PAGE, using anti-49 kDa (A) and anti-51 kDa (B). A total of 150 μg of proteins was loaded per lane. The figures show the most representative blots of three independent experiments. The solid and shaded arrowheads indicate the position of mature complex I and the 700-kDa subcomplex, respectively. Equal loading was tested by Coomassie blue staining (not shown) and the presence of a nonspecific band revealed by the anti–49-kDa antiserum (Figure S2B for an example). (C) Membrane fractions were separated by SDS–PAGE and immunoblot analyses were performed using polyclonal antibodies against subunit-specific peptides. The subunits are 51 kDa, TYKY, and 49 kDa. A total of 10 μg of total protein per lane was loaded. Antibody specificity was evaluated by peptide competition assays (not shown). Only the top band is specific for the 49-kDa subunit. Plastid cytochrome *f* was used as a loading control. The results are representative from at least three independent membrane extractions.

of the membrane arm is compromised. In *amc5* and *amc7*, the identification of molecular lesions in the *NUOB10* gene encoding PDSW, a subunit of the membrane arm, validates our mutant screen strategy. This illustrates the potential of Chlamydomonas as an experimental model system to dissect the molecular basis of complex I assembly.

MATERIALS AND METHODS

Strains and culture conditions: Strains were grown at 22–25° in Tris-acetate-phosphate (TAP), arginine-supplemented TAP liquid, or solid medium (HARRIS 1989) under continuous light (50 $\mu E \cdot m^2 \cdot sec^{-1}$) as described in HOWE and MERCHANT (1992). Wild-type (wt) strains $3A^+$ and $4C^-$ were used for transformation (J. D. Rochaix, University of Geneva, Switzerland) and strains $137c^+$ and $137c^-$ were used for backcrosses. Strain dn26 169 (*mt*$^-$) and a *mt*$^+$ derivative, renamed here *amc2*, were used for crosses. Genetic analyses were performed as in HARRIS (1989).

Insertional mutagenesis and identification of the *amc* mutants: $3A^+$ (*mt*$^+$ *arg7-8*) or $4C^-$ (*mt*$^-$ *arg7-8*) cells were electroporated with 100 ng of either a hygromycin B (iHyg) or paromomycin (iPm) resistance cassette as in SHIMOGAWARA *et al.* (1998). The iHyg and iPm cassettes were amplified by PCR from the pHyg3 (BERTHOLD *et al.* 2002) or pSL18 (DEPÈGE *et al.* 2003) plasmids, respectively. Oligonucleotides Aph7-F (5′-TCGATATCAAGCT

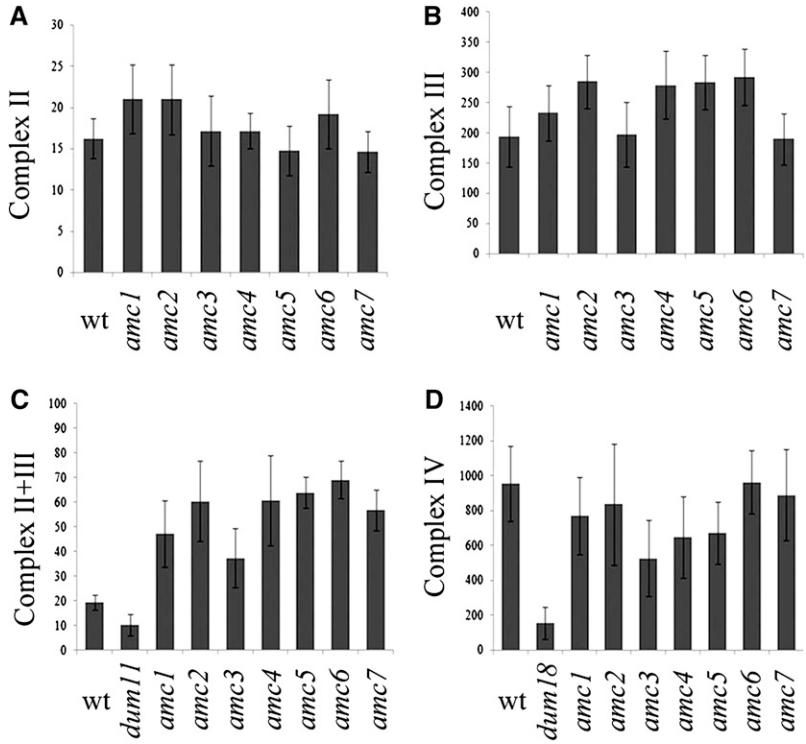

FIGURE 3.—Effects of the *amc* mutations on other respiratory complexes. (A) Complex II activity (succinate:2,6-dichlorophenolindophenol (DCIP) oxidoreductase). Three independent determinations were averaged and activities are expressed as nmol DCIP reduced/min/mg of protein. (B) Complex III activity (decylubiquinol: cytochrome *c* oxidoreductase). Averages from three independent measurements are shown in nmol of cytochrome *c* reduced/min/mg of protein. (C) Complexes II + III combined activities (succinate:cytochrome *c* oxidoreductase) of 10 independent determinations (nmol of cytochrome *c* reduced/min/mg of protein). *dum11* is a complex III mutant (DORTHU *et al.* 1992). (D) Complex IV activity (cytochrome *c* oxidase). *dum18* is a complex IV mutant (REMACLE *et al.* 2001b). Average activities of 10 independent determinations are expressed in nmol cytochrome *c* oxidized/min/mg of protein. Error bars indicate SE.

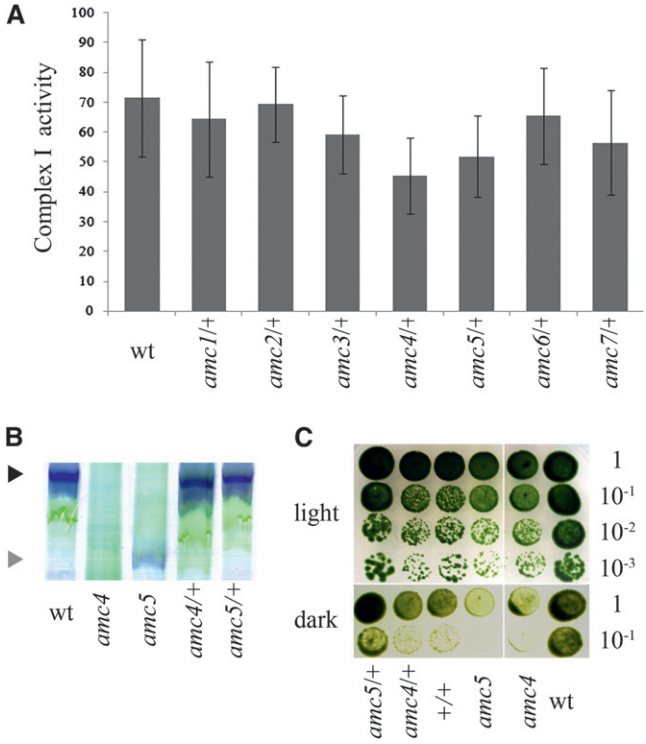

FIGURE 4.—The *amc* mutations are recessive. Heterozygous (*amc*/+) and homozygous (+/+) diploid strains were generated by crosses. (A) Complex I activities (nmol NADH oxidized/min/mg of protein). Columns represent the average of 10 independent determinations, with error bars representing SE. The +/+ diploid activity is not shown here and was 92 ± 8 nmol NADH oxidized/min/mg of protein. (B) BN-PAGE and in-gel NBT staining of NADH dehydrogenase activity. The black and gray arrowheads indicate the position of mature complex I and 700-kDa subcomplex, respectively. (C). Light/dark growth comparison of wild type (wt), *amc4* and *amc5* mutants, and their respective diploids (+/+, *amc4*/+, and *amc5*/+). Dilution series were plated on acetate containing medium and incubated 6 days in the light or 12 days in the dark. For B and C, only representative *amc*/+ diploids are shown. All *amc*/+ diploids exhibit restoration of the growth in the dark and complex I assembly phenotypes (not shown).

TCTTTCTTGC-3′) and Aph7-R (5′-AAGCTTCCATGGGAT GACG-3′) were used for amplification of the iHyg cassette. Aph8-F2 (5′-TCAGGCAGACGGGCAGGTG-3′) and Aph8-R (5′-TCAGGCAGACGGGCAGGTG-3′) were used to amplify the iPm cassette. Transformants were selected on TAP + arginine solid media with 25 μg/ml of hygromycin B or paromomycin in the light. After a 7- to 10-day incubation under light, transformants were transferred individually to 96-well plates and grown in selective liquid media. Individual colonies were replica plated on solid TAP + arginine medium and incubated in the dark and in the light for 7 days before scoring.

Identification of flanking sequences by thermal asymmetric interlaced-PCR: Amplification of insertion-linked sequence by thermal asymmetric interlaced (TAIL)-PCR was as described in DENT *et al.* (2005). Four different degenerate primers, AD1 and AD2 (LIU *et al.* 1995), RMD227 and RMD228 (DENT *et al.* 2005), were tested. iPm-specific primers were PmR1 (5′-GAC CCCGCAGTGCACGCAAC-3′), PmR2 (5′-GCTTGAGACAGC GACAGAGGAGCC-3′), and PmR3 (5′-GCAAGTCAAATCTGC AAGCACG-3′) for the primary, secondary, and tertiary reactions, respectively. The following *NUOB10*-specific primers were used: PDSW-1F (5′-CACCTGGTGCACATTGCTGTA-3′), PDSW-2R (5′-TTCATGCTTGCCCGAGAAG-3′), PDSW-5F (5′-GACCAA GGGCTTTCTGACTG-3′), PDSW-7F (5′-AGCCTCAGGCAT ATTGCGC-3′), PDSW-6R (5′-ATCGCACATGACGGCAG-3′), PDSW-8R (5′-GTAGATGAAGCCCACGTCG-3′), PDSW-9F (5′-GTCATGAAGCGCCTGCAGG-3′), PDSW-10R (5′-GGAAC TGGGTAAGGTTCTAAGC-3′), PDSW-11F (5′-CACTGCCTGA AAGCCTGCC-3′), and PDSW-12R (5′-GAGGAGAGCAGTGGC ATCGTG-3′).

Protein extraction, SDS–PAGE, and immunoblot analyses: Total protein was isolated from 2-day-old light-grown plates using the freeze/thaw method (HOWE and MERCHANT 1992). SDS–PAGE was performed using standard protocols (SAMBROOK *et al.* 1989).

Measurement of respiratory enzyme activities: Respiratory activities were measured as described previously (REMACLE *et al.* 2001a, 2004; CARDOL *et al.* 2002) using partially purified membranes extracted from 2- to 3-day-old cultures.

Antibody production: Protein A purified polyclonal antibodies directed against complex I subunit-specific peptides were custom synthesized in rabbits by GeneScript (Piscataway, NJ) using the following peptide antigens: NUO7 (49 kDa), CGIDWDLRKTQPYDA; NUO6 (51 kDa), SLEGKQGKPRLKPPC; NUO8 (TYKY), YASDWENDPTFKRTC. To assess antibody specificity, protein extracts were competed with the peptides used to generate the polyclonal antibodies (HOWE and MERCHANT 1992). For NUO7 (49 kDa), only the upper band detected with the antiserum is specific.

BN-PAGE and in-gel activity: Separation of protein complexes was done by BN-PAGE (SCHÄGGER and VON JAGOW 1991). Detection of in-gel NADH dehydrogenase activity was performed as in RASMUSSON and MØLLER (1991).

RESULTS

Identification of complex I-deficient (*amc*) mutants via insertional mutagenesis: Only one nuclear mutation (*amc2*) resulting in a complex I defect has been previously described in Chlamydomonas (REMACLE *et al.* 2001a; CARDOL *et al.* 2002, 2008). To uncover additional *AMC* loci, we undertook a screen for nuclear mutants, resulting in complex I dysfunction. Out of ~50,000 insertional transformants, 22 showed slow growth in the dark (*sid* for **s**low growth **i**n the **d**ark; Figure 1A), a feature of complex I mutants. A defect in complex I activity was verified for six *sid* mutants and we named them *amc1* and *amc3*–*7* (Figure 1B). The other *sid* mutants were either not significantly or only marginally reduced in complex I activity (*P*-value > 0.05) (not shown). In-gel staining for NADH dehydrogenase activity was performed for all *amc* mutants (Figure 1C). Note that wild-type complex I is often resolved in more than one purple band of high molecular weight, depending on solubilization conditions. The purple bands, observable at ~950 kDa, correspond to the holoenzyme. These were only present in *amc3* and *amc4*. In the case of *amc3*, while a wild-type–size complex I was detectable, the staining was reduced (Figure 1C and supporting information, Figure S1B). We also noted that very low levels of complex I accumulated in *amc4* (Figure S1B and Figure S2A). This accumulation appeared to be dependent

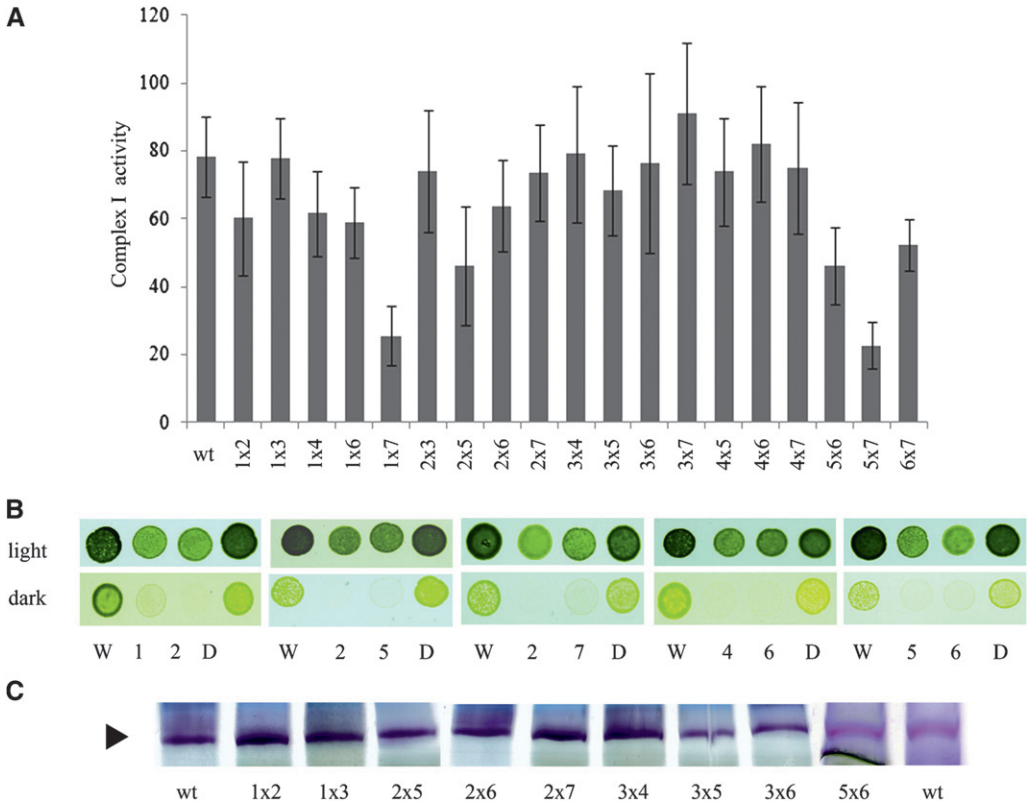

FIGURE 5.—Complementation experiments define alleles of *AMC* loci. (A) Complex I activity of the wild type (wt) and the 19 diploids strains (*amc* × *amc*). The average of 10 independent replicas is indicated. The error bars represent standard error. The activity is expressed in nmol NADH oxidized/min/mg of protein. (B) Light/dark growth comparison of wild type (W), *amc* mutants (*1*, *2*, and *4*–*7*) and respective heterozygous diploids (D). Cells were plated on acetate-containing medium and incubated 3 days in the light or 5 days in the dark. (C) BN-PAGE and in-gel NBT staining of NADH dehydrogenase activity. The black arrowhead indicates the position of mature complex I. For B and C, only representative *amc* × *amc* diploids are shown. All diploids exhibiting restoration of the growth in the dark phenotype also showed restoration of complex I assembly as assessed by NBT staining (not shown). Only the top part of the BN gel is shown in C.

upon extraction, an indication that complex I is not stable in the presence of the *amc4* mutation. All other *amc* mutants accumulated a 700-kDa subcomplex that stained for NADH dehydrogenase activity (Figure 1C and Figure S1, A and B). A similar 700-kDa subcomplex is also detected in Chlamydomonas *nd4* or *nd5* mitochondrial mutants (REMACLE *et al.* 2006; CARDOL *et al.* 2008) (Figure S1B). The subcomplex in *amc2* is labile, as it was not always detected in independent extractions (Figure S1A).

To further evaluate the complex I NADH oxidoreductase function in the mutants, total NADH:ferricyanide oxidoreductase activity was determined. Total NADH:ferricyanide oxidoreductase activity reflects not only the activity of complex I but also that of other cellular NADH dehydrogenases (QUILES *et al.* 1996). However, because most of the NADH:ferricyanide oxidoreductase activity in Chlamydomonas is due to the NADH dehydrogenase activity of the peripheral arm of complex I, the level of this activity is reduced in complex I mutants. A strong reduction was observed in all mutants except *amc3* (Figure 1D). Overall, the levels of reduction correlated with reduced in-gel staining for NADH dehydrogenase activity in the *amc* mutants (Figure 1, C and D). All *amc* mutants showed decreased whole-cell respiration when compared to wild type (Table 1). In accord with the complex I defect, the respiration in the *amc* mutants was rotenone insensitive.

The *amc* mutants display complex I assembly defects: To verify that the 700-kDa subcomplex observed in *amc1*, *-2*, and *-5*–*7* was a *bona fide* subcomplex of complex I, we carried out immunoblot analyses using antibodies against two subunits of the hydrophilic arm (49 and 51 kDa). As expected for an assembly intermediate of complex I, the 700-kDa subcomplex was detected by the two complex I-specific antibodies (Figure 2, A and B). In *amc2*, the putative subcomplex was very rarely detected (not shown). On the basis of the fact that the 700-kDa band was also rarely detected by in-gel staining, we reasoned that this subcomplex is highly unstable in *amc2*.

Using an antiserum against TYKY, another subunit of the hydrophilic arm, we also consistently detected the 700-kDa subcomplex in *amc1* and *amc5*–*7* and in some instances in *amc2* (not shown). Intermediate assembly subcomplexes of similar size were not observed in the wild type or in the *amc3* and *amc4* mutants. In addition, low levels of complex I was evidenced in *amc4* using the anti–49-kDa antibody in instances where NADH

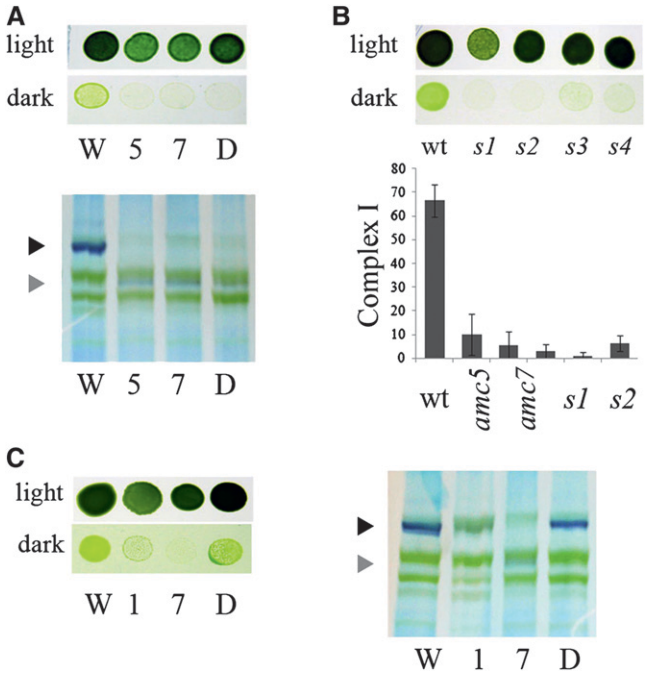

FIGURE 6.—*amc5* and *amc7* define alleles of the same AMC locus. (A, top) Light/dark growth comparison of wild type (W), *amc5* and *amc7*, and the *amc5*/*amc7* diploid (D). (A, bottom) Detection of complex I and the 700-kDa subcomplex via NADH dehydrogenase activity on membrane fractions separated by BN-PAGE. (B, top) Light *vs.* dark growth of wild type (wt) and representative spores (*s1*–*s4*) from the *amc5* × *amc7* cross. Note that the strains used in our study are not isogenic and it is possible that other genetic factors segregate and modulate the slow growth phenotype (compare *s1* and *s2* to *s3* and *s4*). (B, bottom) Complex I activities (nmol NADH oxidized/min/mg of protein) of wild type (wt), *amc5*, *amc7*, and two *sid* spores (*s1* and *s2*) originating from the *amc5* × *amc7* cross. Columns represent the average of 10 independent determinations, with SE as the error bars. (C, left) Light *vs.* dark growth of wild type (W), *amc1* and *amc7* mutants, and the *amc1*/*amc7* diploid (D). (C, right) Detection of complex I and the 700-kDa subcomplex via NADH dehydrogenase activity on membrane fractions separated by BN-PAGE. (A, B, and C) Cells were plated on acetate-containing medium and incubated 3 days in the light or 5 days in the dark. (A and C) The black and gray arrowheads indicate the position of mature complex I and the 700-kDa subcomplex, respectively.

dehydrogenase activity was also detected by in-gel staining (Figure S2, A and B).

Low accumulation of structural subunits has been observed in several cases of complex I deficiency (SAADA *et al.* 2009). We decided to evaluate the steady-state levels of the three complex I subunits (49 kDa, 51 kDa, and TYKY) in membrane extracts of the *amc* mutants, separated under denaturing conditions. As shown in Figure 2C, the accumulation of TYKY and other subunits was severely reduced in all mutants, with the exception of *amc3*.

Impact of the *amc* mutations on respiratory complexes II, III, and IV: To determine whether the *amc* mutations resulted in a pleiotropic defect, activities of other respiratory complexes were measured. All *amc* mutants showed increased levels of complex II + III combined activity, an indication that they compensate for a defect in complex I (Figure 3C). Such a compensatory effect has already been observed in complex I mutants of mitochondrial origin (REMACLE *et al.* 2001a; CARDOL *et al.* 2002). Individually, complex II and complex III activities were not significantly affected (Figure 3, A and B). On average, the augmented complex II + III combined activity correlated with the level of reduction of complex I in all mutants. All *amc* mutants with the exception of *amc3* displayed wild-type levels of complex IV activity (Figure 3D). However, we noted that the complex IV defect seen in *amc3*, unlike the complex I phenotype, was not inherited in the progeny (not shown). We concluded that all *amc* mutants resulted in isolated complex I deficiency.

Genetic analyses reveal six *AMC* loci: All *amc*/+ diploid strains displayed restored levels of complex I activity (Figure 4A). In addition, complex I assembly, assessed via BN-PAGE, was also wild type (not shown and Figure 4, B and C). Therefore, we concluded that all *amc* mutations were recessive. While all the *amc* mutations were found to be monogenic, the antibiotic resistance did not cosegregate with the *sid* phenotype for any except *amc5* (not shown). We concluded that the *AMC5* locus was tagged with the insertional marker. To establish the number of complementation groups defined by the *amc* mutations, we performed allelism tests. We were unable to obtain diploids from *amc1* × *amc5* and *amc2* × *amc4* crosses. The other 19 diploids could be constructed and were further analyzed. Although complex I activity levels did not always reach those of the wild-type strain, we observed restoration of a fully active complex I in most diploids (Figure 5A). This restoration correlated with the recovery of wild-type–like growth in the dark (Figure 5B) and NADH dehydrogenase activity (Figure 5C). Only two combinations of diploids (*amc5* × *amc7* and *amc1* × *amc7*) showed low levels of complex I activity (Figure 5A).

The *amc5* × *amc7* diploid accumulated the same subcomplex as each single mutant and displayed a *sid* phenotype (Figure 6A). Moreover, all the progeny (100 meiotic products) from the *amc5* to *amc7* cross had a *sid* phenotype and was deficient in complex I activity (Figure 6B). Thus, we concluded that *amc5* and *amc7* are alleles of the same locus.

The *amc1* × *amc7* diploid displayed a low level of complex I activity (Figure 5A) but was restored for the growth in the dark and complex I assembly when assessed by BN-PAGE (Figure 6C). We concluded that *amc1* and *amc7* define distinct loci.

We were not able to generate meiotic products of the *amc1* × *amc5* cross. However, if *amc5* and *amc7* define the same locus and *amc1* and *amc7* are alleles of distinct loci, then *amc1* and *amc5* are nonallelic. Similarly, because we were unable to generate *amc2* × *amc4* diploids,

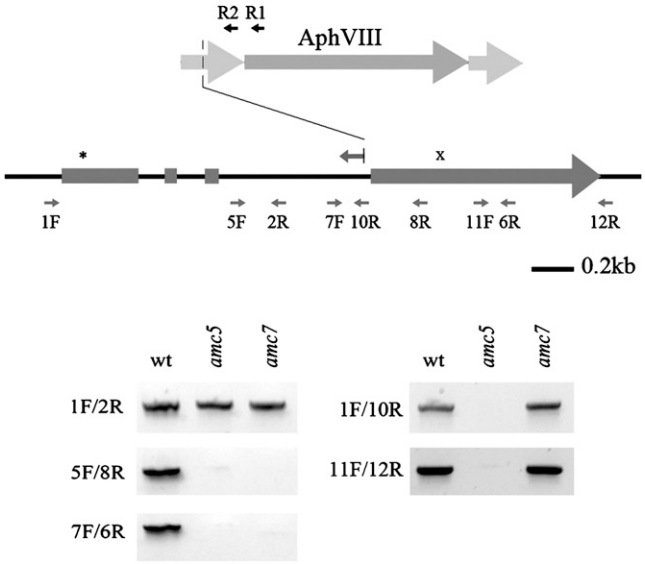

Figure 7.—The *amc5* and *amc7* mutants carry molecular lesions in the *NUOB10* gene encoding the noncore subunit *PDSW*. (Top) Schematic representation of the *NUOB10* gene structure showing the four exons (rectangles) and three introns (thin lines). The asterisk denotes the position of the ATG codon while the "x" symbol indicates the position of the stop codon. The insertional cassette (AphVIII) is represented as an arrow (dark shading) flanked by the promoter and terminator regions (light shading). The positions of primers used to diagnose the molecular lesion are indicated by small arrows. The leftward arrow with a vertical line above intron 3 indicates the extent of the *NUOB10* sequence obtained by TAIL-PCR (Figure S3). R1 and R2 indicate the positions of the AphVIII-specific primers used in the TAIL-PCR to recover the sequence flanking the cassette. A deletion of the promoter occurred upon integration of the cassette in the *NUOB10* locus. (Bottom) Molecular lesions in *NUOB10* were analyzed by PCR using *NUOB10*-specific primers. PCR products were stained by ethidium bromide and imaged using an imaging system (Kodak Image Station 2000R).

we analyzed the segregation of the meiotic spores originating from this cross. Several spores from the *amc2* × *amc4* cross displayed faster growth in the dark (Figure S3, top). Wild-type levels of complex I activity were also confirmed in spores exhibiting a wild-type–like growth phenotype in the dark (Figure S3, bottom). Therefore, we concluded that *amc2* and *amc4* belong to different complementation groups. In summary, the seven *amc* mutants define six nuclear loci.

The *AMC5/7* locus encodes the NUOB10/PDSW encoding gene: Since the *amc5* mutant was tagged with the insertional marker (iPm), we sought to identify the interrupted locus by TAIL-PCR. The iPm was mapped to intron 3 of the *NUOB10* gene encoding PDSW (according to the nomenclature of bovine complex I), a noncore subunit of the membrane arm of complex I (Hirst *et al.* 2003) (Figure 7 and Figure S4). Mitochondrial complex I is composed of core subunits required for the catalytic activity and noncore subunits believed to play a role in the assembly and/or regulation of the activity of the enzyme (Remacle *et al.* 2008). While core subunits are common to both bacterial and mitochondrial complex I, noncore subunits are only found in the eukaryotic enzymes. On the basis of the failure to amplify the region with specific primers, the insertion of the marker occurred at the 3′ end of intron 3 and was accompanied by a deletion of the entire coding sequence and the 3′-UTR downstream of the integration site (Figure 7). In *amc7*, PCR analyses revealed that a molecular lesion had also occurred in the *NUOB10* gene. (Figure 7). Unfortunately, despite several attempts, we were unable to transform the *amc5/amc7* mutants with a *NUOB10*-containing cosmid (not shown). Almost all the *amc* mutants were found to be very recalcitrant to transformation, regardless of the transformation method employed.

The *AMC1* locus does not map to a gene encoding a subunit of the membrane arm: As *amc1* also accumulated a 700-kDa subcomplex, we reasoned that the mutation could affect 1 of the 12 nuclear-encoded subunits of complex I present in the membrane arm (Lazarou *et al.* 2009), the *NUOP1* and *NUOP4* genes encoding small hydrophobic subunits (Cardol *et al.* 2004) or the *NUOAF1* gene encoding the CIA30 assembly factor whose defect results in the accumulation of a subcomplex (Kuffner *et al.* 1998; Vogel *et al.* 2005; Dunning *et al.* 2007). The sequencing of these loci did not reveal any mutation that could account for the phenotype of *amc1* (Table 2). The finding of a wild-type *NUOB10* sequence in *amc1* further confirms that this mutation is not allelic to *amc5* and *amc7* mutations. We concluded that the *amc1* mutation did not map to a gene encoding a complex I subunit that is part of the membrane arm.

DISCUSSION

The assembly of mitochondrial complex I is an intricate process involving coordination of multiple factors to yield a holoenzyme with at least 40 subunits, one FMN molecule, and eight FeS clusters. We aimed to further dissect this process and pursued a forward genetics approach in the green alga *C. reinhardtii* to uncover mutations (*amc*) resulting in loss of complex I function. We took advantage of the fact that in this alga, mutations inactivating complex I are not lethal and result in a slow growth in the dark phenotype, which facilitates visual screening of candidate mutants (Remacle *et al.* 2008). We isolated six new complex I-deficient mutants, four of them resulting in the formation of intermediate assembly subcomplexes. On the basis of our genetic analyses, all *amc* mutations are recessive and fall into six complementation groups (Table 3). The finding that *amc5* and *amc7* are the only recovered allelic mutations indicates that our screen is not saturated. Even though all *amc* mutations result in a defect in complex I activity, they appear different when

TABLE 2

Sequencing of complex I structural genes and NUOAF1 in the *amc1* mutant

Chlamydomonas subunit	Accession no.	Subcomplex	*H. sapiens*	*B. taurus*	Sequence obtained		Sequencing result
					bp from ATG	bp from STOP	
ACP1	AAQ73138	α, βS	NDUFAB1	SDAP	−57	+32	wt
NDUFA11	AAS58499	α, λ	NDUFA11	B14.7	−99	+372	wt
NUO17	AAS48192	β	NDUFB11	ESSS	−109	+130	wt
NUOA8	AAQ55460	α, λ	NDUFA8	PGIV	−8	+43	wt
NUOB10	AAQ55459	βL, βS	NDUFB10	PDSW	−57	+611	wt
NUOB12	AAS48194	β	NDUFB3	B12	−140	+338	wt
NUOB18	AAQ73135	βS	NDUFB7	B18	−13	0	wt
NUOB22	AAQ73134	βS	NDUFB9	B22	−3	+20	wt
NUOP2	AAS48193	α, βS	NDUFB4	B15	−182	+69	wt
NUOP1	AAS58501	ND	NI	NI	−133	+570	wt
NUOP4	AAS58498	ND	NI	NI	−18	+215	wt
NUOA1	AAS48198	α	NDUFA1	MWFE	−63	+488	wt
NUOA9	AAQ55458	α	NDUFA9	39	−233	+177	wt
NUOB16	AAQ64637	α, λ	NDUFA13	B16.6	−151	+656	wt
NUOB8	AAQ63699	α, λ	NDUFA2	B8	−75	+527	wt
Assembly factor							
NUOAF1	ACN88152		NDUFAF1	CIA30	−13	+288	wt

The assignment of the subunits in the various subcomplexes (α, β, βS, βL, and λ) is based on the predicted organization shown in Lazarou *et al.* (2009). NI, not identified; ND, not determined. The location of NUOP4, only identified in Chlamydomonas complex I, has not yet been determined experimentally (Cardol *et al.* 2004). NUOP1 was found in the membrane arm of Arabidopsis complex I (Klodmann *et al.* 2010).

examined at the level of complex I assembly (Table 3). While *amc1*, *-2*, *-5/7*, and *-6* accumulated a complex I assembly intermediate in the form of a subcomplex, *amc3* and *amc4* did not accumulate any subcomplexes. The 700-kDa subcomplex was shown to contain the 51 kDa, 49 kDa, and TYKY subunits that are components of the hydrophilic arm of complex I. Defects in ND4 and ND5 subunits of the membrane arm also yield a 700-kDa subcomplex (Remacle *et al.* 2006; Cardol *et al.* 2008). Overall, the accumulation of the subcomplex indicates that *amc1*, *-2*, *-4*, *-5/7*, and *-6* are disrupted for the assembly of the membrane arm of complex I. The *amc4* mutant appears to accumulate lower amounts of a wild-type–size complex I and is probably not deficient in the assembly process *per se* but rather for the stability of the holoenzyme. The fact that *amc3* displays a reduced complex I activity but no change in the abundance of the TYKY, 49-, and 51-kDa subunits suggests that the enzyme is fully assembled but less active.

The *amc5/7* locus was cloned and shown to correspond to the NUOB10/PDSW encoding gene (Figure 7). The NUOB10/PDSW family is a poorly characterized protein family characterized by the presence of a C $(X)_{11}$C motif (Cardol *et al.* 2004). The bovine PDSW has been found in the membrane arm (Hirst *et al.* 2003) and the Neurospora ortholog is an integral membrane protein, on the basis of its lack of extractability in alkali-treated samples (Videira *et al.* 1993). In

TABLE 3

Phenotype and molecular genetics of *amc* mutants

Strain	CI activity	Fully assembled (950 kDa)	Subcomplex (700 kDa)	Allelism	Monogenic	Tagged
wt	100	++++	—	—	—	—
amc1	12	—	+	No	Yes	No
amc2	4	—	+	No	Yes	No
amc3	41	+++	—	No	Yes	No
amc4	14	+	—	No	Yes	No
amc5	14	—	++	Yes (*amc7*)	Yes	Yes
amc6	22	—	++	No	Yes	No
amc7	14	—	++	Yes (*amc5*)	Yes	No

Complex I activity (CI) is indicated as % of wild type (100). +, ++, +++, ++++, and — indicate the relative levels of fully assembled complex I or 700-kDa subcomplexes.

Arabidopsis, NUOB10/PDSW is found in the membrane arm of complex I, associated with the hydrophobic subunit ND5 (Klodmann *et al.* 2010). This result is in accord with the fact that single mutants affected in ND5 (*dum23*, Figure S1) or NUOB10/PDSW (*amc5*, Figure S1) generate the same subcomplex, lacking the distal part of the membrane domain (this study and Hirst *et al.* 2003; Cardol *et al.* 2008). The finding that the *amc5* and *amc7* mutants carry mutations in the *NUOB10* gene validates our screening strategy for complex I defects.

The identities of the other *AMC* gene products revealed through our mutant screen are unknown and can only be speculated upon at the present time. One possibility is that the *AMC* loci correspond to genes encoding *bona fide* subunits. Alternatively, *AMC* loci could encode factors required for expression or assembly of complex I. Orthologs for identified assembly factors, NDUFAF3 (Saada *et al.* 2009), CIA30 (Dunning *et al.* 2007), C20ORF7 (Sugiana *et al.* 2008; Gerards *et al.* 2009), IND1 (Bych *et al.* 2008; Sheftel *et al.* 2009), C8ORF38 (Pagliarini *et al.* 2008), FOXRED1 (Fassone *et al.* 2010), GLDH (Pineau *et al.* 2008), and candidate assembly factors (C7ORF10, MCCC2, AMACR, LYRM5, DCI, and IVD) (Pagliarini *et al.* 2008) are present in the predicted proteome of Chlamydomonas. Whole-genome sequencing of the *amc* mutants should enable the rapid identification of the molecular lesions causing complex I defects. Such a technology is currently being tested in Chlamydomonas (O. Vallon, personal communication).

To our knowledge, this is the first description of a mutant screen in any organism yielding mutants with an isolated complex I deficiency. The variety of assembly defects in the *amc* mutants suggests the corresponding gene products (either structural subunits or assembly factors) act at different steps of the assembly process. This further stresses the value of Chlamydomonas as an experimental model to address the question of complex I assembly. This question has now received considerable attention in plants, where complex I displays unique features compared to its mammalian/fungal counterpart (Braun and Zabaleta 2007; Pineau *et al.* 2008; Klodmann *et al.* 2010) and also in humans, as most of complex I-linked diseases still have no molecular explanation (Lazarou *et al.* 2009).

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GENETICS

Supporting Information
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A Forward Genetic Screen Identifies Mutants Deficient for Mitochondrial Complex I Assembly in *Chlamydomonas reinhardtii*

M. Rosario Barbieri, Véronique Larosa, Cécile Nouet, Nitya Subrahmanian, Claire Remacle and Patrice P. Hamel

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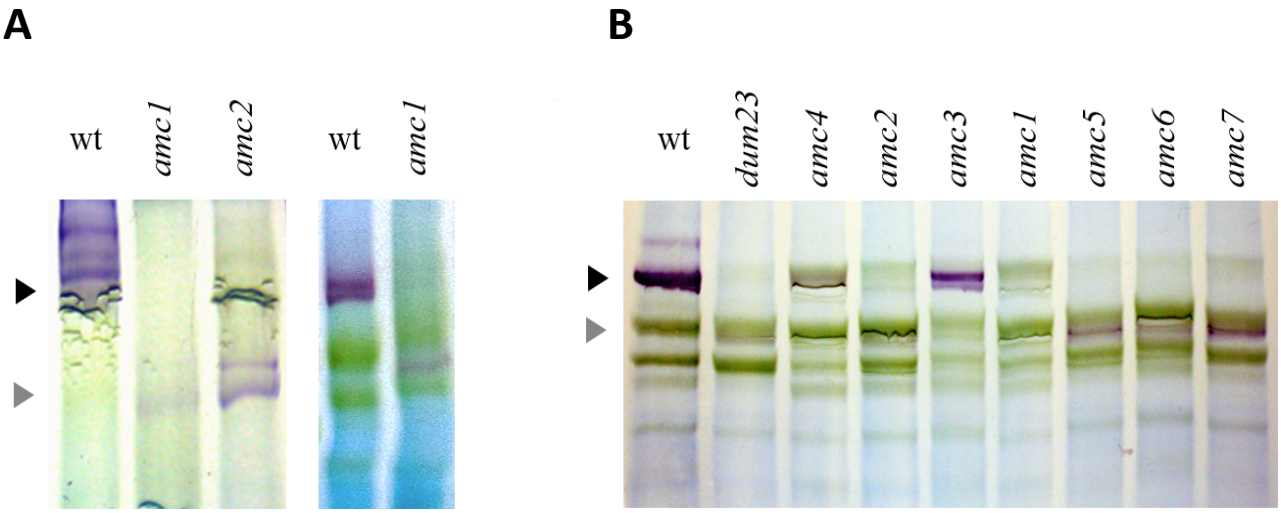

FIGURE S1.—Accumulation of a 700 kDa subcomplex in the *amc* and *dum23* mutants. (A). Detection of Complex I activity in wild type (wt), *amc1* and *amc2* strains. The 700 kDa subcomplex is detected in *amc1* and *amc2*. (B). Detection of Complex I in wild type (wt), *amc1* to *7* and *dum23*. The 700 kDa subcomplex that is detected in the *dum23* mutant, which carries a molecular lesion in the *nd5* gene, also accumulates in *amc1*, *2*, *5*, *6* and *7*. Note the low accumulation of Complex I holoenzyme in *amc4*. (A) and (B). Detection of Complex I and the 700 kDa subcomplex via NADH dehydrogenase activity *in-gel* staining on membrane fractions separated by BN-PAGE. 150 μg of protein (partially purified membranes were loaded per lane). The black and grey arrowheads indicate the position of mature Complex I and the 700 kDa subcomplex, respectively.

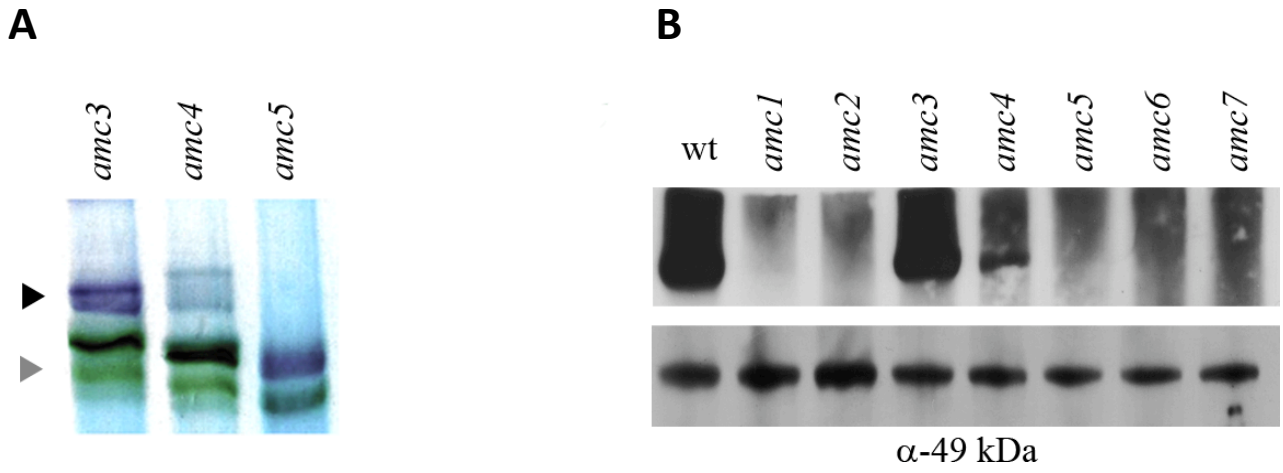

FIGURE S2.—The *amc3* mutant accumulates low amounts of Complex I. (A). Detection of Complex I and the 700 kDa subcomplex via NADH dehydrogenase activity. Membrane fractions of *amc3*, *4* and *5* were separated by BN-PAGE and stained *in gel* for NADH dehydrogenase activity. The black and grey arrowheads indicate the position of mature Complex I and the 700 kDa subcomplex, respectively. (B). Immunoblot analysis of membrane fractions from wild type *amc1* to *7* separated by BN-PAGE using anti-49 kDa. 150 μg protein of partially purified membranes were loaded per lane. The bottom panel shows a non specific band detected by the anti-49 kDa that serves as a loading control.

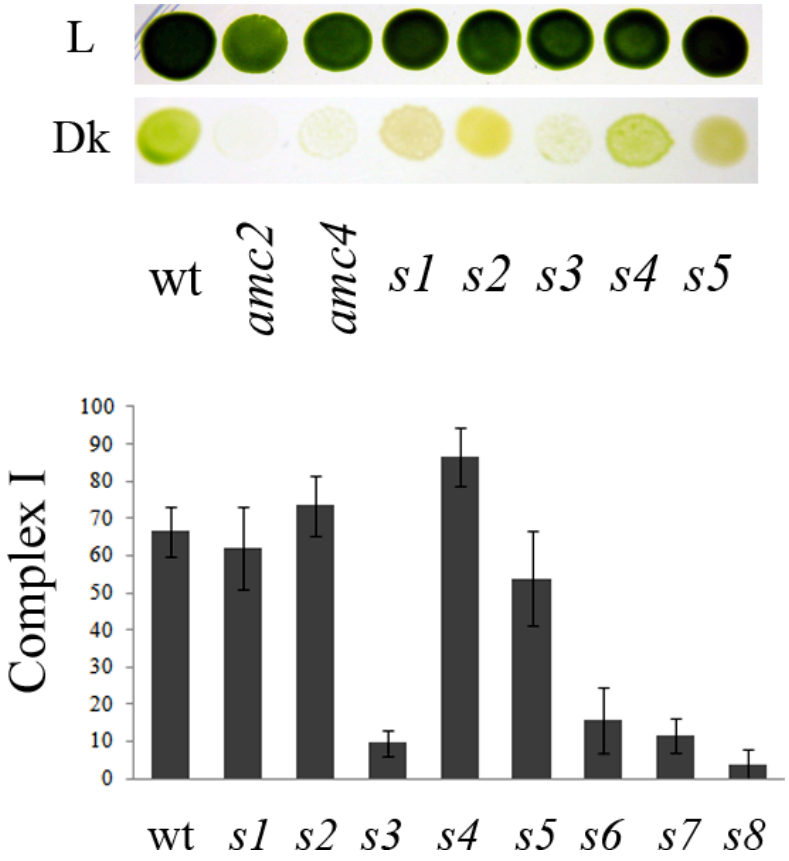

FIGURE S3.—*amc2* and *amc4* define alleles of different *AMC* loci. *(top)* Light (L) versus dark (DK) growth of wild type (wt), *amc2*, *amc4* and several representative spores (*s1* through *s5*) from the *amc2 x amc4* cross. Cells were plated on acetate containing medium and incubated three days in the light and five days in the dark. (*bottom*) Complex I activities (nmol NADH oxidized per minute per mg protein) of wild type (wt) and representative wild type (*s1*, *s2*, *s4*, *s5*) and *sid* spores (*s3*, *s6*, *s7*,*s8*) originating from the *amc2 x amc4* cross. Columns represent the average of ten independent determinations, with S.E. as the error bars.

GCATTGTGTCGACGAAGGCTTTTGGCTCCTCTGTCGC
TGTCTCAAGCAGCATCTAAagtAGCAGGGGGCGGCGGA
GAGCAGGGGCGGGAGGGCGGGAACTGGGTAAGGTTCTA
AGCGGAGGAGGGGCCAGGAGGAGAGGAGGAGCGGGAG
AAAATAGGTGGAGCGCGATTACTGCAGGAGTGCCCGGT
GCTGTCGTGAGCCCGCCCATCGTTGC

FIGURE S4.—Sequence of TAIL-PCR product obtained from the *amc5* mutant. The capitalized letters in bold correspond to the sequence of the *HSP70/RBCS2* promoter in the insertional cassette interrupting the *NUOB10* gene. The small letters indicate additional nucleotides that are not part of the insertional cassette or the *NUOB10* genomic sequence and probably were incorporated upon insertion at the integration site. The underlined capitalized letters correspond to the reverse complement sequence of bases 419 to 573 of the *NUOB10* intron 3.