The mTERF protein MOC1 terminates mitochondrial DNA transcription in the unicellular green alga *Chlamydomonas reinhardtii*

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**ABSTRACT**

The molecular function of mTERFs (mitochondrial transcription termination factors) has so far only been described for metazoan members of the protein family and in animals they control mitochondrial replication, transcription and translation. Cells of photosynthetic eukaryotes harbour chloroplasts and mitochondria, which are in an intense cross-talk that is vital for photosynthesis. *Chlamydomonas reinhardtii* is a unicellular green alga widely used as a model organism for photosynthesis research and green biotechnology. Among the six nuclear *C. reinhardtii* mTERF genes is MTERF-like gene of *Chlamydomonas* (MOC1), whose inactivation alters mitorespiration and interestingly also light-acclimation processes in the chloroplast that favour the enhanced production of biohydrogen. We show here from in vitro studies that MOC1 binds specifically to a sequence within the mitochondrial rRNA-coding module S3, and that a knockout of MOC1 in the mutant *stm6* increases read-through transcription at this site, indicating that MOC1 acts as a transcription terminator in vivo. Whereas the level of certain antisense RNA species is higher in *stm6*, the amount of unprocessed mitochondrial sense transcripts is strongly reduced, demonstrating that a loss of MOC1 causes perturbed mitochondrial DNA (mtDNA) expression. Overall, we provide evidence for the existence of mitochondrial antisense RNAs in *C. reinhardtii* and show that mTERF-mediated transcription termination is an evolutionary-conserved mechanism occurring in phototrophic protists and metazoans.

**INTRODUCTION**

Most of our knowledge about mitochondrial gene expression and its regulation results from research carried out with non-phototrophic organisms, especially mammalian and yeast cells (1,2). In the case of phototrophic eukaryotes, there is an additional level of complexity, as metabolism in the mitochondria needs to be coordinated to that in the chloroplast (3). As yet the molecular mechanisms underpinning the regulation of gene expression in the mitochondria of plant cells, including the unicellular green alga *Chlamydomonas reinhardtii*, which is an established model organism widely used to study the regulation of nuclear and organelle gene expression, are unclear.

Mitochondria of *C. reinhardtii* retain a small, but information-dense, genome (4), which contains genes encoding eight proteins, including the complex IV subunit 1, five complex I subunits, apocytochrome b of complex III, a reverse transcriptase-like protein and a strain-dependent number of introns (5). The rRNA genes encoding large (L) and small (S) ribosomal rRNAs are split into modules encoding rRNA segments (eight L and four S modules), which are interspersed with one another or protein-coding and tRNA genes (6). All the mitochondrial rRNAs are encoded by the mitochondrial genome of *C. reinhardtii* (4), but the majority of tRNAs have to be imported from the cytosol (7). In vitro labelling experiments and physical mapping indicated that the mitochondrial DNA (mtDNA) of *C. reinhardtii* is a linear monomeric molecule (8), but electron microscopy (9) demonstrated that mtDNA preparations contain a small fraction of circular molecules. The telomere structure, known to be decisive for mtDNA architecture (10), of the *C. reinhardtii* mtDNA is unusual and differs from structures described for other organisms (11). Both strands of the *C. reinhardtii* mtDNA contain transcription units (12–14), and two sequences identified in the intergenic region located between the transcription units might act as a bi-directional promoter (15). Transcription of the *C. reinhardtii* mtDNA generates long polycistronic transcripts (12–14), which are further processed to yield the mature mRNAs. The use of few transcription initiation sites to drive the expression of large transcription units is a feature

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frequently found in mitochondria of protists (16) and mammals (1), whereas higher plant chondromes represent a more complex system with multiple transcription initiation sites (17).

Recent work has identified a nuclear gene of \textit{C. reinhardtii} termed mTERF-like gene of Chlamydomonas \textit{MOC1}, which plays a role in fine-tuning mitochondrial transcription on changes in illumination. Absence of \textit{MOC1} in the \textit{stm6} mutant causes a pleiotropic phenotype characterized by perturbed mitorespiration (18) and multiple effects on the physiological state of the plastid resulting in light-sensitivity (18,19) and, interestingly, the enhanced production of biohydrogen (20).

MOC1 is a member of the mTERF (mitochondrial transcription termination factor) family of transcription factors, which are found in metazoans (21) including higher plants (mono- and dicotyledonous) (22), but which are absent in fungal and prokaryotic genomes (21). Current knowledge about mTERF function was mainly obtained by the characterization of metazoan mTERF proteins (23). All vertebrate mTERF factors are targeted to the mitochondrial, where they are implicated in the regulation of transcription (24–28), translation (29) and replication (30,31). The knockout of mTERF2 (27) and tissue-specific inactivation of mTERF3 (26) in mice caused severe mitochondrial phenotypes resulting from aberrant transcription of the organellar genome, possibly at the level of transcription initiation. To date, three mTERF factors from different organisms have been shown to terminate mtDNA transcription (23). Human mTERF1 binds specifically to a sequence within the \textit{tRNA\textsubscript{Leu(UUR)}} gene and arrests RNA polymerase progression \textit{in vitro} (24,32). Simultaneous binding of mTERF1 to the termination site and the heavy strand promoter \textit{in vitro} (25) was proposed as a mechanism facilitating rapid re-initiation of transcription after termination at the \textit{tRNA\textsubscript{Leu(UUR)}} site, but evidence that the higher amount of rRNA relative to mRNA \textit{in vivo} depends on this mechanism remains to be provided. Another well-described mTERF protein is the \textit{Drosophila} homologue of human mTERF named DmTTF, which possesses two specific binding sites in the \textit{Drosophila} mtDNA and terminates transcription \textit{in vitro} (33) and \textit{in vivo} (34).

Given the current interest in developing strains of green algae with enhanced production of biohydrogen, we decided to investigate the molecular function of MOC1. To this end, we analysed possible DNA-binding properties of recombinant MOC1 and investigated the phenotypic consequences of an \textit{MOC1} knockout in the mutant \textit{stm6}. The sum of our data shows that mTERF-mediated transcription termination occurs in mitochondria of lower photosynthetic eukaryotes, for the first time demonstrating that this mechanism is not restricted to animal mitochondria. MOC1 binds specifically to an octanucleotide sequence within the mitochondrial rRNA-coding module \textit{S2}, and a loss of MOC1 increases read-through transcription at the \textit{S3}-binding site thereby causing elevated antisense RNA levels in the mutant \textit{stm6}. Impaired transcription termination in \textit{stm6} alters the mitochondrial transcriptome leading to a growth defect under strictly heterotrophic conditions.

**MATERIALS AND METHODS**

**Strains and culture conditions**

The MOC1 knockout mutant \textit{stm6} and the complemented strain B13 were generated as described by Schönfeld \textit{et al.} (18). Liquid cultures of \textit{C. reinhardtii} were cultivated mixotrophically (50 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1}) and heterotrophically (dark cultivation) in Tris–acetate phosphate medium [TAP, (35)] or phototrophically in high-salt medium (35). Dark growth as well as phototrophic low light (50 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1}) and high-light growth (1500 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1}) was analysed in an FMT 150 photobioreactor (Photon Systems Instruments, Brno, Czech Republic) with continuous monitoring of the optical density at 735 nm as a measure for cell density.

**Plasmid construction**

To construct expression vector pRSET-A(Thr) \textit{MOC1ΔN46}, the \textit{MOC1} cDNA [(36); clone HCL008g04; Accession No. AV640010; Kasuza DNA Research Institute (Kisarazu, Japan)] was first amplified with primers MOC1-\textit{MTS_A} fw/rev (Supplementary Table S1), and the resulting polymerase chain reaction (PCR) product was sub-cloned into vector pGem T-Easy (Promega). The MOC1-coding sequence lacking the first 46 amino acids of the N-terminus was then either cloned into a derivative (kind gift of Dr Ernesto Cota Segura, Imperial College London) of expression vector pRSET A (Invitrogen) or vector pGEX-6P3 (GE Healthcare). For the construction of \textit{pQE-80 L-MOC1}, the full coding sequence of \textit{MOC1} was amplified with primers MOC1Ex fw/rev (Supplementary Table S1) using the \textit{MOC1} cDNA clone as a template and sub-cloned into vector pGem T-Easy before cloning into the \textit{Escherichia coli} expression vector pQE80L (Qiagen).

**Production of recombinant MOC1 in \textit{E. coli} for binding studies**

Expression of recombinant MOC1 in \textit{E. coli} KRX cells (Promega) transformed with plasmid pRSET-A(Thr)MOC1\textit{ΔN46} was induced by adding 0.01% (w/v) 1(+) -rhamnose to cultures grown at 37°C until OD\textsubscript{600} = 0.5. Subsequently cultures were transferred to 18°C and cultivated for 16h before the cells were harvested by centrifugation. For the expression of glutathione S-transferase (GST)-MOC1 in \textit{KRX} cells, cultures were grown at 37°C to an OD\textsubscript{600} of 0.4 before 1 mM IPTG (isopropyl \beta-D-thiogalactopyranoside) was added. Expression was carried out at 37°C overnight. Recombinant His-MOC1 was purified by nickel-nitritotriacetic acid (Ni-NTA) chromatography and GST-MOC1 affinity purified on glutathione-cellulose (Supplementary Methods).

**Generation of an antiserum directed against MOC1**

For the production of an MOC1-specific antiserum, full-length MOC1 was expressed in \textit{E. coli} KRX cells (Promega) transformed with plasmid \textit{pQE-80 L-MOC1}. The His-tagged protein was purified from inclusion bodies according to standard protocols for denaturing
Ni-NTA chromatography and used to raise an antiserum in rabbits at Seqlab (Göttingen, Germany).

Isolation of mitochondria from *C. reinhardtii* cells

Mitochondria were purified from the cell wall-deficient *C. reinhardtii* strain cc400 as described by Atteia et al. (37) using the BioNeb cell disruption system (Glas-Col, Terre Haute, USA) with N₂ (20 psi).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting

Protein samples were separated on 12.5% (w/v) denaturing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels containing 6 M urea and stained with Coomassie blue R-250 or electroblotted onto nitrocellulose membrane (0.2-µm pore size; GE Healthcare, Germany). Immunoblotting analyses were performed using specific primary antibodies and a horse-radish peroxidase-conjugated secondary antibody (GE Healthcare, Germany). Signals were visualized using the FUSION-FX7 detection system (Peqlab, Germany) or ECL Western Blotting Substrate (Pierce, Germany) in combination with X-ray films. The following polyclonal antiseras were used as primary antibodies: rabbit anti-MOC1, rabbit anti-histone H3 (Agrisera, Sweden, product AS10710), rabbit anti-LHCBM4/6 (kindly provided by M. Hippler, Institute of Plant Biology and Biotechnology, University of Muenster, Muenster, Germany) and anti-COXIIb (Agrisera, Sweden, product AS06 151).

*In vitro* binding studies with dsDNA–cellulose

Recombinant MOC1 (1.8 µM) or bovine serum albumin (BSA) (1.8 µM) in DNA-cellulose binding buffer (DCBB) buffer [50 mM hydroxyethyl piperazinethanesulfonic acid (HEPES), pH 8.0, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA); 1 mM β-mercaptoethanol and 1 mM Pefabloc® SC protease inhibitor] were incubated for 2 h with 60 mg double-stranded DNA–cellulose resin (Sigma, D8515) at room temperature. The resin was washed using DCBB buffer, and a step gradient (100, 300, 600 and 1000 mM NaCl in DCBB) was used to elute bound protein.

Electrophoretic mobility shift assays

Double-stranded DNA probes (500–550 bp) for the screening of the entire mtDNA of *C. reinhardtii* were prepared by PCR using primers labelled with the fluorescent dye Cy-3 (Supplementary Table S1). PCR products were purified using the peqGOLD Cycle-Pure Kit (peqlab, Germany). Recombinant MOC1 (39 pmol) was incubated for 20 min with Cy-3-labelled probes (0.12 pmol) in 25 µl of electrophoretic mobility shift assays (EMSA) binding buffer [10 mM HEPES, pH 7.5, 100 mM KCl, 5 mM dithiothreitol (DTT), 0.1% (w/v) Tween 20, 20% (w/v) glycerol, 12.5 mM MgCl₂ and 0.2 mg/ml of BSA] containing unspecific competitor (salmon sperm DNA added in mass excess relative to probe) at room temperature. After 20 min, samples were immediately put on ice before they were loaded onto pre-cooled 1.5% (w/v) agarose or 6% (w/v) polyacrylamide gels prepared in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris, pH 7.5, and 2.5 mM EDTA), which were pre-run in the cold room for 30 min before sample loading. Electrophoresis (3 min/300 V followed by 45–60 min/90 V) was carried out in the cold room. For the EMSA with probes S3, S2/nd1 IGR and LHCBM6 NG (0.36 pmol each), 26 pmol recombinant MOC1 was added, and complexes were separated from free probe in a 2% (w/v) agarose gel. Small (24 bp) double-stranded oligonucleotides were prepared by annealing equal molarities of complementary single-stranded oligonucleotides (Supplementary Table S1), which were modified with Cy-3 at their 5'-end. Binding reactions contained 17 pmol recombinant MOC1 and 1.5 pmol Cy-3-labelled probes. Poly dI:dC (Sigma) was added as an unspecific competitor (mass excess relative to probe), and unlabelled probes were added to the labelled probe in molar excess. Cy-3 fluorescence was detected using a Typhoon scanner (GE Healthcare).

Determination of the relative mtDNA content in *stm6*

Total DNA samples (5 ng) extracted from *stm6* and the MOC1-complemented strain B13 were used as a template for quantitative PCR (Q-PCR) using the KAPA SYBR FAST Universal reagent (peqlab, Germany) and the DNA Engine Opticon (Bio-RAD, Germany). The primer pair Chlamy nd1 fw/rev (Supplementary Table S1) was used to amplify part of the mitochondrial *nd1* gene, and the primers TBP for and TBP rev (Supplementary Table S1) were applied to amplify a sequence of the nuclear *TBP* (TATA-box–binding protein) gene (NCBI Gene ID: 5716470). Cycle threshold (Ct) values were used to calculate ΔCt values [Ct(*nd1*)–Ct(*TBP*)] and the relative mtDNA content in each strain (2–ΔCt). DNA was extracted from two different cultivations per strain, and for each DNA sample, three technical Q-PCR replicates were used.

Extraction of nucleic acids

Total RNA was extracted according to Chomczynski and Sacchi (38), and total cellular DNA was isolated using the cetyltrimethylammonium bromide method (39).

Reverse transcriptase–Q-PCR

Real-time reverse transcriptase (RT)–PCR was performed with total RNA samples, which were subjected to DNaseI (RQ1 RNase-free DNase, Promega) digest before reverse transcription and PCR amplification using the SensiFAST™ SYBR No-ROX One-Step Kit (BIOLINE, Germany). SYBR Green I fluorescence was recorded on a DNA Engine Opticon (Bio-RAD, Germany). For the quantification of mature transcripts, 100 ng of total RNA was used, and CYN19-3 (Gene ID: 5719445) served as a housekeeping gene (primers CYN 19-3 fw/rev). The amount of unprocessed transcripts was quantified using 400 ng of total RNA, and *TBP* (TATA-box–binding protein) served as a reference gene. Relative expression values for mitochondrial transcripts in mutant *stm6* and the complemented strain (B13) were calculated.
according to Pfaffl (40). For the quantification of antisense transcripts, strand-specific reverse transcription was carried out, and RT reactions in the absence of primer served as controls to exclude primer-independent cDNA synthesis. Primer sequences are given in Supplementary Table S1.

### Cyclic amplification and selection of targets

The randomized DNA library was prepared as described by Blackwell (41) using a 56-nt-long oligonucleotide template (Supplementary Table S1) containing 20 internal random nucleotides, which were flanked on either side by 18 nt representing BamHI and HindIII restriction sites. A double-stranded and Cy-3-labelled library was prepared by PCR using 1.13 pmol single-stranded template and 30 pmol of primers SELEX Cy-3 fw and SELEX rev. Excess primers were removed from the library by gel purification using 14% native polyacrylamide gels. For sequence selection, recombinant MOC1 (3.3–17.5 pmol) was incubated with the double-stranded library (1.65 pmol) for 20 min in 25 μl of EMSA binding buffer [10 mM HEPES, pH 7.5, 100 mM KCl, 5 mM DTT, 0.1% (w/v) Tween 20, 20% (w/v) glycerol, 12.5 mM MgCl2 and 0.2 mg/ml of BSA] at room temperature before binding reactions were stopped on ice. Unbound library was separated from complexes by electrophoresis (5 min at 300 V then 90 min at 120 V) in 10% native polyacrylamide gels, which were run in the cold room using pre-cooled 1 × TAE buffer (40 mM Tris, pH 7.5, and 2.5 mM EDTA) as the running buffer. Complex bands were excised from the gel, and DNA was extracted according to Blackwell (41). Extracted DNA served as a PCR template for the generation of a target-enriched library used in the subsequent round of selection. During the selection process, the amount of recombinant MOC1 was gradually reduced, and unspecific competitor (poly dl:dC) was added to increase selection stringency. The progress of selection was analysed by sequencing of library sequences after cloning into vector pGem-T-Easy (Promega).

### Detection of a transcript containing cob in sense and L2b in antisense orientation

First 2µg of total RNA extracted from the wild-type strain cc400 was reverse transcribed using M-MLV reverse transcriptase (Promega) and 170 pmol of primer cob-L2b as N1 rev (Supplementary Table S1) according to the manufacturer’s instructions. In a control reaction, reverse transcriptase was replaced by nuclease-free water (−RT control). Excess primer was removed from the cDNA using the peqGOLD Cycle-Pure Kit (peqlab, Germany). Diluted (1:20 in nuclease-free water) cDNA (2 μl) from ± RT reactions was used as a template for the first PCR with primers cob-L2b as N1 fw/rev. Purified and diluted (1:10) products (2 μl ± RT) from the first PCR were then used in a second nested PCR with primers cob-L2b as N2 fw/rev. PCR products were analysed in a 1.5% (w/v) agarose gel, which was stained with SYBR Gold (Invitrogen, Germany). The dominating band migrating at 700 bp was excised, and DNA was purified using the peqGOLD gel extraction kit (peqlab, Germany) before cloning into pGem-T-Easy and sequencing.

### RESULTS

#### MOC1 is targeted to the mitochondrion

As reported before (22), the nuclear genome of *C. reinhardtii* encodes six distinct members of the mTERF family (Table 1). Two of the *C. reinhardtii* mTERF proteins (MOC5 and MOC6) contain putative transit peptides for chloroplast targeting and four mTERFs (MOC1–4) contain mitochondrial targeting sequences according to the sub-cellular prediction tool PredaGlo (43), which was developed for use with proteins from *C. reinhardtii* or other green algae. Among the four proteins is MOC1, which is in the centre of interest in this study. *In silico* analyses performed with the amino acid sequence of MOC1 (Figure 1A) and the sub-cellular prediction tool MitoProtII (44) predicted that this protein localizes to the mitochondrion (probability of

### Table 1. The six distinct mTERF factors encoded by the *C. reinhardtii* nuclear genome

| UniProtKB | Gene   | No. of mTERF motifs | Sub-cellular localization prediction by Predalgo (score) | Closest homologue A. thaliana (max score) | Closest homologue Homo sapiens (max score) | Closest homologue Drosophila melanogaster (max score) |
|-----------|--------|---------------------|--------------------------------------------------------|------------------------------------------|------------------------------------------|-------------------------------------------------|
| MOC1      | A8IXZ5 | Cre12.g5425008      | 3                                                     | Mitochondrion (1.54)NM_116533 (164)       | mTERF1 (mTERF) (73.9)D-mTERF3 (78.6)     |
| MOC2      | E1VD14 | Cre10.g427000       | 5                                                     | Mitochondrion (1.25)NM_129964 (342)       | mTERF1 (mTERF) (112)D-mTERF3 (87.4)     |
| MOC3      | A8HP31 | Cre12.g560750       | 6                                                     | Mitochondrion (1.54)NM_129964 (273)       | mTERF1 (mTERF) (115)D-mTERF3 (73.6)     |
| MOC4      | E1VD13 | Cre09.g408050       | 4                                                     | Mitochondrion (0.78)NP_566005.1 (316)     | mTERF1 (mTERF) (99)D-mTERF3 (80.9)     |
| MOC5      | E1VD13 | Cre14.g611200       | 2                                                     | Chloroplast (3.51)NM_116533 (126)        | mTERF1 (mTERF) (49.3)D-mTERF3 (52.0)    |
| MOC6      | A8IH52 | Cre07.g330600       | None                                                   | Chloroplast (0.99)NP_566005.1 (92)       | mTERF1 (mTERF) (45.4)D-mTERF3 (35)      |

For each of the mTERF genes, the locus name (*C. reinhardtii* v5.3; http://www.phytozome.com/) and available UniProtKB entries (http://www.uniprot.org/) are given. PredaGlo (42), https://giavap-genomes.ibpc.fr/cgi-bin/predalgodb.perf?page = main, was used to predict the sub-cellular localization in *silico*. The number of mTERF motifs was determined using the SMART tool (http://smart.embl-heidelberg.de/). In addition the closest (highest Max score) homologue from *H. sapiens*, D. melanogaster and A. thaliana is indicated for each *C. reinhardtii* mTERF.

*Predicted protein truncated.*
biochemical localization studies with purified organelles which was not related to mTERF domains. Consequently, of 373 amino acids and containing a C-terminal part, amino acid sequence (UniProtKB Q8LJS6_CHLRE)
drial localization of MOC1 was reported (18), but the as recombinant MOC1. In a previous study, the mitochon-
sequence (amino acids 1–45; Figure 1A) predicted by MitoProtII. This recombinant protein was used through-
duality predictions (amino acid 46 MitoProtII/ export to mitochondria 0.53; cleavage site 46) (Figure 1A). Cleavage site predictions (amino acid 46 MitoProtII/ amino acid 51 Predalgo) are in good agreement with the observed apparent molecular weight of mature MOC1 detected in a C. reinhardtii crude protein extract by immunoblotting (Figure 1B; WT) using an antiserum directed against histone H3 (αH3) and light-harvesting proteins (αLHCBM4/6) were performed to assess the purity of the mitochondrial fraction. COXIIb served as a mitochondrial marker protein. A Coomassie brilliant blue (CBB) stain of mitochondrial fractions and the whole-cell extract served as a loading control.

Figure 1. In silico prediction and biochemical evidence for mitochondrial targeting of MOC1. (A) Amino acid sequence (UniProtKB A8IXZ5) of MOC1. The mitochondrial targeting sequence is highlighted in grey, and cleavage sites predicted by MitoProtII or Predalgo are underlined. The three mTERF motifs are highlighted in black. (B) Comparison of the apparent molecular weight of recombinant His-tagged MOC1 lacking the mitochondrial targeting sequence predicted by MitoProtII (rMOC1) and mature MOC1 in a C. reinhardtii wild-type protein extract (WT) by immunodetection. An arrow indicates the position of recombinant and native MOC1. The MOC1 knockout mutant stom6 served as a control to assess the specificity of the antiserum raised against MOC1. (C) Immunodetection of MOC1 (αMOC1) in whole-cell extracts (WCE) and purified mitochondria (M). Immunoblots using antibodies directed against histone H3 (αH3) and light-harvesting proteins (αLHCBM4/6) were depleted during mitochondria purification. A mitochondrial marker (Figure 1C; M) were compared regarding their MOC1 content by immunoblot experiments (Figure 1C; αMOC1) clearly demonstrating that MOC1 becomes highly enriched during mitochondria purification. A mitochondrial marker (Figure 1C; αCOXIIb), the nucleus-encoded cytochrome c oxidase subunit IIb, showed a comparable enrichment during mitochondria purification, whereas chloroplast-targeted light-harvesting proteins (Figure 1C; αLHCBM 4/6) and the nuclear histone H3 (Figure 1C; αH3) were depleted during mitochondria isolation. In summary, in silico studies and the enrichment of MOC1 during mitochondria purification demonstrate that this protein resides in C. reinhardtii mitochondria.

In vitro MOC1 binds specifically to mitochondrial DNA
Most of the mTERF factors characterized to date bind double-stranded DNA, thereby regulating the transcription or replication of mtDNA (23), but additional functions such as a role in translational regulation have been reported (29). To investigate whether MOC1 binds to double-stranded DNA (dsDNA), as is suggested by the existence of mTERF motifs (Figure 1A), its binding to double-stranded DNA cellulose was assessed (Figure 2A). The recombinant MOC1 bound to dsDNA–cellulose (Figure 2A; input versus unbound; MOC1), whereas a non–DNA-binding control protein (Figure 2A; input versus control 26) and a new antiserum (raised against full-length MOC1; UniProtKB A8IXZ5_CHLRE) were repeated to confirm that this protein indeed localizes to algal mitochondria. To confirm a mitochondrial localization of MOC1, whole-cell extracts (Figure 1C; WCE) and mitochondrial extracts (Figure 1C; M) were compared regarding their MOC1 content by immunoblot experiments (Figure 1C; αMOC1) clearly demonstrating that MOC1 becomes highly enriched during mitochondria purification. A mitochondrial marker (Figure 1C; αCOXIIb), the nucleus-encoded cytochrome c oxidase subunit IIb, showed a comparable enrichment during mitochondria purification, whereas chloroplast-targeted light-harvesting proteins (Figure 1C; αLHCBM 4/6) and the nuclear histone H3 (Figure 1C; αH3) were depleted during mitochondria isolation. In summary, in silico studies and the enrichment of MOC1 during mitochondria purification demonstrate that this protein resides in C. reinhardtii mitochondria.
Binding of MOC1 to the DNA resin could be reversed by high ionic strength (Figure 2A; 300 mM–1 M NaCl; MOC1), typical for DNA–protein interactions (45). The wash–stable interaction of MOC1 with dsDNA–cellulose indicated a dsDNA-binding activity of the protein. Against the background that structure and gene content of mtDNAs vary enormously between eukaryotic organisms (1,2,16), the already identified binding sites for mammalian or insect mTERFs (23) cannot be used to identify respective binding sites in C. reinhardtii mtDNA. With the aim to identify genome regions containing binding sites for MOC1, the entire mtDNA of C. reinhardtii was systematically screened for putative binding sites (Supplementary Figure S2). For this, 30 overlapping and fluorescently labelled DNA probes (550–600 bp) covering the entire mitochondrial genome were used in EMSA. Among the tested probes, an mtDNA fragment No. 26 (NCBI Accession NC_001638; nt 13064–13604), containing parts of the ribosomal L8 and S3 rRNA-coding modules, showed binding to recombinant MOC1 even in presence of excess unspecific competitor (Supplementary Figure S2 and Figure 2B; +MOC1; 57–144 × unsp. comp.).

Identification of an MOC1-binding site within the mitochondrial rRNA-encoding module S3

Because of the fact that the probes used within the EMSA screen of the mitochondrial genome of C. reinhardtii (Supplementary Figure S2 and Figure 2B) were 550–600-bp long and thus contained many potential binding sites, an additional strategy had to be applied to narrow down the actual binding site on probe 26 (Figure 2B). CAST [cyclic amplification and selection of targets; (46)] was conducted to derive a consensus sequence (Figure 3A and Supplementary Figure S3), which could be used to identify the binding site on the mtDNA fragment representing parts of the L8 and S3 rRNA-coding modules. After 10 rounds of selection, 14 of 29 sequences (Figure 3A) contributed to a motif (Supplementary Figure S3) identified by the software.
tool MEME (multiple EM for motif elicitation) as part of the MEME suite (47). Two identical octanucleotide sequences (Figure 3A; S3_1 and S3_2) showing similarity to the motif obtained by the CAST experiment were identified within the mtDNA fragment (No. 26), which showed binding to MOC1 in the EMSA screen [Supplementary Figure S2 and Figure 2B]. Both sequences GTGAACAC are located in the small-subunit rRNA-encoding module S3 of the mitochondrial genome (S3_1 nt position 13308–13315 and S3_2 nt position 13406–13413; NCBI Accession NC_001638). In a first step, the part of probe 26 harbouring the octanucleotide sequences was analysed by EMSA and exclusive binding of this probe (Figure 3B; S3) but not of control probes (Figure 3B; S2/nd1 IGR and LHCBM6 NG) to recombinant MOC1 in the presence of unspecific competitor further suggested that the sequences S3_1 and S3_2 represent MOC1-binding sites. The sequences S3_1 and S3_2 were further analysed by mobility shift assays using oligonucleotide probes in which the octanucleotide GTGAACAC was flanked by eight nucleotides at either side [Figure 3C; S3_1 (probe 3) and S3_2 (probe 2)]. An additional oligonucleotide served as a control and was derived from S3_2 with the exception that the GTGAACAC sequence was disrupted by shuffling the eight nucleotides contained in the motif [Figure 3C; S3_2 Mut (probe 1)]. All three probes showed binding to recombinant MOC1 in the absence of unspecific competitor (Figure 3C, lanes A–C), but only probes having an intact GTGAACAC motif bound to MOC1 in the presence of excess unspecific competitor (Figure 3C; lanes D–L; poly dI:dC). Similar results were obtained using a GST-tagged version of recombinant MOC1 (Supplementary Figure S4). To confirm specific recognition of the identified octanucleotide motif further, specific and unspecific competition experiments were carried out with the fluorescently-labelled probe S3_1 (Figure 3D). When the unlabelled probe S3_1 (Figure 3D; S3_1 unl.) was added in excess as a specific competitor to the labelled probe S3_1, a 20-fold excess abolished complex formation almost completely (Figure 3D; lane E–G). Further evidence that the motif GTGAACAC is specifically recognized by MOC1 was obtained by binding studies with mutants of the motif (Figure 3E). When motif positions 7 and 8 (Figure 3A; ultimate and penultimate positions AC) were permuted to yield probe S3_1 MutI binding to recombinant MOC1 was no longer stable in the presence of unspecific competitor (Figure 3E; lane A versus B). Replacement of thymine at position two by cytosine had a smaller effect on complex stability, but decreased complex formation compared with the probe containing the wild-type sequence (Figure 3E; lane A versus C). When single mutations introduced into the S3_1 motif did not completely abolish complex formation, additional single mutations were tested (Figure 3E; sequences 4–13). A systematic analysis of all possible octanucleotide motifs derived from S3_1 and deviating from the wild-type motif at only a single position led to the identification of 10 single mutants, which can be found in the mtDNA of C. reinhardtii. For these mutated motifs, binding to MOC1 could not be detected in the presence of unspecific competitor (Figure 3E; 20× and 5× poly dI:dC; lanes D–F, H—L, N and O). These data allow us to conclude that the octanucleotide motif GTGAACAC represents a target binding site for MOC1, and that similar motifs found in the mitochondrial genome of C. reinhardtii are bound with less affinity in vitro.

**Phenotypic consequences of an MOC1 knockout**

The knockout mutant *stm6*, which is devoid of a functional MOC1 gene, and which was identified in a forward genetics screen aiming at the identification of mutants affected in short-term light acclimation mechanisms (18), was used to analyse the consequences caused by loss of MOC1. Localization studies demonstrated that this protein is targeted to the mitochondrial of *C. reinhardtii* (Figure 1C), and *in vitro* DNA-binding studies (Figures 2 and 3) revealed that MOC1 binds specifically to an octanucleotide motif, which can be found twice in the mitochondrial rRNA-encoding module S3. Consequently, replication and transcription of the mitochondrial genome were investigated in mutant *stm6* (Figure 4), as mTERF proteins are known to regulate both processes (23–34).

The relative mtDNA content was analysed (Supplementary Figure S5) because a role of mTERF proteins in mitochondrial genome maintenance has been reported previously (31). The knockout of MOC1 in *stm6* (Supplementary Figure S5; MOC1 k.o.) resulted in a 30% reduction [MOC1 k.o. 71.2% ± 9.8% (SD.)] of the relative mtDNA content compared with the MOC1-complemented strain [Supplementary Figure S5; MOC1 cs; strain B13 (18)]. In general, this reduction can be either because of a reduction in the number of mitochondria in the algal cell or because of a decreased mtDNA copy number in mitochondria of *stm6*.

The mitochondrial genome of *C. reinhardtii* (Figure 4A) is expressed by the generation of long co-transcripts, which are subsequently processed by precise endonucleolytic cleavages (12–14). Two bi-directional transcriptional units exist (13), whose expression could be driven by two potential promoters (one for the leftward and one for the rightward unit) with similarity to the consensus promoter sequence found in fungal mtDNA (A/T)TA(T/G)(T/A)RR(T/G)N (48) and located in the intergenic region between *cox1* and *nd5*. There is evidence in favour (15) and against (13) a function of these sequences serving as a bi-directional promoter, but to date, there is no reported identification of transcription initiation sites for the mtDNA of *C. reinhardtii*. The rightwards unit (Figure 4A) starts with the *cox1* gene and the leftwards unit begins with the gene *nd5*. For the rightwards transcription unit (RTU), four different polycistronic transcripts were identified (13) by transcript mapping studies (*cox1-nd2-nd6-trnW, L6-S1-L5, L7-S2-nd1-L3-rtl-L8-S3 and trnM-L4-S4-L1-L2*). A longer precursor covering the entire rightwards unit could not be identified, but our RT–PCR data demonstrated co-transcription of *nd6*-L6, *L5*-L7 and *S3*-L4 providing evidence that a long precursor covering the entire rightwards unit is generated.
Expression of the leftwards transcription unit was shown to result in co-transcripts containing the open reading frames of \textit{nd5} and \textit{nd4} (12), and transcriptional linkage of \textit{cob} and \textit{nd4} (Supplementary Figure S6) might indicate that all three genes of the leftwards unit are transcribed as a long polycistronic message.

Analysis of mature transcript levels (Figure 4B) in \textit{stm6} (\textit{MOC1} k.o.) showed that disruption of the \textit{MOC1} gene leads to changes in the mitochondrial transcriptome, when it is compared with that of the \textit{MOC1}-complemented strain (\textit{MOC1} c.s.). The mRNA amount of protein-coding genes was reduced in the MOC1-free mutant (Figure 4B; black bars) with reductions in the 10–30\% range for most of the genes except for \textit{nd1} [53.6\% ± 1.6\% (SE)] and \textit{rtl} [42.5\% ± 0.9\% (SE)], which showed the strongest reduction. Levels of rRNA-coding modules were less affected by a loss of MOC1 with either no change compared with the complemented strain (Figure 4B; grey bars; \textit{L5}, \textit{L3}, \textit{L8} and \textit{S4}) or slight reductions (Figure 4B, grey bars; \textit{L6}, \textit{L7} and \textit{S3}). Similar to the mode of mtDNA transcription found in other protists (16), the mitochondrial genes in \textit{C. reinhardtii} are co-transcribed to yield polycistronic RNAs, which are then processed to generate mature monocistronic RNAs (12–15). If RT–Q-PCR is conducted using primers binding within single-coding sequences (Figure 4B), therefore, polycistronic RNA species will serve as templates for cDNA synthesis in addition to mature RNAs. Because of the far lower abundance of polycistronic species, however, analyses shown in Figure 4B will primarily detect changes in mature RNA levels. Against the background that polycistronic RNAs are the primary products of \textit{C. reinhardtii} mtDNA transcription, exclusive quantification of such species seems to
be more informative than a quantification of mature RNAs (Figure 4B), if changes in transcriptional activity (initiation rates) are in the focus of interest. Recent studies have shown that the relative abundance of mature RNAs encoded by distinct mitochondrial genes shows a wide variation, despite the fact that they originate from a common precursor (49–51), thus reflecting the importance of post-transcriptional regulation of mature transcript abundance in mammalian mitochondria. A similar variation of mature transcript abundance has been reported for the mitochondrial genome of C. reinhardtii with rRNAs being much more abundant than mRNAs (13).

To address effects on mtDNA transcription in \( \text{stm6} \) more directly, additional RT–Q-PCR analyses were conducted (Figure 4C). Because of their length and extremely low abundance (49), full-length unprocessed precursor transcripts are normally not amenable to quantification by RT–Q-PCR. Therefore, primers were designed to bind in two adjacent coding sequences to exclude fully processed monocistronic mRNAs from the analysis. When RT–Q-PCR was carried out with primers spanning intergenic regions (Figure 4C), Ct (cycle threshold)-values increased by \( \approx 10–14 \) cycles compared with analyses mainly detecting mature RNAs (Figure 4B), demonstrating that the abundance of mature RNAs is far higher. These analyses revealed more pronounced transcriptomic differences between \( \text{stm6} \) (MOC1 k.o.) and the MOC1-complemented strain for both transcription units (60–80% reduction for most of the analysed genes). The subtle differences in mature RNA levels between \( \text{stm6} \) and complemented strain (Figure 4B) along with strong reductions in the level of unprocessed transcripts in \( \text{stm6} \) (Figure 4C) suggest that the efficiency of RNA processing could be altered in \( \text{stm6} \). If the relative expression level ratio of mature to unprocessed (bicistronic) RNAs is determined for selected genes in total RNA preparations of mutant and complemented strain a significantly higher ratio can be observed for \( \text{stm6} \) (Figure 4D). This is indicative of an altered processing activity in the mutant, which could act as a compensatory mechanism helping to maintain normal levels of mature RNA under conditions of impaired mitochondrial transcription control. In summary, the data clearly demonstrate that a loss of MOC1 strongly reduces the level of unprocessed RNAs derived from both transcription units without having strong effects on mature RNA abundance for most of the protein-coding genes except for \( \text{nfl} \) and \( \text{rtl} \) (Figure 4B and C). A changed mature RNA level of \( \text{nfl} \) is in agreement with a previous less comprehensive study (18), which mainly focused on light-induced changes of the mitochondrial transcriptome in \( \text{stm6} \). The reduced mtDNA content (Supplementary Figure S5) in \( \text{stm6} \) might indicate that genome maintenance is affected in this mutant. Changes in the relative abundance of mature versus unprocessed transcripts in \( \text{stm6} \) (Figure 4D) demonstrate that transcriptomic differences cannot be simply attributed to the reduced mtDNA content. Against the background that MOC1 binds to mtDNA in a specific fashion, it seems more reasonable to assume a deregulated transcription initiation or termination as the primary cause. Growth of the MOC1 knockout mutant \( \text{stm6} \) is severely impaired under strictly heterotrophic conditions (Supplementary Figure S7), indicating that normal mitochondrial function requires MOC1.

### The absence of MOC1 causes aberrant transcription of the non-coding mtDNA strand

Analysis of the mitochondrial transcriptome in the MOC1-free mutant \( \text{stm6} \) and the fact that MOC1 binds to mitochondrial DNA suggest that the transcription of both transcription units could be impaired. The two binding sites, which were identified \textit{in vitro}, are both located in the rRNA-coding module S3 (Figure 4A; GT GAACAC), a region distal to the mtDNA region harbouring potential promoters for both units. A direct effect of MOC1 on transcription initiation rates, therefore, seems to be an unlikely consequence of MOC1 binding to the S3 sites. Among the described functions of mTERF factors is the termination of mtDNA transcription at specific sites (32,33), so that a potential terminator function of MOC1 was investigated (Figure 5). Impaired termination of transcription in a knockdown cell line depleted of the \textit{Drosophila} mTERF protein DmTTF (34) resulted in a higher level of transcripts encoded by genes located downstream of the termination site, as a result of read-through transcription. Similar observations have recently been made for human mTERF (28), which showed that manipulation of mTERF protein levels affected read-through transcription of the antisense mtDNA strand. An investigation of sense-transcript levels in \( \text{stm6} \) did not reveal a higher amount of transcripts encoded by genes located downstream of the S3-binding sites (Figure 4B and C), which challenges a function of MOC1 as a terminator of sense-transcription of the rightwards transcription unit. The existence of antisense (as) transcripts in the mitochondrial transcriptome of \( \text{C. reinhardtii} \) has never been reported before, but strand-specific RT–PCR demonstrated the existence of antisense transcripts derived from the non-coding strand of the right mtDNA arm (Figure 5A; strand-specific RT–PCR). The gene cluster \( \text{nd1-L3-rtl-L8} \) is located downstream of the MOC1-binding sites, which are contained in the module S3 (Figure 5A; upper part), if transcription proceeds in the antisense direction. In contrast, parts of the S3 module and the cluster \( \text{M-L4-S4-L1-L2} \) are located upstream of the GTGAACAC binding sites. For all rRNA modules situated upstream of S3, antisense RNA could be detected by RT–PCR in wild-type total RNA samples (Figure 5A; a+b, c+d, e+f and g+f), whereas for the cluster \( \text{nd1-L3-rtl-L8} \), only antisense RNA derived from \( \text{rtl} \) and \( \text{L8} \) was detectable (Figure 5A; h+i and j+k). Longer polycistronic antisense transcripts are of very low abundance, and detection by RT–PCR required two successive PCRs [Figure 5A; g-i+a and g+f (N)]. As shown by these RT–PCR experiments, only parts of the non-coding strand of the right mtDNA arm are transcribed to an extent allowing their detection. Furthermore, the results indicate that transcription of the non-coding strand ends in the \( \text{rtl-L8} \) region adjacent to the S3 module harbouring the two MOC1-binding sites identified \textit{in vitro}. 
More detailed analyses of antisense RNA levels in \textit{stm6} (MOC1 k.o.) and complemented strain (MOC1 c.s.) by RT-Q-PCR (Figure 5B) revealed dramatic differences between both strains. For antisense transcripts containing \textit{rtl} and \textit{L8} sequences, a 12-fold higher level was found in \textit{stm6} [Figure 5B; \textit{rtl-L8} as; 12.3 ± 3.2 (SE)] compared with the complemented strain. The amount of antisense transcripts derived from \textit{L8} and \textit{S3} was also higher in the MOC1-free mutant [Figure 5B; \textit{L8-S3} as; 2.8 ± 0.7 (SE)], whereas quantification of antisense transcripts encoded by genes located upstream of the \textit{S3}-binding sites (Figure 5B; \textit{L4-S4} as; \textit{L1-L2a} as) showed that their amount in \textit{stm6} was far lower than in the complemented strain [\textit{L4-S4} as 0.14 ± 0.05 (SE); \textit{L1-L2a} as 0.31 ± 0.08 (SE)]. In addition, the relative expression level ratio of sense to antisense transcripts was determined in an independent experiment for both strains (Figure 5C). The relative expression level of sense RNA was in both strains higher than the level of corresponding antisense RNAs, but \textit{stm6} (Figure 5C; black bars; 11- to 49-fold) showed in general a lower sense-to-antisense ratio than the complemented strain (grey bars; 80- to 140-fold). Importantly, the relative difference between \textit{stm6} and complemented strain ratios (Figure 5C; white bars) was greater for \textit{L8-S3} and \textit{rtl-L8} RNA species (7- to 8-fold higher ratios in the complemented strain), which is in agreement with the strong accumulation of these antisense RNAs in the mutant (Figure 5B). To correlate the amount of MOC1 in the complemented strain with the level of antisense RNA, cell cultures were shifted from low to high light under phototrophic conditions (Figure 5D). In previous studies (18,19), a light-sensitive phenotype was reported for the MOC1 knockout mutant and interestingly MOC1 protein accumulates when cell cultures are transferred from low-light to high-light conditions (Figure 5D; \textit{\$MOC1 1B}). In the complemented strain, \textit{MOC1} expression is driven by the endogenous promoter (18), and after 6 h of high-light exposure, MOC1 started accumulating to reach a plateau about 24 h after the onset of high-light stress. Accumulation of MOC1 in the complemented strain was accompanied by a reduction in the level of \textit{rtl-L8} antisense RNA (Figure 5D; grey bars; 1 at \textit{t0} versus 0.21 ± 0.05 at \textit{t24h}), and after 50 h of high-light exposure, this RNA species was no longer detectable. When the MOC1-free mutant (Figure 5D; black bars) was cultivated under these conditions, \textit{rtl-L8} levels first declined within the first 24 h (4.8 ± 0.5 at \textit{t0} versus 2.9 ± 0.4 at \textit{t24h}) and re-accumulated again to reach the pre-stress level (4.4 ± 1.1 at \textit{t50h}). Although antisense RNAs derived from regions downstream of the MOC1-binding site

Figure 5. Continued and RNA extraction during the LL-to-HL shift experiment are indicated on the x-axis. The RNA level at time point \textit{t0} in the complemented strain was set to 1. Error bars indicate the standard error derived from four technical replicates (\textit{n} = 4). (E) SYBR Gold-stain of RT-PCR products separated in an agarose gel after 44 PCR cycles. RNA extracted from complemented strain and knockout mutant after 50 h of high-light exposure was used to detect two antisense RNA species encoded by sequences located downstream of the MOC1-binding sites.
could be detected in the mutant (Figure 5E; RT–PCR; MOC1 k.o.; rrl-L8 as and nd1-L3 as) after 50 h of high-light exposure, the amount of these transcripts dropped below detection limits in the complemented strain (Figure 5E; MOC1 c.s.). A higher amount of transcripts corresponding to genes located downstream of the S3-binding sites in the MOC1-free mutant under distinct growth conditions (Figure 5B, C and D) indicates that absence of MOC1 increases read-through transcription at this site, which provides evidence that MOC1 functions as a terminator of mtDNA transcription in vivo. The lower level of transcripts encoded by genes located upstream of the termination site (Figure 5B) is in agreement with a previous study reporting in vivo termination of mtDNA transcription by dmTTF in Drosophila (34). Furthermore, the negative correlation between MOC1 protein levels and the level of antisense transcripts (Figure 5D and E) is in support of the view that MOC1 is needed to terminate transcription of the non-coding strand belonging to the right mtDNA arm. In summary, quantification of transcripts resulting from antisense transcription downstream or upstream of the S3-binding sites in an MOC1-containing and an MOC1-free cell line suggests that MOC1 acts as a terminator at the S3 sites in vivo. Transcription of the non-coding strand of the right arm of the mitochondrial genome is a novel finding and raises the question of how such transcripts might be generated.

Transcriptional linkage of the cob and L2b gene indicates that the leftwards transcription unit extents to L2b of the right mtDNA arm

One possible explanation for the finding that the non-coding (antisense) strand of the right mtDNA arm is transcribed (Figure 5A) is the existence of promoter sequences at the right end of the mitochondrial genome (Figure 4A). Transcription initiation at these sites would...

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**Figure 6.** Evidence for co-transcription of cob and L2b and a functional model for MOC1. (A) Upper panel: Structure of a transcript containing the coding sequence of cob and L2b in antisense (as) orientation, which results from the linkage of the left and right arm of the C. reinhardtii mitochondrial genome. The cob sequence and L2b as flank an inverted repeat region (IR) and the 86-bp region (rr) according to Vahrenholz et al. (4). TAA indicates the stop codon of the cob open reading frame. Detection of the transcript by RT–PCR involved RT with a gene-specific primer binding to the L2b as part of the transcript in the first step and two successive PCR reactions with nested primers (white arrows) used in the second PCR step. Lower panel: SYBR Gold stain of an agarose gel used to separate the PCR products of the first and second PCR reaction. Reverse transcription was carried in the absence (−RT) or presence (+RT) of reverse transcriptase. The main PCR product of the second PCR amplification (black arrow) was gel extracted and sub-cloned for sequencing. (B) Detection of antisense RNA derived from the non-coding strand of the left mtDNA arm by RT–PCR. RNA was converted to cDNA by strand-specific reverse transcription to detect antisense RNA (primers a and c; upper part) or sense RNA (primers b and d). PCR (primers a + b or c + d) was used to amplify cDNA before gel separation of products and staining (lower part). As a control, reverse transcription was performed in the absence of primers (−P). (C) A model depicting the function of MOC1 as it is suggested by the sum of data. Upper part: termination of LTU (left part of the circle) transcription by MOC1 at the S3-binding sites in a wild-type (WT) mitochondrial containing MOC1. The mtDNA of C. reinhardtii is presented as a circle. Black arrows indicate the direction of transcription, and dotted lines indicate the length of LTU/RTU-derived transcripts. Abbreviations: TSS_LTU/RTU: transcription start sites; IGR: intergenic region between nd5 and cox1; gene names as given in Figure 4A. Lower part: Lack of MOC1-mediated transcription termination in stm6. Read-through at the S3 site potentially reduces transcription of both units.
generate mainly non-coding RNA, so that another potential explanation was investigated (Figure 6). Most of the in vitro studies conducted on the configuration (linear versus circular molecules) of C. reinhardtii mtDNA supported the view that the vast majority of mtDNA molecules in this green alga are linear (8,9). However, the existence of a small fraction of circular molecules in preparations for electron microscope studies has been reported (9), and successful PCR amplification of a sequence containing the right and left end of the linear C. reinhardtii mitochondrial genome further indicated that mtDNA populations contain at least small amounts of circular molecules (15,52). The sequence [GenBank Accession No. M83998, (52)] obtained by PCR amplification of mtDNA preparations from C. reinhardtii using primers binding to the 3’-ends of cob and L2a and published by Ma et al. (52), suggested that in a fraction of mtDNA molecules, the cob-coding sequence is fused to L2b in antisense orientation. The part connecting both genes contained only one of the inverted repeat regions present in each of the two linear molecule ends and was proposed to result from homologous recombination events (52). Transcription of such molecules starting upstream of nd5 (Figure 4A) could result in a transcript extending to L2b in antisense orientation (Figure 6A; upper part) instead of terminating downstream of cob (Figure 4A). To detect such transcripts in total RNA preparations of C. reinhardtii RT–PCR was applied and confirmed that transcripts comprising cob and L2b do indeed exist (Figure 6A; upper part). The product obtained after the second nested PCR amplification (Figure 6A; lower part; second nested PCR; +RT) of the cDNA resulting from reverse transcription using a primer specifically binding to the L2b part of the transcript was sequenced. Sequencing revealed fusion of cob and L2b, and the sequence corresponded to the one published by Ma et al. (52). In agreement with a fusion of both genome arms, antisense transcripts derived from the left arm could also be detected by strand-specific RT–PCR (Figure 6B; RT–PCR; as).

**DISCUSSION**

The sum of data obtained during the characterization of MOC1 function indicates that this protein acts as a factor required for terminating transcription of the leftwards mtDNA in wild-type C. reinhardtii mitochondria (Figure 6C; WT). In vitro binding studies led to the identification of two octanucleotide sequences in the rRNA module S3, which bound specifically to recombinant MOC1 (Figure 3). At least in vitro, the two GTGAACA C motifs are bound by MOC1 with a strong preference (Figure 3E), suggesting that the S3 motifs represent prime targets for recognition in vivo. Analyses performed to investigate potential differences between the mitochondrial transcriptome of wild-type cells and that of mutant cells devoid of MOC1 (Figure 4) revealed a general reduction of unprocessed transcript amounts (Figure 4C) in the MOC1-free mutant with both transcription units of the mitochondrial genome being affected. Although levels of unprocessed transcripts are reduced in stm6, the effects on mature RNA abundance are for most of the genes small (Figure 4B), which indicates an altered processing activity in the mutant (Figure 4D). A lower amount of unprocessed transcripts in the mutant could be caused by an increased processing activity alone, or might represent a combination of pleiotropic effects on RNA processing and reduced transcription initiation rates as a consequence of impaired termination at the S3 site. Considering that the S3 module is located in a promoter-distal area of the genome, direct modulation of transcription initiation rates seemed to be unlikely as a function of MOC1. As reported before (28,34), a consequence of impaired mTERF-mediated transcription termination is read-through transcription at respective sites. Inspection of sense transcripts levels in stm6 and comparison with the levels found in the complemented strain (Figure 4C) did not indicate that read-through occurs at the S3 site as far as coding strand transcription is concerned. Interestingly, there is to date no in vivo evidence for the function of human mTERF1 as a terminator acting on the heavy strand, which encodes the majority of sense transcripts in human mitochondria. In contrast, termination of light-strand transcription yielding mainly non-coding RNAs has recently been supported by in vivo data (28) and is in good agreement with a preferential termination of light-strand transcription in vitro (53,54).

Unexpectedly, stable antisense transcripts derived from the right arm of the mitochondrial genome could be detected by RT–PCR (Figure 5A) so that a more detailed analysis of antisense transcript levels encoded by mtDNA sequences directly upstream or downstream of the MOC1-binding site was performed (Figure 5B and C). The higher level of antisense transcripts in stm6 (Figure 5B and C), which are encoded by genes downstream of the S3-binding site (antisense orientation of transcription) can be explained by read-through at the S3 site in the absence of MOC1 (Figure 6C; lower part; stm6). In the complemented strain, the extent of read-through at this site is negatively correlated with the amount of MOC1 (Figure 5D and E). The existence of antisense RNAs in the mitochondrial transcriptome of C. reinhardtii is a novel finding, and an intriguing question raised by the detection of such transcripts was how they are generated. If transcription initiation sites at the right end of the mitochondrial genome in its linear configuration exist, the use of such promoters would generate transcripts mainly containing non-coding information. An alternative explanation considers circular templates formed by end-to-end fusion of the linear genome, thus connecting the coding strand of the lightwards transcription unit (LTU) to the non-coding strand of the RTU. This idea was supported by the detection of a transcript that comprised cob in sense and L2b in antisense orientation, demonstrating that previous models (13) regarding the transcription of C. reinhardtii mtDNA need to be refined. The most reasonable explanation for the observed transcriptional linkage of the RTU and non-coding RNA derived from the RTU is that circular mtDNA molecules (Figure 6C) serve as a template for mtDNA transcription in C. reinhardtii, and that transcription of the leftwards transcription unit extends beyond cob.
has an overall repressive effect on transcription initiation. A recent finding is that impaired termination of LTU transcription in C. reinhardtii encoded by sequences located upstream and downstream of stm6 (Figure 6C; WT; MOC1), and this view is supported by S3 might be terminated at the right inverted repeat (Figure 4A; IR2). In further analyses, it was proposed that in analogy to their nuclear counterparts, t-loops form by base pairing of single-stranded overhangs at the extremities of linear molecules and internal complementary sequences in the terminal inverted repeats (57). It was also proposed that in analogy to their nuclear counterparts, they protect linear mtDNA from exonucleolytic attack. Both ends of the C. reinhardtii mitochondrial genome contain terminal inverted repeats and single-stranded protrusions, but only for the single-stranded overhang of the right mitochondrial genome arm, a complementary structure within an 86-bp internal repeat exists (4), which would allow t-loop formation at the right end of the mtDNA. The left arm of the genome lacks an internal repeat precluding t-loop formation. Interestingly, the 86-bp internal repeat of the right mtDNA arm partly overlaps with the gene L2b, so that, although the overhangs found at both sides are not cohesive, (t)-loop formation involving the left single-stranded overhang and the internal repeat within and immediately downstream of L2b would lead to circularization. The structure of the detected transcript (Figure 6A) further suggests that such a circular template indeed exists because the left terminal inverted repeat (Figure 4A; 1R1), including the 86-bp region (Figure 4A; rr) downstream of cob, is directly fused to L2b in antisense orientation omitting the right terminal 86-bp region (Figure 4A; right telomere; rr) and the right inverted repeat (Figure 4A; 1R2). In further support for the existence of circular end-to-end fused mtDNA molecules, antisense transcripts could also be detected for the non-coding part of the left genome arm (Figure 6B). Transcripts, whose synthesis is initiated in the nd5-cox1 region and comprises the nd4-nd5-cob cluster, might be terminated at the S3-binding sites by MOC1 (Figure 6C; WT; MOC1), and this view is supported by the differential expression of non-coding information in stm6 encoded by sequences located upstream and downstream of the S3 region (Figure 5B).

Besides the strong effect seen for antisense RNA transcription in stm6, expression of coding information was also found to be affected in stm6 (Figure 4 and 6C; stm6; black arrow). A potential explanation for this finding is that impaired termination of LTU transcription has an overall repressive effect on transcription initiation of both LTU and RTU. Similar observations were made after manipulating the levels of dmTTF in Drosophila (34) and were explained by a reduced availability of transcription machinery components at initiation sites because of their engagement in read-through transcription at unoccupied termination sites. It should, however, be noted that post-transcriptional mechanisms have a major impact on mitochondrial RNA abundance (59), and many direct effects on mtDNA transcription might be obscured by pleiotropic effects on RNA processing, stability or translation efficiency. Although the level of certain antisense RNAs is higher in the MOC1-free mutant (Figure 5B), their abundance is still low in comparison with sense transcripts (Figure 5C). It, therefore, seems unlikely that the phenotype of stm6 is directly caused by detrimental effects of antisense RNA on mitochondrial gene expression (e.g. by dsRNA formation with sense counterparts). The low accumulation of these RNA species can probably be explained by the fact that RNA surveillance mechanisms like those described for higher plant organelles (60,61) are still active in the mutant. It is currently difficult to depict all the consequences of impaired transcription termination for mitochondrial gene expression, but a loss of MOC1 clearly impedes mitochondrial function (18) and heterotrophic growth (Supplementary Figure S7).

With MOC1, the first mitochondrial mTERF protein from a phototrophic organism has been characterized regarding its molecular function. Besides MOC1, only four other mTERFs from a phototrophic organism, namely, Arabidopsis thaliana, have been characterized (62–66), but for all four factors, the precise molecular function remains to be elucidated. The mTERFs SOLDAT10 (64) and MDA1 (62) are both targeted to the chloroplast, whereas SHOT1 (66) was found to reside in mitochondria, and RUG2 (65) represents an example of dual targeting to chloroplast and mitochondrion. Aberrant expression of chloroplast genes caused by the BELAYA SMERT/RUGOSA2 (63) and SOLDAT10 (64) mutations together with proteomic analyses of higher plant chloroplast nucleoids (67,68) suggest that mTERFs are crucial components of the plastid transcriptional machinery and required for unperturbed expression of organelle genomes. For the recently identified SHOT1 protein, the phenotype caused by the shot1–2 mutation indicated that higher plant mTERFs also regulate mitochondrial gene expression (66). Changes in organelle gene expression are known to be a source for retrograde signals (69) communicating the physiological state of an organelle to the nucleus where most of the mitochondrial/chloroplast proteins are encoded. mTERF proteins might, therefore, be implicated in the modulation of retrograde signals, and a changed expression of nuclear genes in A. thaliana mTERF mutants supports this idea (64). The loss of a functional MOC1 gene also results in a changed expression of nucleus-encoded genes. Among them are genes encoding subunits of mitochondrial respiratory complexes (18) or stress-related light-harvesting proteins targeted to the chloroplast (19). Perturbed mitochondrial function in stm6 affects the physiological state of the plastid causing an increased light-sensitivity of the mutant (18,19) and enhanced hydrogen production capacity in the chloroplast under anaerobic conditions.
conditions (20). Against this background, *stm6* represents a valuable tool for the analysis of the complex interplay between the chloroplast, the mitochondria and the nucleus.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Table 1, Supplementary Figures 1–7 and Supplementary Methods.

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