A high-affinity molybdate transporter in eukaryotes

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Molybdenum is an essential element for almost all living beings, which, in the form of a molybdopterin-cofactor, participates in the active site of enzymes involved in key reactions of carbon, nitrogen, and sulfur metabolism. This metal is taken up by cells in form of the oxyanion molybdate. Bacteria acquire molybdate by an ATP-binding-cassette (ABC) transport system in a widely studied process, but how eukaryotic cells take up molybdenum is unknown because molybdate transporters have not been identified so far. Here, we report a eukaryotic high-affinity molybdate transporter, encoded by the green alga *Chlamydomonas reinhardtii* gene *MoT1*. An antisense RNA strategy over the *MoT1* gene showed that interference of the expression of this gene leads to the inhibition of molybdate transport activity and, in turn, of the Mo-containing enzyme nitrate reductase, indicating a function of *MoT1* in molybdate transport. MOT1 functionality was also shown by heterologous expression in *Saccharomyces cerevisiae*. Molybdate uptake mediated by MOT1 showed a K_m of ≈6 nM, which is the range of the lowest K_m values reported and was activated in the presence of nitrate. Analysis of deduced sequence from the putative protein coded by *MoT1* showed motifs specifically conserved in similar proteins present in the databases, and defines a family of membrane proteins in both eukaryotes and prokaryotes probably involved in molybdate transport and distantly related to plant sulfate transporters SULTR. These findings represent an important step in the understanding of molybdate transport, a crucial process in eukaryotic cells.

micronutrient transport | molybdenum | molybdenum cofactor | nitrate assimilation

Molybdenum is one of the least abundant elements in organisms and is essential for molybdenum cofactor (Moco) biosynthesis. Moco is present in almost all living beings, taking part, as a prosthetic group, in the active site of key enzymes such as nitrate reductase, aldehyde oxidase, xanthine dehydrogenase, and sulfite oxidase. These enzymes participate in crucial processes for life such as nitrate assimilation, phytohormone biosynthesis, purine metabolism, and sulfite detoxification in plants, animals, and microorganisms (1, 2).

Moco biosynthesis is a conserved pathway that consists of four main steps (3), conversion of GTP into cyclic pyranopterin monophosphate, synthesis of the molybdopterin dithiolate, adenylation of molybdopterin, and molybdenum-insertion reaction. In the fourth and final step, mature Moco is formed by the ligation of a single Mo atom to adenylated molybdopterin; this process directly depends on the intracellular availability of molybdenum. Deficiency in Moco biosynthesis results in a pleiotropic loss of all Mo-enzymes activity, which leads in humans to a severe metabolic disorder, and affected patients die in early childhood (4).

In prokaryotes, molybdenum is taken up in an energy-dependent process by high-affinity molybdate transporters belonging to the ABC family (5). This system is formed by a periplasmic molybdate-binding protein (ModA), a membrane channel protein (ModB), and an energy-transducing ATPase protein (ModC). In *Escherichia coli*, the molybdate transport system is encoded by the *modABC* operon, exhibits a K_m of 50 nM, and is transcriptionally regulated by intracellular levels of molybdate in a process mediated by the protein ModE (6–9).

In contrast to bacteria, molybdate transporters in eukaryotes are unknown, and genes similar to the bacterial molybdate channel *modB* have not been found in sequenced eukaryotic genomes. Physiological data from the green alga *Chlamydomonas reinhardtii* suggest the presence of at least two molybdate transport systems that are related to the unlinked genetic *loci Ni5* and *Nit6* (10). Mutants defective at one of these *loci* are phenotypically wild type but have a reduced molybdate transport activity; double mutants at both loci lack Moco and, thus, activity of the molybdoenzyme nitrate reductase (11).

Proteins from the ABC family are widely distributed in bacteria and participate in the transport of an ample variety of substrates (12), but in eukaryotes, these transport systems have a particular protein structure and seem to be more specialized in mediating the export of different substrates (13). On the other hand, anions such as molybdate, sulfate, and selenate are similarly shaped anions sharing some physicochemical characteristics and might well be transported by carriers from related families. In fact, a cross-inhibition of sulfate transport by molybdate and selenate has been related to the interactions of these anions in different eukaryotic systems (14–16).

We have carried out an expression silencing of the *C. reinhardtii* gene *MoT1* (molybdate transporter, type 1), showing that *Chlamydomonas* strains with reduced expression of *MoT1* exhibit a diminished molybdate transport and nitrate reductase activities, pointing to a molybdate transport function of *MoT1*. *MoT1* encodes a protein with homologous ones in other eukaryotes and also in prokaryotes; these proteins share highly conserved motifs that define a previously uncharacterized family of transporters probably involved in molybdate uptake. Our findings could allow the understanding of molybdate transport in other eukaryotes, in which this crucial process is unknown.

Results

Identification of *MoT1*; the MOT1 Family. Because of the possible relationship between molybdate and sulfate transport, we performed a search in the *Chlamydomonas* Genome Database for sulfate transporter-like proteins whose functionality had not been shown and that were different enough from the typical proteins described for this family of transporters (17). Among five members found, two of them were highly homologous to the SULTR sulfate transporters from plants and two other to SulP from bacteria and corresponding to plastidic sulfate transporters (18). There appeared a fifth one that showed a deduced amino acid sequence with only a conservation of ≈13% with the other *Chlamydomonas* sulfate transporters. Thus, we focused our

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The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper have been deposited in the GenBank database (accession no. EF437943).

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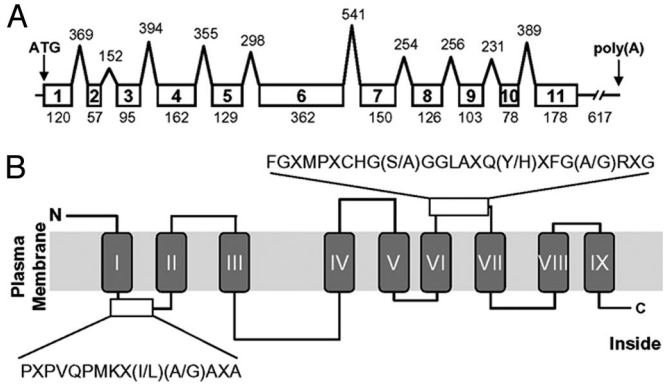

Fig. 1. Analysis of the *MoT1* gene. (*A*) Schematic representation of the *MoT1* gene. Exons are represented in boxes, and introns are represented in lines. The number in boxes is the exon number from initial ATG. Numbers under each exon and above each intron are its length in nucleotides. The 3′ UTR is represented as a broken horizontal line with its length in nucleotides. (*B*) Schematic representation of the predicted transmembrane topology of MOT1. White boxes indicate the position of conserved motifs. Transmembrane topology has been predicted by using the MOT1 deduced amino acid sequence with HMMTOP software.

efforts on this putative molybdate transporter and verified subsequently its functionality as such. Therefore, we have named it *MoT1* (molybdate transporter, type 1).

The *MoT1* cDNA was isolated by PCR amplifications and its sequence annotated in the GenBank database (accession no. EF437943). *MoT1* genomic DNA, available in the *Chlamydomonas* Genome Database, was analyzed and shows the presence of 11 exons with a long sixth intron and 3′ UTR end (Fig. 1*A*).

The deduced MOT1 is a 519-aa protein that contains nine predicted hydrophobic membrane-spanning domains (Fig. 1*B*) and shows a significant conservation to other homologous proteins (35.2–25.4% identity) of unknown function in algae, plants, fungi, and bacteria (Fig. 2*A*). Alignment of proteins similar to MOT1 showed two sequence conservation motifs (Fig. 2*B*): PXPVQPMKX(I/L)(A/G)AXA, after the first membrane-spanning fragment, and FGXMPXCHG(S/A)GGLAXQ(Y/H)XFG(A/G)RXG, after the sixth membrane-spanning fragment (Fig. 1*B*). These motifs are exclusive of proteins homologous to MOT1, defining a previously uncharacterized family of membrane proteins.

Isolation of *Chlamydomonas* Strains with Reduced *MoT1* Expression. To elucidate the function of MOT1, we have used a *MoT1* antisense strategy. We transformed two *C. reinhardtii* strains, 704 (wild type) and 21gr (*Nit5*$^{-}$) using the plasmid pRBCMoT1as. This plasmid contains a paramomycin resistance cassette used for selection of transformants (19) and a *MoT1* antisense construction under the control of the *Chlamydomonas RbcS2* gene promoter. Antisense construction consists of a 2,2 kb *MoT1* genomic fragment including the initial ATG followed by a 0.8-kb *MoT1* cDNA fragment corresponding to this processed genomic fragment (supporting information (SI) Fig. 7).

Transformation of strain 704 resulted in ≈500 paramomycin-resistant single transformants per plate (2,500 transformants per microgram of pRBCMoT1as plasmid). A total of 200 transformants was rescued, and all of them were capable of growing in 4 mM nitrate-containing media. PCR assays confirmed the presence of *MoT1* antisense construction in 3 of 25 randomly selected transformants. The confirmed antisense mutants were used in this work and were named 7i, 8i, and 15i.

Transformation of strain 21gr resulted in ≈20 paramomycin-resistant single transformants per plate (20 transformants per microgram of pRBCMoT1as plasmid). A total of 100 transformants were rescued, and 6 of them showed a deficient growth in 4 mM nitrate-containing media. PCR assays confirmed the presence of *MoT1* antisense construction in these six mutants; three of them were used in this work, and are named 1gr, 2gr, and 6gr.

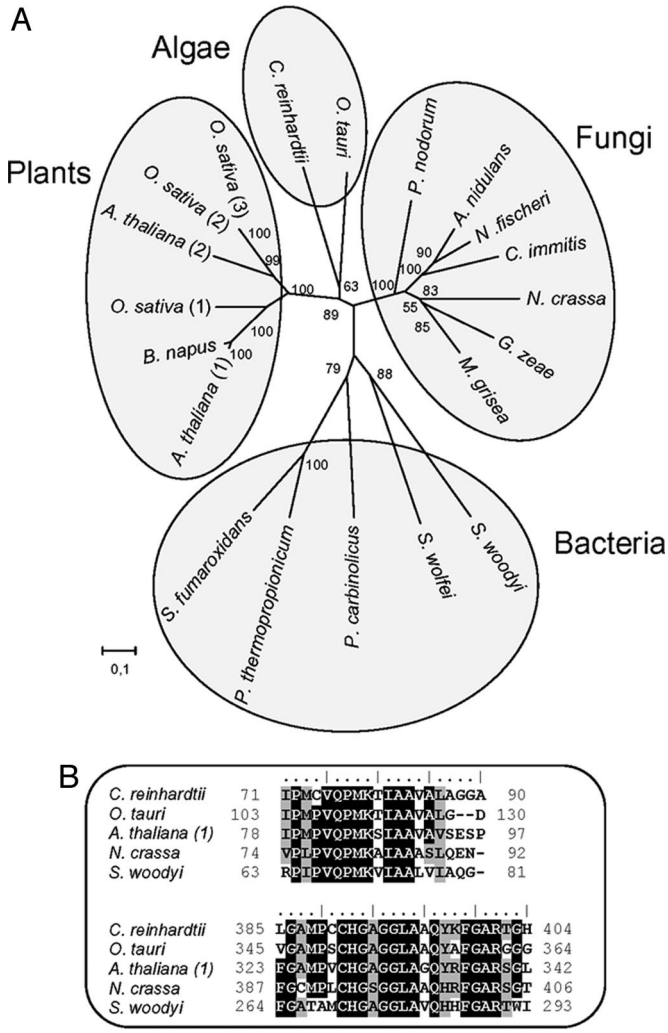

Fig. 2. The MOT1 family. (*A*) Phylogenetic tree of MOT1 homologous proteins; the tree was drawn by using the Mega3 program. (*B*) Alignment of two motifs conserved specifically in the MOT1 family. Sequences were aligned by using the ClustalW method and taken from a larger alignment that included all sequences shown on the phylogenetic tree. Accession nos: *C. reinhardtii*, EF437943; *O. tauri*, CAL56055; *A. thaliana* (1), BAF01113; *A. thaliana* (2), AAD31368; *Oryza sativa* (1), BAB40169; *O. sativa* (2), BAD03554; *O. sativa* (3), EAZ05271; *Brassica napus*, CAC39421; *Phaeosphaeria nodorum*, EAT78207; *Aspergillus nidulans*, EAA60447; *Neosartorya fischeri*, EAW23840; *Coccidioides immitis*, EAS34579; *Neurospora crassa*, EAA31472; *Gibberella zeae*, EAA67679; *Magnaporthe grisea*, XP_364700; *Shewanella woodyi*, EAV38087; *Syntrophomonas wolfei*, ABI67693; *Pelobacter carbinolicus*, ABA87935; *Pelotomaculum thermopropionicum*, GAA02550; *Syntrophobacter fumaroxidans*, ABK15972.

Molybdate Transport in Strains with Underexpression of *MoT1*. We studied molybdate uptake in the mutants previously characterized

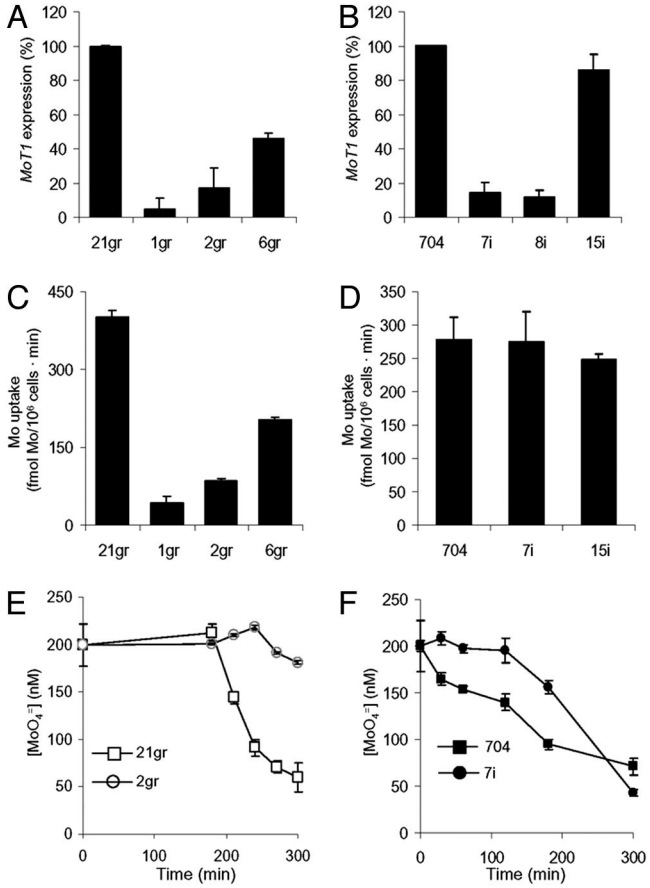

Fig. 3. Silencing of *MoT1* expression by antisense RNA and effect on molybdate transport. (*A* and *B*) Quantization of *MoT1* transcription was carried out by real-time PCR. Total RNA was isolated from mutants coming from the strains 21gr (*A*) or 704 (*B*) incubated during 1.5 h in a 4 mM nitrate medium. Expression of parental strains was taken as 100%. (*C–F*) Molybdate uptake in *Chlamydomonas* mutants derived from the strains 21gr (*C* and *E*) or 704 (*D* and *F*) was determined in a 4 mM nitrate medium supplemented with 200 nM molybdate. Triplicate samples were used in each experiment and repeated three times. Error bars indicate the standard deviation.

as defective in *MoT1* expression. Molybdate uptake was determined by measuring disappearance of this anion in the medium. Mutants 1gr, 2gr, and 6gr showed a reduced molybdate uptake compared with the parental strain 21gr, which was proportional to the *MoT1* expression found in each mutant (Fig. 3*C*). In contrast, mutants 7i and 15i had a molybdate uptake level comparable with that observed in the parental strain 704 (Fig 3*D*).

Mutant 8i lost its *MoT1*-underexpression phenotype during this work, which is a common phenomenon described in other *Chlamydomonas* antisense mutants (20). This is why we did not use mutant 8i in this study.

A second transporter present in 704 (wild-type strain) but not in 21gr (*Nit5*$^-$) (10) might explain the apparent normal molybdate uptake level exhibited by the mutants 7i and 15i. To check this hypothesis, we studied the evolution of molybdate transport along time in strains 21gr and 704 and in the *MoT1* antisense mutants 2gr and 7i. This study revealed a single Mo transport activity in the strain 21gr that was dramatically affected by the interference of *MoT1* expression (Fig. 3*E*); and two Mo transport activities in the strain 704, one of which was not affected in strain 7i carrying *MoT1* antisense (Fig. 3*F*), suggesting that this other transport activity is not related to the gene *MoT1*.

These results indicate that molybdate uptake in *Chlamydomonas* directly depends on the expression of *MoT1* gene in strain

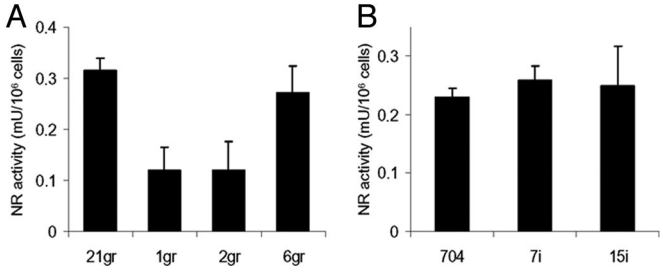

Fig. 4. Effect of *MoT1* underexpression on nitrate reductase activity. *Chlamydomonas* mutants derived from the strains 21gr (*A*) or 704 (*B*) were incubated for 3 h in a 4 mM nitrate medium supplemented with 200 nM molybdate. One unit of nitrate reductase activity was defined as the amount of enzyme catalyzing reduction of 1 μmol of nitrate in 1 min. Experiments were repeated at least three times. Error bars indicate the standard deviation.

21gr, pointing to a role of this gene in molybdate transport. Nevertheless molybdate transport activity associated to *MoT1* is not the only Mo transport activity present in *Chlamydomonas*, and an alternative transport system ensuring the provision of molybdenum to the cell has also been put forward.

Nitrate Reductase (NR) Activity in Strains with Underexpression of *MoT1*. NR is a molybdoenzyme whose activity requires the presence of Mo in its active site (21). To study the effect of a reduced molybdate uptake on NR activity, we measured it in the mutants that showed a reduced expression of *MoT1* and, therefore, a reduced molybdate transport. The NR activity found was concomitant with molybdate uptake in each strain. Mutants derived from the strain 21gr exhibited a reduced NR activity compared with the parental strain; probably caused by a deficient entry of Mo (Fig. 4*A*). However, mutants 7i and 15i showed NR levels similar to the parental strain 704, because of the molybdate transport activity independent of *MoT1* (Fig. 4*B*).

Thus, a deficiency in molybdate uptake caused by interference in *MoT1* expression in strain 21gr affects to NR activity, supporting the role of *MoT1* in molybdate transport and the relationship of nitrate reduction and molybdenum availability.

Heterologous Expression of *MoT1* in *S. cerevisiae*. To ensure that MOT1 is a real molybdate transporter and not just an activator of the transport process, we carried out a heterologous expression of MOT1 in the yeast *S. cerevisiae*. This yeast lacks the Moco biosynthetic pathway and also a *MoT1* homologous gene; therefore, *Saccharomyces* is a good model to study the functionality of MOT1 by means of heterologous expression. We transformed the *Saccharomyces* strain 31019b (22) using the *MoT1* cDNA cloned in the yeast expression plasmid pDR196 (23). The transformation resulted in ≈1,000 transformants capable of growing in a medium without uracil (10^6 transformants per microgram of pDR96 plasmid); we randomly selected 10 of them and one was used to study the MOT1 activity. The *Saccharomyces* strain transformed with *MoT1* exhibited a molybdate transport activity that was not present in a strain transformed with the empty pDR196 plasmid (Fig. 5); this result confirms the functionality of MOT1 as a molybdate transporter.

Using the *Saccharomyces* strain expressing MOT1, we investigated the energy requirement and possible inhibitors of the MOT1 activity. We have measured molybdate uptake in the presence of 1 mM sulfate or 20 μM tungstate as putative inhibitors and in a medium without glucose to study the energy requirement. We found that molybdate uptake was partially inhibited by tungstate (60%) but not by sulfate (Fig. 5). The transport mediated by MOT1 seems to be energy-dependent because it was reduced (62%) in a medium lacking glucose (Fig. 5).

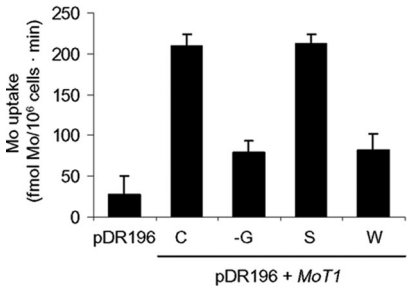

Fig. 5. Heterologous expression of MOT1 in *S. cerevisiae.* Yeast cells were grown in standard YNB-rich medium. Molybdate uptake was determined in a Mes buffer (pH 5.8) containing 2% glucose and 200 nM molybdate. C, control; −G, without glucose; S, plus 1 mM sulfate; W, plus 20 μM tungstate. Experiments were repeated at least three times. Error bars indicate the standard deviation.

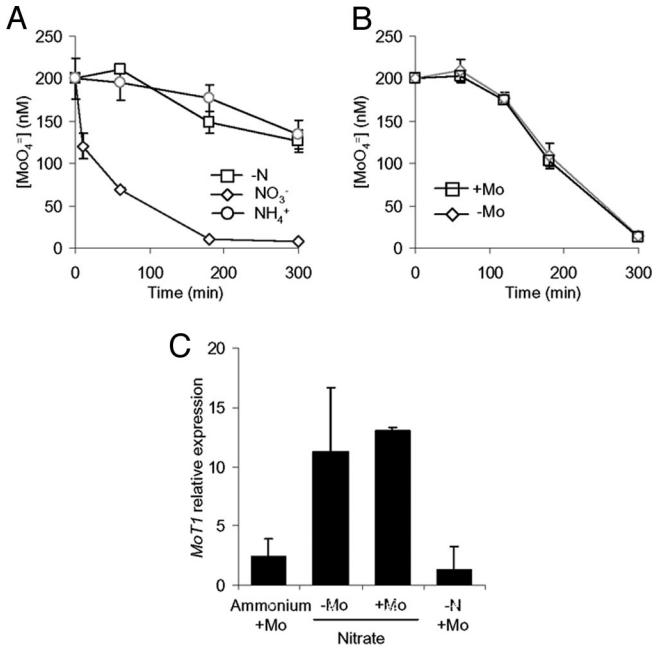

Fig. 6. Role of nitrate and molybdate in *MoT1* expression and MOT1 activity. (*A*) Effect of the nitrogen source on MOT1 activity. (*B*) Effect of molybdate availability on MOT1 activity. (*C*) *MoT1* expression levels in response to the nitrogen source and the presence of molybdate. Incubation time for RNA extraction was 1.5 h. The value 100 was assigned to the expression level of internal standard gene ubiquitin ligase in each condition. Experiments were repeated at least three times. Error bars indicate the standard deviation.

Characterization of MOT1 Activity. To know more about molybdate transport activity of MOT1, we determined the kinetic parameters for MOT1 in strain 21gr to avoid interferences of the other Mo transport system. A K_m of 6.7 ± 0.6 nM and a V_{max} of 1.7 pmol of $MoO_4^=$ per 10^6 cells · min were obtained. This K_m value indicates a very high affinity for molybdate, ensuring the provision of an essential micronutrient from miniscule amounts of this element in the medium. This K_m is comparable with those reported for the molybdate ABC transport system from *E. coli* (50 nM) (9).

To characterize the activation of MOT1 activity, we studied the effect of the nitrogen source and molybdate availability. Strain 21gr, having MOT1 as a single Mo transporter activity, was incubated during 3 h in a Mo-free medium containing nitrate, ammonium, or no nitrogen; and then 200 nM molybdate was added to the media, and molybdate uptake was studied (Fig. 6*A*). Cells in nitrate medium started to take up molybdate very rapidly after the addition of this anion, whereas cells from ammonium or no-nitrogen medium needed a long incubation time after molybdate addition to start the transport of the anion. To study the effect of molybdate on MOT1 expression, strain 21gr was incubated for 3 h in a nitrogen-free medium with or without 200 nM molybdate, after which cells were transferred to a nitrate-containing media supplemented with 200 nM molybdate. Cells incubated with or without molybdate did not show differences in molybdate uptake (Fig. 6*B*). These results point to nitrate as a strong activation signal of MOT1 activity, whereas the presence of molybdate does not seem to affect it appreciably.

Transcription levels of *MoT1* in strain 21gr were also determined by real-time PCR in response to the nitrogen source and molybdate availability (Fig. 6*C*). We found that *MoT1* expression was activated in the presence of nitrate but not by molybdate. These findings are in agreement with the activation pattern of MOT1 activity, suggesting a strong transcriptional control over the regulation of MOT1 activity.

Discussion

The activity of fundamental enzymes for life in eukaryotes depends on the capacity of the cells to acquire molybdenum; however, eukaryotic molybdate transporters are poorly understood. The *Chlamydomonas MoT1* gene encodes a high-affinity molybdate transporter from a eukaryotic organism that has not been previously reported. Using an RNA antisense strategy, we have silenced *MoT1* expression in two *Chlamydomonas* strains. Interference of *MoT1* expression in strain 21gr, which showed only one molybdate transport activity, leads to the inhibition of molybdate uptake and, in turn, of the molybdoenzyme nitrate reductase activity. However, in strain 704, underexpression of *MoT1* does not affect either molybdate uptake or nitrate reductase activity; this fact can be explained by the presence of a second molybdate transport activity that is independent of *MoT1* expression. The molecular nature of the second molybdate transporter is unknown, although it does not seem to be related to *MoT1* because Southern blot analysis and PCR experiments using degenerated primers did not reveal a putative second member of the MOT1 family (*SI Text*).

To confirm the MOT1 functionality as a molybdate transporter, we carried out a heterologous expression in the yeast *S. cerevisiae.* Yeast strains carrying the *MoT1* cDNA were able to transport molybdate from 200 nM, whereas the strain carrying the empty expression vector showed a residual uptake.

Molybdenum is a microelement, and its availability to the organisms as molybdate in most of the media is low (2). To ensure the provision of this metal, bacterial molybdate transport systems exhibit transporters with a K_m of ≈50 nM (9). MOT1 shows a K_m of 7 nM, supporting the need of a high-affinity transport system to guarantee the maintenance of intracellular levels of molybdenum. This very low K_m value is comparable with those reported for uptake of manganese in barley (3–5 nM) (24), folate in the protozoan parasite *Leishmania major* (84 nM) (25), or calcium in the mitochondrial uniporter (2 nM) (26), illustrating important processes that need to be mediated in an efficient way even from miniscule substrate concentrations.

In *Saccharomyces* expressing *MoT1*, molybdate transport is diminished in a medium lacking glucose, pointing to an energy-dependent process. The analogous oxyanion tungstate can partially inhibit the MOT1 activity, but it does not occur with another molybdate analogous as sulfate.

Both *MoT1* expression and MOT1 activity are activated by the presence of nitrate, but are not affected by molybdate availability, indicating that regulation of the molybdate transport process mediated by *MoT1* might occur mostly at the transcriptional level. Bacterial molybdate transporters also have transcriptional regulation; however, it depends on intracellular levels of molyb-

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date (5, 6), suggesting a different regulation process for MOT1 activity. The role of nitrate in *MoT1* activation points to a direct connection between molybdate transport and nitrate assimilation and corresponding to the Mo requirement for an efficient nitrate reduction. Interestingly, among >1,000 genes showing a rapid response to nitrate found by microarray analysis in *Arabidopsis* roots and shoots, there appears the gene that encode an *Arabidopsis* MOT1 family member. It is remarkable that in addition to this putative molybdate transporter, two other genes for Moco biosynthesis, *Cnx2* and *Cnx6* from *Arabidopsis thaliana*, also respond rapidly to nitrate (27). This transcriptional regulation of the first steps of Moco biosynthesis by nitrate in *Arabidopsis* reinforces the intimate connection of Mo transport to the needed Moco production for nitrate assimilation, as we have suggested herein. In addition, the *Ostreococcus tauri MoT1* homologous gene is clustered together with genes for nitrate assimilation and molybdenum metabolism (28).

The protein encoded by *MoT1* has homologs in other organisms; these proteins are not related to the bacterial ABC systems and share exclusive conserved sequence motifs defining a previously uncharacterized family of transporters that includes proteins from plants, algae, fungi, and bacteria. The finding of this family might allow the characterization of molybdate transport in other eukaryotes and also a better understanding of this process in bacteria. Finally, it should be pointed out that, although Moco, and therefore molybdenum, is also essential in animals (29), MOT1 family members have not been found in these organisms so far, indicating that putative molybdate transporters from animals might belong to a family of proteins not closely related to MOT1, probably suggesting an additional mechanism for molybdate transport.

Materials and Methods

Algal Strains. Details of used algal strains are listed in SI Table 1.

Chlamydomonas Transformation. Strains 704 and 21gr were efficiently transformed by the glass beads method (30) with some modifications: polyethylene glycol 8000 was used at a final concentration of 2.5% (wt/vol); $\approx 10^8$ cells were shaken for only 30 s, and the pRBCMoT1as plasmid DNA concentration was from 0.2 to 1 μg per transformation reaction. Transformants were selected in ammonium-containing medium supplemented with 25 μg/ml paramomycin.

Quantitative Real-Time PCR. Quantization of *MoT1* transcripts was performed by real-time PCR. Reverse transcription of 2 μg of total RNA was carried out by using SuperScript II Reverse Transcriptase (Invitrogen) by following manufacturer instructions. Quantitative real-time PCR was performed on an iCycler iQreal-time PCR detection system (Bio-Rad) by using SYBR green I (Molecular Probes) as a fluorescent dye. RNA levels were normalized by using the ubiquitin ligase gene as an internal standard (31).

Primer sequences for *MoT1*: forward, 5′-GTGCAGCCGATGAAGACCATT-3′; reverse, 5′-CAACGCCATGTCTACACCCTTCA-3′.

Molybdate Determination. Determinations of molybdate were carried out in 10 ml of cell-free growth medium, by following the spectrophotometric method described by Cardenas and Mortenson (32). To avoid interferences in final measurements, samples were previously extracted with 0.8 ml of isoamyl acetate.

NADH-Nitrate Reductase Activity. Extracts from 10 ml of algal culture ($3–4.5 \cdot 10^6$ cells per milliliter) were prepared 20-fold concentrated in 50 mM Tris·HCl buffer, pH 7.5. NADH-Nitrate reductase assay was carried out as reported (33).

Note Added in Proof. The group led by Toru Fujiwara recently cloned a molyodate transporter from *A. thaliana*.

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