A spontaneous tRNA suppressor of a mutation in the Chlamydomonas reinhardtii nuclear MCD1 gene required for stability of the chloroplast petD mRNA

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ABSTRACT
Numerous nuclear gene products are required for the correct expression of organellar genes. One such gene in the green alga Chlamydomonas reinhardtii is MCD1, whose product is required for stability of the chloroplast-encoded petD mRNA. In mcd1 mutants, which are non-photosynthetic, petD mRNA is degraded by a 5′–3′ exonuclease activity, resulting in a failure to synthesize its product, subunit IV of the cytochrome b6/f complex. Here, we report the sequence of the wild-type MCD1 gene, which encodes a large and novel putative protein. Analysis of three mutant alleles showed that two harbored large deletions, but that one allele, mcd1-2, had a single base change resulting in a nonsense codon near the N-terminus. This same mutant allele can be suppressed by a second-site mutation in the nuclear MCD2 gene, whereas mcd2-1 cannot suppress the deletion in mcd1-1 (Esposito, D. Higgs, D.C. Drager, R.G. Stern, D.B. and Girard-Bascou, J. (2001) Curr. Genet., 39, 40–48). We report the cloning of mcd2-1, and show that the mutation lies in a tRNA_Ser(CGA), which has been modified to translate the nonsense codon in mcd1-2. We discuss how the existence of a large tRNA_Ser gene family may permit this suppression without pleiotropic consequences.

INTRODUCTION
In photosynthetic eukaryotes, a significant portion of the nuclear genome encodes proteins required for correct functioning of the plastid. A subset of these genes encodes factors that participate in gene expression, either at the transcriptional or post-transcriptional level. Both forward and reverse genetic approaches have been used to identify and study these nuclear gene products, primarily in maize, Arabidopsis, and the unicellular green alga Chlamydomonas reinhardtii [reviewed in (1–3)].

One class of mutations that is largely unique to Chlamydomonas affects the stability of individual chloroplast mRNAs. This has led to the identification and in some cases cloning of the corresponding nuclear genes, including NAC2, required for the stability of psbD mRNA (4), MCA1, which stabilizes petA mRNA (5), MBB1, which stabilizes psbB mRNA (6) and MDB1, which stabilizes atpB mRNA (7). We have previously reported that mutations in the MCD1 gene lead to instability of the petD transcript, and that because degradation in this background can be blocked by a 5′-untranslated region (5′-UTR) polyguanosine sequence, a 5′–3′ exonucleolytic mechanism is involved (8).

The mechanism by which the nucleus-encoded proteins prevent this degradation is still unresolved, but available data suggest that multiprotein complexes containing the genespecific factors bind to sequence elements in the 5′-UTR to prevent degradation and perhaps, stimulate translation. These data include the demonstration that Mbb1 and Nac2 are members of RNA-containing complexes (4,6), and the finding that cis element mutations in the 5′-UTRs of psbD (9) and petD (10), for example, can phenocopy the cognizant nuclear mutants.

One way to identify additional proteins that participate in RNA stability regulation is to screen for second-site suppressors of the above-mentioned mutants, and such a screen was carried out previously by plating non-photosynthetic mcd1-2 cells on minimal medium requiring photosynthesis. A spontaneous semi-dominant suppressor was isolated, and the mutation in this unlinked nuclear suppressor was termed mcd2-1 (11). Curiously, mcd2-1 suppressed mcd1-2 but not mcd1-1, defining an allele-specific interaction. Since both mcd1-1 and mcd1-2 were expected to be point mutants as they resulted from chemical and UV mutagenesis, respectively, mcd2-1
was hypothesized to encode a change-of-function mutation, perhaps in a protein which interacted with Mcd1.
The study cited above also found that mcd2-1 was tightly linked to the ARG7 gene, which encodes argininosuccinylase, and had been previously cloned. This linkage, along with the newly available Chlamydomonas nuclear genome sequence (12), offered an opportunity to isolate the MCD2 gene, and learn how the mcd2-1 mutation might specifically suppress mcd1-2. Here, we report the cloning of MCD1 from the insertional allele mcd1-3, and the cloning of mcd2-1 based on the analysis of mcd1 mutations and its linkage to ARG7. We show that mcd2-1 is an amber suppressor tRNA, which represents the first mutation of this type to be found in plants using an undirected forward genetic screen.

MATERIALS AND METHODS

Culture conditions and quantification of chlorophyll

Chlamydomonas reinhardtii strains used in this study are listed in Table 1. Unless otherwise noted, cells were grown in Tris–acetate–phosphate (TAP) medium (13) in the light. Chlorophyll was quantified as described previously (13).

Nuclear transformation and molecular genetic analysis

Chlamydomonas cells were transformed either by the glass bead procedure (14) or by electroporation (15), with several modifications. For the glass bead procedure, mcd1-1, cw15 cells were incubated <2 h in either minimal medium, or in N-free TAP medium (13) prior to transformation with 2–5 μg cosmids or plasmid DNA. Cosmids were not digested prior to transformation. For electroporation, CC-125 or mcd1-2 cells were treated with autolysin, centrifuged and concentrated 100-fold in TAP medium containing 60 mM sucrose. DraI-modifications. For the glass bead procedure, bead procedure (14) or by electroporation (15), with several

Table 1. Strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| CC-124 | WT       | Chlamydomonas Stock Center |
| CC-406 | cw15     |        |
| F16.6  | mcd1-1   |        |
| F16.6.A20 | mcd1-1   | This study |
| F16cw  | mcd1-1, cw15 | This study |
| 670.1  | mcd1-2   |        |
| 715.90a | mcd1-3   | This study |
| R1.670 | mcd1-2, mcd2-1 |        |
| R1.670.5 | mcd1-2, mcd2-1 | This study |
| R1.670.cw15.12 | mcd1-2, mcd2-1, cw15 | This study |
| R1.670.cw15.22 | mcd1-2, mcd2-1, cw15 | This study |

*This original mutant and all derivatives (e.g. 7-90.33) have identical genotypes and phenotypic characteristics, except some derivatives exhibited better survival of tetrad progeny.

For genetic analysis of mcd1-3, a better-crossing derivative of strain 7-90 was created by crossing to CC-2986 (arg2, nit1, mt); this strain was named 7-90.33. When 7-90.33 (mt) was crossed to strain F16.6 (mcd1-1, mt), no photoautotrophic (low chlorophyll fluorescence) recombinants were recovered, indicating tight linkage of the mutant loci. When CC-2986 was crossed to an intermediate version of mcd1-3, strain 7-90.1, co-segregation of arginine prototrophy and high chlorophyll fluorescence indicated tight linkage of the inserted ARG7 gene and the mcd1 mutant phenotype. For molecular analysis, total Chlamydomonas DNA was extracted as described previously (16). One microgram of total DNA digested by BamHI was transferred to nylon membranes using standard techniques (17).

RT–PCR

Total Chlamydomonas RNA was extracted with TRI-Reagent according to the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). Poly(A) RNA was isolated using the PolyATtract mRNA Isolation System (Promega, Madison, WI) and was treated with RQ1 RNase-Free DNase (Promega). Poly(A) RNA was used as a template to synthesize cDNA with 10 μl of SuperScript III RNA H− reverse transcriptase (Invitrogen) and 0.25 μM of primer Q (5’-GACTGCGTAGCAATGGGAAGCAGCATCAGCTAA-GCTTTTTTTTTTTTTTTTTTTT-3’) with the buffer containing 1 U/μl recombinant RNasin ribonuclease inhibitor (RNasin; Promega) and 1 μl betaine. The cDNA was used as a template for PCR with Ex Taq DNA polymerase (Takara, Otsu, Japan) with 1 μl betaine. Primer Q (5’-GACTGCGTAGCAATGGGAAGCAGCATCAGCTAA-GCTTTTTTTTTTTTTTTTTTT-3’) and gene-specific primers were used for amplification of the 3’ end, and pairs of gene-specific primers were used for internal regions. PCR conditions were 95°C for 30 s, 60°C for 1 min and 72°C for 1.5 min/kb, for 35 cycles.

Primer extension with dideoxynucleoside triphosphates

The primer tRNA-Ser PPE (5’-ATGCGGGAGATCCCATC-3’) was labeled at its 5’ end with 100 μCi of γ-[32P]ATP and 10 U of T4 polynucleotide kinase and gel-purified. RNA was isolated from Chlamydomonas cells cultured in TAP medium for 7 days and then transferred into minimal medium for 7 days. Five micrograms of total RNA were incubated with 4 μl of 5× first-strand buffer (Invitrogen), 0.5 μl of RNasin (40 U/μl), 2 μl labeled primer (>106 c.p.m.) at 85°C for 15 min and then transferred to 50°C for 30 min. The reaction mixture was supplemented with 1 μl of 0.1 M DTT, 40 U of SuperScript III, 1 μl each of 2 mM dNTPs and 1 μl of 4 mM ddCTP or 2 mM ddTTP. The reaction mixtures with total volume of 20 μl were incubated at 50°C for 20 min, and stopped by addition of 18 μl of formamide and 4 μl of 10× TBE containing loading dyes. Reaction products were resolved in 17.5% denaturing polyacrylamide gels and visualized and quantified using a Storm scanner (Molecular Dynamics, Sunnyvale, CA).

Plasmid construction and PCR

Random Mu transposon insertions into the genomic MCD1 fragment were made using the Finnzymes Template Generation System (MJ Research, Inc., Waltham, MA). To clone the mcd1-2 locus, a 6.47 kb fragment was amplified using Ex Taq
DNA polymerase, 0.25 μM primers (mcd1 5′-2′, 5′-ATGTA-CCGGTCTTGATGTAGCCGT-3′; mcd1 3′-2′, 5′-CCTGC-CCGTTTACATTTCCATCCCA-3′) and total DNA as the template in manufacturer-supplied buffer containing 1 M betaine. PCR conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 50 s, 60°C for 50 s, and 72°C for 9 min, with a final extension step of 72°C for 5 min. We subsequently discovered that 30–35 cycles of 98°C for 5 s, 60°C for 30 s and 72°C for 9 min (~1.5 min per kb) gave more consistent results. Genomic fragments of 1319 bp containing WT or mutant tRNA<sup>Ser</sup> (CGA)-1 were amplified by PCR from total DNA of CC-124 (WT) or R1.670 (mcd2-1) cells to see whether they could complement the mcd1 phenotype. Transformations of two overlapping cosmids, PF28 cosmids 3 and 4, yielded significant and reproducible numbers of photoautotrophic colonies. These two cosmids contained a common 12 kb BglII/HindIII fragment, which was the smallest fragment capable of complementation (data not shown). The sequence of 9.4 kb of this fragment starting at the BglII site was subsequently determined using the Mu transposon to generate randomly located primer binding sites. Once an unambiguous sequence was generated, we used GreenGenie, a gene finder program for *Chlamydomonas* (http://www.cse.ucsc.edu/~dkulp/cgi-bin/greenGenie), to predict any genes. This program indeed generated a gene model, centered within the sequenced region.

To define the borders of *MCD1* within the 12 kb fragment using functional criteria, we took advantage of the Mu insertion clones used for DNA sequencing. A series of clones with insertions spaced throughout the 12 kb region were transformed into F16cw, and photoautotrophic colonies were selected. Because the transposon has two HindIII sites, it could be predicted that if the transposon had inserted outside of *MCD1*, the plasmid could complement the mutation following HindIII digestion, whereas if Mu had inserted with *MCD1*, whether an intron or exon, it could not. Twenty-eight clones were tested in this way, and the results defined the maximum and minimum extent of *MCD1* as 6.2 and 5.6 kb, respectively. This region was mostly consistent with the GreenGenie prediction: if the ‘A’ of the first ATG in gene model is defined as +1, the GreenGenie model covered 1–5365 bp, whereas the maximum and minimum regions deduced from transformations were from −577 to +5701, and from −75 to +5577.

**RESULTS**

**Isolation of the *MCD1* gene**

*C.reinhardtii* mcd1 mutants are non-photosynthetic because of the instability of petD mRNA, which encodes subunit IV of the cytochrome b<sub>6</sub>f complex (SU1V). Several mutant alleles of *mcd1* were available, including the presumed point mutations *mcd1*-1 (strain F16) and *mcd1*-2 (strain CF670), which were described previously (11). A third allele, *mcd1*-3, is defined by the strain 715.90, which was generated from the arginine auxotroph 715 (arg<sup>7</sup>, cw<sup>15</sup>, mt<sup>−</sup>) by random integration of the plasmid pARG7.8p3, which encodes a copy of the ARG7 gene containing a 392 bp HpaI fragment of φX174 DNA (19). Genetic analysis of *mcd1*-3 showed that the non-photosynthetic phenotype co-segregated with the arginine prototrophy conferred by the transformed vector, and that this mutation was closely linked to the *MCD1* locus (see Materials and Methods). We therefore assumed that this strain contained the pARG7.8p3 insertion at *MCD1* locus and named it *mcd1*-3.

To clone the *MCD1* gene, we began by performing DNA gel blot analysis of KpnI-digested *mcd1*-3 DNA, probed with the 392 bp HpaI fragment of φX174 (Hpa-392; data not shown). Fragments of 9.5, 8.5 and 6 kb were detected and this, together with other restriction digestions, suggested that *mcd1*-3 contained tandem insertions of pARG7.8p3, and that a 6 kb fragment represented one of the borders of the insertion. A plasmid library with 5.5–6 kb KpnI fragments was therefore generated from *mcd1*-3 DNA, and screened using the Hpa-392 probe. Plasmids containing the desired insert were purified and sequenced. Interestingly, these clones contained sequences from the *PF28/ODA2* gene, which encodes the dynein gamma chain of flagellar outer arms (20), and is located on linkage group XI (21). This indicated that the *MCD1* locus was close to *PF28*, and genomic clones containing *PF28* might include *MCD1* as well.

We obtained *PF28*-containing cosmids from George Witman (Worcester Foundation for Experimental Biology) and transformed them into F16cw (*mcd1*-1, cw<sup>15</sup>) cells to see whether they could complement the *mcd1* phenotype. Transformations...
positions 1081–1104 (PROSITE accession no. PS00527), however this motif is considered to arise frequently as a false positive. The apparent uniqueness of Mcd1 contrasts with the three previously cloned Chlamydomonas cpRNA stability factors, which possess either tetratricopeptide (TPR) or pentatricopeptide (PPR) repeats.

Analysis of mcd1 mutant alleles

The three mutant alleles of mcd1 mentioned above were mcd1-1, generated by 5-fluorouracil treatment; mcd1-2, which resulted from UV mutagenesis; and mcd1-3, which is the ARG7::MCD1 insertional mutant. As a first step in characterizing the mutation in each allele, DNA from each strain was digested with BamHI and subjected to DNA gel blot analysis (Figure 1A) using the mixed probes indicated in Figure 1B.

Figures 1. Gene structure of MCD1 and its mutants. (A) DNA gel blot analysis of wild-type (WT) and the mcd1 mutants indicated above each lane. Total DNA from WT (CC-124), mcd1-1 (strain F16.A20), mcd1-2 (strain 670.1) and mcd1-3 (strain 7-90.33) were digested with BamHI and the resultant blot was hybridized with the 32P-labeled probes indicated at the top of panel, which also shows a diagram of the WT MCD1 locus. In this diagram, black rectangles represent exons and gray rectangles represent the 5'-UTR, the three introns and the 3'-UTR. BamHI restriction sites 'B' and the sizes of fragments (in kb) identified in panel A are shown. MCD1 is adjacent to PF28, whose C-terminal end on the opposite strand is shown. Below the gene diagram, the nucleotide and amino acid sequences surrounding the mcd1-2 mutation are shown.

Frags of 5.2 and 2.2 kb were detected in the wild-type control and in mcd1-2, whereas mcd1-1 and mcd1-3 samples only gave nonspecific hybridization at the position of undigested DNA. These results suggested that mcd1-1 had suffered a large deletion, and that in addition to the known insertion of ARG7, mcd1-3 had a deletion around MCD1. In contrast, mcd1-2 had a restriction pattern consistent with a point mutation(s) or small insertion/deletion.

To identify the mutation in mcd1-2, the entire mcd1-2 locus was amplified by PCR using primers mcd1 5'-2 and mcd1 3'-2, which yielded a fragment extending from –625 to +5848. This 6.47 kb fragment was cloned and sequenced, revealing an A to T mutation that changed the lysine codon AAG to the amber stop codon TAG at amino acid position 113 (Figure 1B). The existence of this mutation in the genome was verified in two ways. First, multiple, independent PCRs were carried out, and the products always contained this mutation when amplified from mcd1-2 DNA. Second, the A to T mutation creates a restriction endonuclease recognition site for the enzyme AvrII (CCTAGG). When DNA was amplified from mcd1-2, but not from other strains, this restriction site was present in the PCR product (data not shown). Taken together, the sequence suggests that in mcd1-2, translation initiation would result in production of a truncated, and possibly unstable, protein of 112 amino acids, which would be highly unlikely to be functional.

Identification of the suppressor mutation in mcd2-1

As described in the Introduction, mcd2-1 is a semi-dominant mutation closely linked to the ARG7 locus; no recombinants between mcd2 and ARG7 were found in our previous work (11). Because mcd2-1 could suppress mcd1-2 but not mcd1-1, we hypothesized that Mcd2 was most likely a protein factor that interacted with Mcd1, and presumed that mcd2-1 carried a suppressible lesion, whereas mcd1-1 did not. However, the fact that mcd2-1 contains a nonsense mutation very early in the coding region argued against this hypothesis, and raised the possibility that mcd2-1 might encode a nonsense suppressor.

Because MCD2 is linked to ARG7, we examined version 2 of the Chlamydomonas nuclear genome sequence (http://genome.jgi-psf.org/chlre2/chlre2.home.html) around ARG7, and found a predicted tRNASer(CGA) at a 320 kb distance from ARG7 on scaffold 35 (Figure 2A). This tRNA has been previously found to become an amber (UAG) suppressor in other organisms, originally in Escherichia coli [reviewed in (22)]. To determine whether mcd2-1 carried a mutation in this tRNA, we amplified and sequenced this region of the mutant mcd2-1, mcd2-2. This revealed a point mutation that changed the anticodon CGA to CUA, which would allow the mutated tRNA to recognize the amber stop codon. To confirm that this mutation truly represented the mcd2-1 mutation, we sequenced this region in 11 mcd2-1 strains derived from independent backcrosses, and found that all of them had the same mutation. The wild-type gene was designated as tRNASer(CGA)-1, and the mutant allele as tRNASer(CGA)-1mcd2.

Expression of the mutant tRNA

Because the same tRNA point mutation was found in each mcd2 strain, tRNASer(CGA)-1mcd2 was an excellent candidate
for the suppressor. We therefore examined its expression using a primer extension assay. Experimental design was complicated by the fact that the available nuclear genome sequence includes eight other tRNA genes which are nearly identical to tRNASer(CGA)-1 (Figure 2B; Table 2). This was overcome by using appropriate ddNTPs during primer extension, which would terminate elongation by reverse transcriptase at a position in the mutant tRNA calculated to yield a differently sized product than in the wild-type. The primer chosen for this experiment is drawn at the bottom of Figure 2B.

Predicted and actual results from primer extension are shown in Figure 3. Panel A shows that when no ddNTPs are included, the major product from the collective WT tRNA Ser would be 26 nt. This premature termination results from methylation at the cytidine marked by an asterisk in MCD2 and other similar tRNA<sup>Ser</sup> genes. (A) Scaffold 35, which includes ARG7, derived from the Chlamydomonas genome portal v2.0. The positions of ARG7 and the chromosomal marker GP123 (vertical brown lines) are labeled. The five tRNA genes present in the region are shown as vertical brown lines in the lower part of the diagram, and MCD2 is labeled. Red areas are unsequenced regions, whose sizes are estimated. (B) Alignment of selected Chlamydomonas tRNA<sup>Ser</sup> genes. The genes shown are highly similar to MCD2, and needed to be taken into consideration in designing a primer extension expression experiment. Anticodons are boxed, and asterisks indicate positions where a base difference exists in at least one gene. The lower two lines compare the WT and mcd2-1 mutant sequences, with the point mutation shown in bold. The horizontal arrow represents the primer used for the assay shown in Figure 3 and described in the text.

Table 2. Positions of selected tRNA<sup>Ser</sup> genes in the nuclear genome<sup>a</sup>

| Gene name         | JGI Chlamydomonas genome v2.0 |
|-------------------|--------------------------------|
| tRNA<sup>Ser</sup> (CGA)-1 | scaffold_35: 681829–681909 |
| tRNA<sup>Ser</sup> (CGA)-2 | scaffold_10: 21195–21275   |
| tRNA<sup>Ser</sup> (CGA)-3 | scaffold_10: 14902–14822   |
| tRNA<sup>Ser</sup> (CGA)-4 | scaffold_48: 598155–598235 |
| tRNA<sup>Ser</sup> (CGA)-5 | scaffold_48: 598768–598848 |
| tRNA<sup>Ser</sup> (AGA)-1 | scaffold_10: 20500–20580   |
| tRNA<sup>Ser</sup> (AGA)-2 | scaffold_10: 15591–15511   |
| tRNA<sup>Ser</sup> (AGA)-3 | scaffold_13: 711455–711535 |
| tRNA<sup>Ser</sup> (AGA)-4 | scaffold_13: 715098–715178 |

<sup>a</sup>tRNAs were identified using MCD2 in a Blast search of available nuclear genome sequence.

Figure 2. MCD2 and other similar tRNA<sup>Ser</sup> genes. (A) Scaffold 35, which includes ARG7, derived from the Chlamydomonas genome portal v2.0. The positions of ARG7 and the chromosomal marker GP123 (vertical brown lines) are labeled. The five tRNA genes present in the region are shown as vertical brown lines in the lower part of the diagram, and MCD2 is labeled. Red areas are unsequenced regions, whose sizes are estimated. (B) Alignment of selected Chlamydomonas tRNA<sup>Ser</sup> genes. The genes shown are highly similar to MCD2, and needed to be taken into consideration in designing a primer extension expression experiment. Anticodons are boxed, and asterisks indicate positions where a base difference exists in at least one gene. The lower two lines compare the WT and mcd2-1 mutant sequences, with the point mutation shown in bold. The horizontal arrow represents the primer used for the assay shown in Figure 3 and described in the text.

Figure 3. Expression of a tRNA<sup>Ser</sup> encoded by mcd2-1. (A) Design of primer extension experiment using ddNTPs, showing the WT and mutant versions of tRNA<sup>Ser</sup> (CGA)-1 (MCD2). The anticodon is underlined and the mcd2-1 point mutation is shown in bold. The horizontal arrows represent the 3' part of the primer, and numbers to the right predicted product sizes in nucleotides, which are terminated by hydroxy (–OH) or dideoxy (–H) nucleosides. The asterisk indicates a putative methylation site discussed in the text. (B) Primer extension assay without or with ddNTPs, as indicated across the top. The arrowhead indicates the mcd2-1-specific band. Total RNA from cells with WT tRNA<sup>Ser</sup> (CGA)-1 was derived from CC-124 (WT; lanes 2, 8 and 14) and CC-406 (cw15; lanes 5, 11 and 17). tRNAs carrying the mcd2-1 mutation were isolated from the following strains: R1.670 (lanes 3, 9 and 15), R1.670.5 (lanes 4, 10 and 16), R1.670.cw15.12 (lanes 6, 12 and 18) and R1.670.cw15.22 (lanes 7, 13 and 19).
Figure 3A, which has been previously reported for this tRNA in tobacco (23). With the inclusion of ddTTP, all reverse transcription reactions should terminate at the 3'-A of the anticodon, generating 21 nt products. However, the presence of ddCTP should differentiate between tRNA<sup>Ser</sup>(CGA)-1 and tRNA<sup>Ser</sup>(CGA)-1<sup>mcd2</sup>, with the mutant anticodon allowing readthrough to the 26 nt size, rather than ddCTP-mediated termination at 24 nt. Thus, a 26 nt product derived uniquely from mcd2 RNA preparations, in the presence of ddCTP, would be diagnostic of expression of the amber suppressor.

Figure 3B shows the results of primer extension experiments, which bore out the predictions stated above. Without ddNTPs, most of the products were 26 nt, although some longer (>30 nt) products were also observed (lanes 2–7). However, when ddCTP was used, strong signals at 24 nt were detected for each sample (lanes 8–13), whereas a 26 nt band could be detected uniquely in the four independent samples derived from mcd2 cells (lanes 9, 10, 12 and 13). RNAs from mcd2 cells also generated a 24 nt product, derived from non-mutated tRNA<sup>Ser</sup> transcribed from other loci. The fact that only 21 nt products could be detected when ddTTP was used (lanes 14–19) suggested that ddNTP incorporation can fully impede extension under the conditions used. We therefore concluded that the 26 nt signals represented the expression of tRNA<sup>Ser</sup>(CGA)-1<sup>mcd2</sup> in mcd2.

**Ectopically expressed tRNA<sup>Ser</sup>(CGA)-1<sup>mcd2</sup> complements mcd1-2**

To confirm that this mutant tRNA could suppress the amber mutation at a phenotypically significant level, we performed a complementation experiment. To do this, genomic fragments containing tRNA<sup>Ser</sup>(CGA)-1 were amplified from wild-type and mcd2-1 DNA, and inserted into pMS171, a Chlamydomonas vector containing a zeocin resistance marker (Figure 4A). The amplified fragment was large enough to very likely contain complete expression elements for the tRNA gene. The resulting plasmids, or the original vector, were then used to transform strain 670.1 (mcd1-2), and colonies were selected on zeocin-containing TAP medium, to which the ble gene confers resistance. As expected, numerous transformants were obtained with each plasmid, since TAP medium does not select for photosynthesis.

To see whether the ectopically expressed suppressor tRNA could restore photosynthetic activity to mcd1-2, transformants and suitable controls were replica-plated on TAP and minimal media, as shown in Figure 4B. All strains grew on TAP medium, although the non-photosynthetic strains showed less robust growth, a phenomenon which is commonly observed with Chlamydomonas. On the other hand, only five strains were viable on minimal medium. These were CC-406 (MCD1) cells, two independent clones of mcd1-2 and mcd2-1, and two independent strains where mcd1-2 had been transformed with pMS171 co-expressing tRNA<sup>Ser</sup>(CGA)-1<sup>mcd2</sup>. When mcd1-2 was transformed with pMS171 alone, or with pMS171 co-expressing (non-suppressing) tRNA<sup>Ser</sup>(CGA)-1, cells failed to grow on minimal medium. This indicates that the suppressor tRNA confers the biological function of allowing sufficient expression from the mcd1-2 locus to permit photoautotrophic growth.

It should be noted that occasional escapes were seen in the transformation experiments described above. Although 16/16 zeocin-resistant mcd1-2 transformants expressing the suppressor tRNA were able to grow on minimal medium, 1/16 transformants from pMS171 alone, and 2/14 pMS171 transformants co-expressing non-suppressing tRNA<sup>Ser</sup> also exhibited some growth on minimal medium. Although the reasons for these occasional unexpected growth phenotypes was unclear, the fact that all the transformants possessing the mcd2-1 tRNA gene recovered photosynthetic activity strongly suggested that the transformed mutant tRNA could suppress the mcd1-2 mutation.

**DISCUSSION**

In this report, we describe the isolation of MCD1 and MCD2, two genes which were identified by mutations affecting the stability and possibly translation of Chlamydomonas petD mRNA. The predicted MCD1 gene product, a novel 1553
amino acid protein, is posited to interact with of 5'-UTR of petD mRNA and protect this transcript from 5'-3' exoribonucleolytic degradation. mcd1-2, a semi-dominant, allele-specific suppressor of mcd1-2, was found to encode a nonsense suppressor tRNA. In the haploid state, mcd1-2 and mcd2-1 show partial restoration of MCD1 function (11), consistent with incomplete suppression of the mcd1-2 amber mutation.

Several nuclear genes are known whose products are required for chloroplast mRNA stability (see Introduction), but their precise mechanisms of action are poorly understood. To clarify how Mcd1 might protect the petD mRNA 5' end, we sought to identify the MCD1 gene, and ultimately obtained cDNA and genomic sequence. Surprisingly, the deduced Mcd1 protein does not contain any significant identity to proteins in the database, nor any motifs that hint at its mode of action. This contrasts with the three other Chlamydomonas cpRNA stability regulators, which possess TPR (4,6) or PPR (5) protein–protein interaction motifs. These domains are present in vastly expanded numbers in land plants, and are thought or known to be frequent regulators of organellar processes (24,25). Based on the currently available Chlamydomonas nuclear genome sequence, however, they are relatively uncommon in the algae: v2.0 has seven genes annotated as containing PPR motifs, and 80 annotated as containing TPR motifs. This suggests that Mcd1 may contain a previously unknown domain involved in protein–protein and/or protein–RNA interactions.

The lack of relationship between Mcd1 and known chloroplast regulatory proteins may reflect a small sample size, but might also be related to unique features of petD in Chlamydomonas. Located downstream of petB in organisms as diverse as cyanobacteria (26), land plants (27) and the Chlamydomonas relative Chlorella vulgaris (28), petD in Chlamydomonas is located downstream of petA, transcribed from both the petA and its own promoter, and matured to its monocistronic form through uncharacterized RNA processing steps (29,30). One might speculate that Mcd1 evolved to serve dual roles of RNA maturation and 5' end protection, and indirect evidence suggests that Mcd1 may also play a role in translational activation of petD mRNA (10,31). Biochemical analysis of Mcd1, and any homologues discovered in the future, would be highly informative.

We originally hypothesized that the mcd2-1 mutation was in an Mcd1-interacting protein, as it had been obtained in a classical suppressor screen designed to yield such a result. The finding that mcd2-1 possessed a point mutation changing the lysine codon AAG (Lys-113) to the amber stop codon TAG led us to believe that mcd2-1 might be a nonsense suppressor mutation. Although in principle a truncated protein could be reactivated by a second-site suppressor in an interacting protein, K113 is near the N-terminal of Mcd1, and any protein expressed in mcd1-2 would be expected to consist largely of a chloroplast targeting signal. Our finding raises the possibility that suppressors isolated in analogous screens [e.g. (32)] could be of a similar nature.

To test the hypothesis that mcd2-1 expressed a suppressor tRNA, we first examined the genomic sequence around the ARG7 locus, to which mcd2-1 was closely linked. This led to a mutated tRNA<sup>Ser</sup> (CGA), which was subsequently found in all 11 strains carrying mcd2-1. Although the original mcd2 mutation was isolated only once, the 11 strains differ in their sources in that they were derived from a variety of crosses to test specificity or dominance of the mutation. Our hypothesis was supported by expression of an ectopic copy of the suppressor tRNA, which restored photosynthetic activity to the mcd1-2 mutant.

Nonsense suppression is a well-known phenomenon in prokaryotes and eukaryotes, but naturally occurring examples in plants are rare, to our knowledge. In Chlamydomonas, an amber suppressor tRNA was identified in the chloroplast (33), as it restored photosynthesis to a strain bearing a nonsense mutation in the rbcL gene, which encodes the Rubisco large subunit. Although the tRNA<sup>Trp</sup> which was mutated is a single-copy gene, the polyploid nature of the chloroplast allowed heteroplasmic maintenance of the suppressor. Curiously, the frequency of the mutant allele was ~70%, suggesting that a preponderance of tRNA<sup>Trp</sup>(CUA) was required for biological activity. This contrasts with the apparently minor contribution of mcd2-1 to total tRNA<sup>Ser</sup> transcription (Figure 3B). An important class of nonsense suppressors in plants are naturally occurring tRNAs which are able to promote readthrough of stop codons, particularly in single-stranded RNA viruses [reviewed in (34)]. These contrast with mcd2-1 because they possess a wild-type anticodon, but are able to recognize the viral stop codons through sometimes unusual wobble base-pairing mechanisms.

The principle that mutant tRNAs in plants can promote nonsense suppression has been demonstrated through transient or stable transgenic approaches. In one example, tRNA<sup>Ser</sup> was engineered to become an amber suppressor, and transient expression resulted in 10% of β-glucuronidase activity relative to a gene without a nonsense codon (35). Stable expression of amber suppressor tRNA<sup>Ser</sup>, or other tRNAs, were similarly shown to confer GUS (36,37), luciferase (38) or chloramphenicol acetyltransferase (39) activity. As in the case of chloroplast tRNA suppression cited above, mcd2-1 is viable because wild-type tRNA<sup>Ser</sup>(CGA) genes are still expressed. As shown in Figure 2B, available Chlamydomonas sequence information reveals five tRNA<sup>Ser</sup>(CGA) genes, as well as four with an AGA anticodon. This may well be an underestimate; the Arabidopsis genome has 65 loci annotated as encoding tRNA<sup>Ser</sup>; four of which feature a CGA anticodon and the vast majority of the remainder AGA. Assuming that mcd2-1 represents 20% of the tRNA<sup>Ser</sup>(CGA) activity, expression of suppression is reasonable based on the fact that cognizant E.coli amber suppressor, supD, exhibited a 28% efficiency, second only to the 55% conferred by the Tyr amber suppressor [reviewed in (22)].

Our results suggest that Chlamydomonas, like other organisms, will be amenable to site-directed protein mutagenesis through expression of engineered suppressor tRNAs (40,41). The ability to recover MCD2 without a laborious map-based cloning approach also illustrates the utility of the newly available nuclear genome sequence, when combined with the powerful genetic approaches long utilized in Chlamydomonas (42). The tight linkage of MCD2 to ARG7 was indeed fortuitous, but the proliferation of chromosome markers (43) makes mapping of suppressors a fully practical endeavor, which should quickly enhance our understanding of how nuclear gene products (44–47) regulate chloroplast gene expression in this organism.
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