Algae and humans share a molybdate transporter

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Almost all living organisms need to obtain molybdenum from the external medium to achieve essential processes for life. Activity of important enzymes such as sulfite oxidase, aldehyde oxidase, xanthine dehydrogenase, and nitrate reductase is strictly dependent on the presence of Mo in its active site. Cells take up Mo in the form of the oxianion molybdate, but the molecular nature of the transporters is still not well known in eukaryotes. MOT1 is the first molybdate transporter identified in plant-type eukaryotic organisms, but it is absent in animal genomes. Here we report a molybdate transporter different from the MOT1 family, encoded by the *Chlamydomonas reinhardtii* gene *MoT2*, that is also present in animals including humans. The knockdown of *CrMoT2* transcription leads to the deficiency of molybdate uptake activity in *Chlamydomonas*. In addition, heterologous expression in *Saccharomyces cerevisiae* of *MoT2* genes from *Chlamydomonas* and humans support the functionality of both proteins as molybdate transporters. Characterization of CrMOT2 and HsMOT2 activities showed an apparent Km of about 550 nM that, though higher than the Km reported for MOT1, still corresponds to high affinity systems. *CrMoT2* transcription is activated when extracellular molybdate concentration is low but in contrast to *MoT1* is not activated by nitrate. Analysis of protein databases revealed the presence of four motifs present in all the proteins with high similarity to MOT2, that label a previously undescribed family of proteins probably related to molybdate transport. Our results open the way toward the understanding of molybdate transport as part of molybdenum homeostasis and Moco biosynthesis in animals.

molybdenum metabolism | molybdate resistance | molybdenum homeostasis

Molybdenum is an essential micronutrient for most living beings, from bacteria to animals (1). Mo is biologically inactive by itself, but in the form of molybdopterin-cofactor (Moco) it plays a role in the active site of more than 50 enzymes (2). Eukaryotic Mo enzymes are involved in key steps of carbon, sulfur, and nitrogen metabolisms, such as sulfite detoxification (sulfite oxidase), purine catabolism (xanthine dehydrogenase), phytohormone biosynthesis (aldehyde oxydase), and nitrate assimilation (nitrate reductase) (3, 4). Recently, a new Mo enzyme, named mARC (mitochondrial amidoxime reducing component), has been identified in mammals and is involved in the activation of N-hydroxylated prodrugs to the amidine active drug (5).

Organisms belonging to all kingdoms share a well-conserved pathway to synthesize Moco (6). The last step of this pathway consists of the insertion of a single Mo atom into adenylated molybdopterin to convert molybdopterin in active Moco (7). Therefore Moco biosynthesis strongly depends on a precise supply of Mo. Impairment to synthesizing Moco causes the loss of the activity of all the Mo enzymes avoiding a proper development of essential cellular functions (8, 9).

Organisms meet the need of Mo using specific molybdate transporters. Bacterial molybdate transporters are well known and widely described. These transporters belong to the ATP-binding cassette family and mediate a high affinity process (Km about 50 nM) that is regulated by the protein ModE in response to variations in intracellular Mo concentration (10).

In eukaryotes, molybdate transporters are still not well known. Proteins related to the bacterial molybdate transport system are not encoded by eukaryotic genomes. Algae (*Chlamydomonas*) and plants (*Arabidopsis*) are the only eukaryotes where a MOT1 (molybdate transporter, type 1) protein was demonstrated to transport molybdate with high affinity (11, 12). MOT1 mediates a molybdate transport process that exhibits a Km of 7–20 nM and in *Chlamydomonas* is upregulated by nitrate but is insensitive to external Mo availability. Phylogenetically MOT1 belongs to MFS superfamily and is distantly related to the plant sulfate-carrier (SULTR) type proteins.

MOT1 type proteins are present in bacteria, algae, fungi, and plants but not in animal genomes (11), therefore how animal cells satisfy metabolic molybdenum requirements remains unknown. In animals Moco deficiency is especially serious because it causes a severe phenotype characterized by a progressive neurological damage that leads to death shortly after birth (9, 13).

In *Chlamydomonas* physiological data suggest the presence of at least two molybdate transport systems that are related to genetic loci *Nit5 and Nit6* (14). Mutants at one of these loci, *Nit5*, show a reduced molybdate uptake activity together with a tolerance for high concentrations of molybdate or tungstate in presence of any nitrogen source. This phenotype is shown by strain 21gr that has MOT1 as the only molybdate transport activity (11). Mutants at both loci are unable to synthesize Moco and thus are defective in the activity of the molybdoenzyme nitrate reductase (15). In addition, when *Chlamydomonas* gene *MoT1* is knockdown in a wild-type strain, the presence of a putative second transporter balances the uptake of molybdate (11). However, only one member of the MOT1 family is present in the alga genome (11).

In the present work we have identified MOT2 as a putative molybdate transporter present in eukaryotes, including animals. We have performed an antisense RNA strategy over the *Chlamydomonas* gene *MoT2*, which resulted in a diminution of molybdate uptake proportional to the decrease of *CrMoT2* transcription level. *Saccharomyces cerevisiae* cells transformed with *MoT2* cDNA from *Chlamydomonas* or humans (*HsMoT2*) showed a significant and specific molybdate uptake activity, supporting the function of these genes in molybdate transport. Our findings shed light on molybdate transport in animals as a key step of molybdenum homeostasis and Moco biosynthesis processes.

Results

Identification of MOT2 Family. In order to identify a second *Chlamydomonas* molybdate transporter, we performed a search in the *Chlamydomonas* genome database within the ubiquitous

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MFS superfamily among their members for which functionality had not been shown. Nine putative transporters fitting with this condition were found. Each of them was analyzed for sequence conservations and its presence in Moco-containing eukaryotic genomes. Transporters were discarded if a high conservation with prokaryotic and not with eukaryotic proteins was evident, and if present in organisms such as *Saccharomyces* lacking Moco. One transporter (accession no. XP_001693567) showed these selected characteristics and in addition its expression was found to be upregulated in the absence of molybdate. We focused our efforts on this protein whose functionality as molybdate transporter was later verified and therefore named CrMOT2.

We isolated the *CrMoT2* cDNA by PCR amplifications, and its sequence was obtained and verified by successive reactions. *CrMoT2* genomic DNA was analyzed and showed the presence of 12 exons that result in a 2208 bp cDNA (Fig. S1). The deduced CrMOT2 is a 535 amino acid protein that contains 11 predicted hydrophobic transmembrane domains and shows a significant conservation to other homologous proteins (51 – 26% identity) of unknown function in algae, plants, and animals (Fig. 1*A*). MOT2 shares four motifs highly conserved with similar proteins of the database (Fig. S2), ($_{100}$DWLQGPYV/LYXLY$_{111}$) in the second transmembrane fragment, ($_{177}$GRXLGGXS/ATSLLFSV/AFES/AWL/YXXEH$_{200}$) in the fourth one, ($_{243}$GPVAPF$_{248}$) before the sixth one, and ($_{299}$LGXXQA/SLFEG/SXM/IYI/TFVFL/EWTPV/AL$_{321}$) in the seventh one (Fig. 1*B*). These four motifs are proposed to be characteristic of the family of proteins MOT2. CrMOT2 was analyzed for structure prediction using HHpred software showing high probability of identical structure to several proteins belonging to the MSF superfamily such as the multidrug resistance protein D (99.88%), glycerol-3-phosphate transporter (99.87%), and lactose permease (99.70%). This result suggests that proteins from the MOT2 family also belong to the MSF superfamily. Proteins in the database belonging to the MOT2 family have been named before as protein containing domain of unknown function DUF791. Thus, CrMOT2 and CrMOT1 are different types of proteins, and in fact they present a low sequence identity (11–14%).

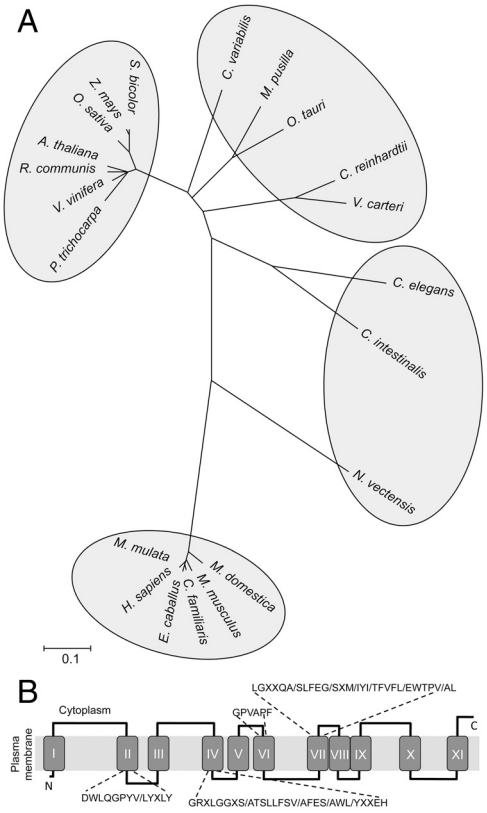

Fig. 1. Analysis of the MOT2 protein. (*A*) Phylogenetic tree of MOT2 homologous proteins; alignments were performed using ClustalW method, and the tree was drawn using Mega4 software. Accession numbers: *Arabidopsis thaliana*, AAF19685; *Caenorhabditis elegans*, NP_500274; *Canis familiaris*, XP_849836; *Ciona intestinalis*, XP_002129829; *Chlamydomonas reinhardtii*, XP_001693567; *Chlorella variabilis*, EFN51339; *Equus caballus*, XP_001504553; *Homo sapiens*, BAC11137; *Monodelphis domestica*, XP_001364537; *Macaca mulatta*, XP_002798648; *Mus musculus*, NP_598861; *Micromonas pusilla*, EEH53273; *Nematostella vectensis*, XP_001629395; *Oryza sativa*, NP_001065080; *Ostreococcus tauri*, CAL56055; *Populus trichocarpa*, XP_002318449; *Ricinus communis*, XP_002518895; *Sorghum bicolor*, XP_002466934; *Volvox carteri*, XP_002948878; *Vitis vinifera*, XP_002280860; *Xenopus tropicalis*, NP_001015939; *Zea mays*, ACG37381. Circles include plants, algae, worms, and animals. (*B*) Schematic representation of predicted CrMOT2 transmembrane topology. Dashed lines indicate the location of conserved motifs. Transmembrane topology has been predicted using TMPRED software.

The *CrMoT2* transcription expression pattern was checked in connection with the nitrogen source and molybdate availability. As shown in Fig. 2, *CrMot2* is upregulated after molybdate shortage but not in the presence of nitrate. This situation is different to the reported regulation of *Chlamydomonas MoT1*, which is activated in presence of nitrate and not affected by molybdate availability (11).

CrMoT2 transcript accumulation was also measured in the strain 21gr (Fig. S3). This strain showed a low *CrMoT2* expression in all conditions checked. This underregulation could be involved in the absence of CrMOT2 activity in this strain (11).

***CrMoT2* Knockdown by Antisense RNA.** With the purpose of defining the function of CrMOT2 protein we carried out an RNA antisense strategy over the *Chlamydomonas* gene *MoT2* in the strain 704 (wild type). The construct used (pGENDP-Mot2as) consists of a 270 bp *CrMoT2* antisense-cDNA under the control of the *Chlamydomonas PsaD* gene promoter (16) and contains the paromomycin resistance cassette as selectable marker (17) (Fig. S4). Using the plasmid pGENDP-Mot2as we obtained 200 single transformants resistant to the antibiotic paromomycin. Among these transformants we found 32 that acquired the ability to grow in media with a high concentration of molybdate (50 mM). Tolerance to molybdate was shown before for the strain 21gr (14)

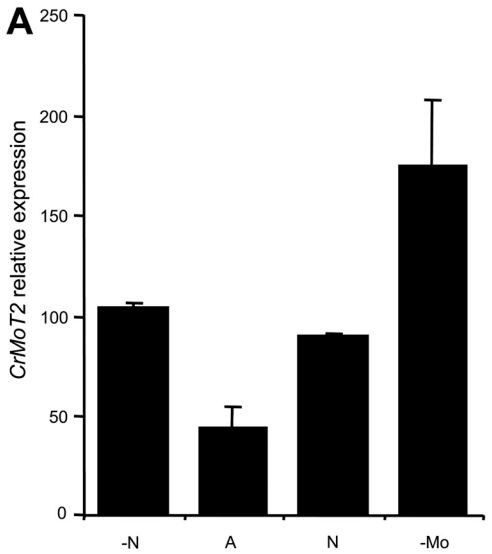

Fig. 2. Regulation of *CrMoT2* transcription in response to nitrogen source and molybdenum availability. A, 8 mM ammonium; N, 4 mM nitrate; -N, nitrogen-free medium; -Mo, molybdenum-free medium. The value 1 was assigned to expression level of internal standard gene ubiquitin-ligase in each condition. Triplicate samples were used in each experiment and the experiments were repeated three times. Error bars indicate the standard deviation.

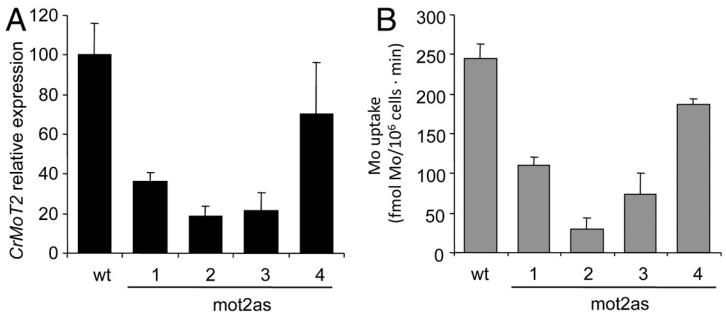

Fig. 3. Effect of reduced *CrMoT2* expression on molybdate transport in *Chlamydomonas*. (*A*) *CrMoT2* transcription in antisense transformants. Quantization was made by real-time PCR. Total RNA was isolated from cells induced during 1.5 h in an 8 mM ammonium-containing medium. (*B*) Molybdate uptake in *CrMoT2* antisense transformants. Experiments were performed in triplicate and repeated at least three times. Error bars indicate the standard deviation.

that is defective in a molybdate transport activity (11). To continue this study we randomly selected four antisense transformants able to grow in the presence of 50 mM molybdate (mot2as1, mot2as2, mot2as3, mot2as4).

CrMoT2 transcription in the selected antisense transformants was measured by real-time PCR and compared to the parental strain. All the antisense transformants showed a decreased expression of Cr*MoT2* (36, 18, 21 and 70% of parental strain, respectively) (Fig. 3*A*).

To know the effect of a low *CrMoT2* expression in molybdate transport rate, we measured molybdate uptake capability in the *CrMoT2* antisense transformants. All the antisense mutants showed a reduced molybdate uptake compared to parental strain; this reduction was proportional to the decreased *CrMoT2* transcript level in each mutant (Fig. 3*B*). In order to avoid MOT1 activity present in the wild-type strain 704, molybdate uptake experiments were performed in media containing ammonium (8 mM) as nitrogen source and using incubation times shorter than 180 min. Under these conditions MOT1 is practically inactive because 3 h are needed by MOT1 to show molybdate transport activity (11). In fact, under conditions where CrMOT1 is active, strains carrying antisense CrMOT2 are not defective in molybdate transport (Fig. S5).

Heterologous Expression of *CrMoT2* in *Saccharomyces cerevisiae*. To confirm the function of CrMOT2 as a molybdate transporter *CrMoT2* cDNA was expressed in *Saccharomyces cerevisiae*. The lack of Moco biosynthesis pathway, including molybdate transport and also *MoT2* homologous genes, makes *Saccharomyces* a good system to study MOT2 function by heterologous overexpression. *CrMoT2* cDNA was cloned in the *Saccharomyces* strain 31019b (18) using the expression vector PDR196 (19). The yeast transformation resulted in approximately 500 transformants able to grow in a uracil free medium. We randomly selected 10 of them to carry out the CrMOT2 functionality study. *Saccharomyces* strains expressing *CrMoT2* cDNA showed a significant molybdate transport activity and comparable to the activity shown by *Saccharomyces* strains transformed with *CrMoT1* cDNA; this activity was not present in a strain carrying the empty PDR196 vector (Fig. 4*A*).

Saccharomyces strains expressing Cr*MoT2* were used to learn the specificity of CrMOT2 to transport molybdate. We determined the molybdate uptake in the presence of 1 mM of sulfate or 10 μM of tungstate; sulfate and tungstate are similar to molybdate in terms of charge and shape. Molybdate uptake mediated by CrMOT2 was inhibited by tungstate (25–40%), but it was practically insensitive to high concentration of sulfate (Fig. 4*B*). These characteristics concerning substrate specificity are similar to those shown by MOT1 (11).

Activity of *HsMoT2* in *Saccharomyces cerevisiae*. In order to elucidate if the MOT2 protein from animals also mediate molybdate transport, we chose a human MOT2 (*HsMoT2*) to be overexpressed in *Saccharomyces*. HsMOT2 studied in this work corresponds to the 450 aa protein encoded by the cDNA clone "MAMMA1001893" isolated from mammary gland (accession no. BAC11137). The gene mapped in the chromosome 12 position 12q13.3 and has been named MFSD5 for major facilitator superfamily domain-containing protein 5.

HsMoT2 corresponds to a 1,353 bp cDNA and its deduced protein HsMOT2 contains a 450-amino-acid sequence that shows the typical hydrophobicity pattern of a membrane protein (12 transmembrane regions are deduced in this case) and 34.5% identity to CrMOT2 (Figs. S2 and S6).

Among the yeast transformants, we selected 10 of them to study the ability to take up molybdate. *Saccharomyces* strains transformed with *HsMoT2* cDNA exhibited a significant molybdate uptake activity comparable with the activity shown by *CrMoT2* expressed in *Saccharomyces* (Fig. 5*A*).

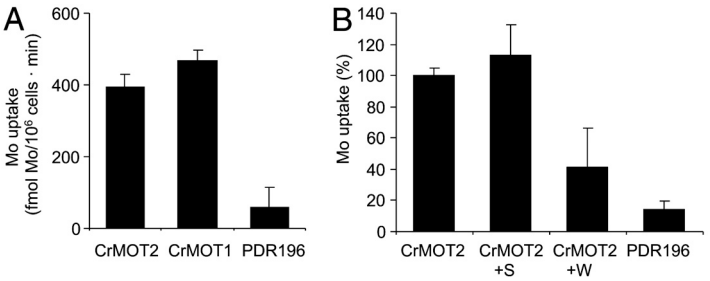

Fig. 4. Activity of CrMOT2 expressed in *Saccharomyces cerevisiae*. (*A*) Molybdate uptake mediated by CrMOT2 compared with molybdate uptake mediated by *Chlamydomonas* MOT1. (*B*) Effect of the presence of molybdate analogue anions in molybdate transport activity mediated by CrMOT2. CrMoT2, *Saccharomyces* transformed with *CrMoT2* cDNA; CrMoT1, *Saccharomyces* transformed with *Chlamydomonas MoT1* cDNA; PDR196, *Saccharomyces* transformed with the empty expression plasmid PDR196. +S, plus 1 mM sulfate; +W, plus 20 μM tungstate. Triplicate samples were used in each experiment and the experiments were repeated three times. Error bars indicate the standard deviation.

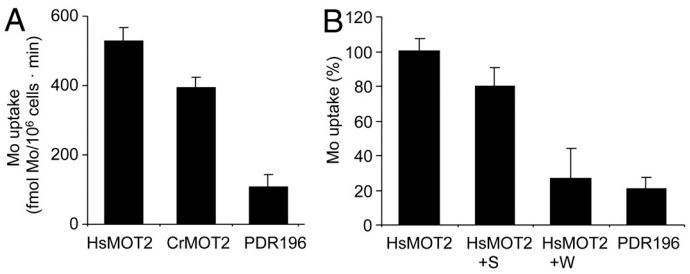

Fig. 5. Activity of protein HsMOT2 expressed in *Saccharomyces cerevisiae*. (*A*) Molybdate uptake mediated by HsMOT2 compared with molybdate uptake mediated by CrMOT2. (*B*) Effect of the presence of molybdate analogue anions in molybdate transport activity mediated by HsMOT2. HsMOT2, *Saccharomyces* transformed with *HsMoT2* cDNA; CrMOT2, *Saccharomyces* transformed with *CrMoT2* cDNA; PDR196, *Saccharomyces* transformed with the empty expression plasmid PDR196. +S, plus 1 mM sulfate; +W, plus 20 μM tungstate. Triplicate samples were used in each experiment and the experiments were repeated three times. Error bars indicate the standard deviation.

The specificity of HsMOT2 was also investigated. As occurred with CrMOT2, molybdate uptake by HsMOT2 was inhibited by 10 μM tungstate, but it was nearly insensitive to 1 mM sulfate (Fig. 5*B*). This result strongly suggests that molybdate transport is the function of the HsMOT2 protein as well as of other proteins belonging to MOT2 family.

Molybdate Affinity MOT2 Transporters. Kinetic parameters for MOT2 were determined using *Saccharomyces* transformed with *CrMoT2* cDNA. The apparent Km obtained was 550 ± 46 (s.d.) nM (Fig. S7*A*). Despite the Km obtained is higher than the values reported for MOT1 (20) and for the bacterial molybdate transport system (10); we can consider CrMOT2 as a high affinity molybdate transporter.

We also measured the Km of the process mediated by *HsMoT2* cDNA expressed in *Saccharomyces* that corresponded to 546 ± 54 (s.d.) nM (Fig. S7*B*). The affinity shown by HsMOT2 is very similar to that shown by CrMOT2 as expected for two proteins belonging to the same family.

Discussion

Homeostasis of micronutrients is a complex mechanism that requires the coordination of a number of cellular processes that maintain their proper intracellular needs (21, 22). Among the different micronutrients molybdenum is the least abundant, estimated in only 10^5 atoms per *Chlamydomonas* cell (23). Notwithstanding, molybdenum is a key metal in the active site of important oxidoreductases (1, 2) and requires specific transporters contributing to its particular cell homeostasis. Molybdate is used in the final step of Moco biosynthesis when it is incorporated into molybdopterin catalyzed by CNX1 (7). However, molybdate transporters are poorly understood in eukaryotes and especially in animals, because the only molybdate transporter named MOT1 was identified in plant-type organisms (*Arabidopsis* and *Chlamydomonas*) that is even present in some bacterial genomes but not in animals (11, 12, 20). The *Chlamydomonas* MOT1 was successfully identified by considering the close chemical similarity between sulfate and molybdate and then looking for putative sulfate transporters with a nondemonstrated function (11). In this work, we identify a second molybdate transporter whose existence was predicted in *Chlamydomonas* from physiological, biochemical, and genetic data (14). The search for this second system was based on the fact that *Chlamydomonas* has only one *MoT1* gene and so another gene family would account for it. Probably, it might correspond to genes from the ubiquitous MFS superfamily with unknown function, which in turn would be absent in organisms unable to metabolize molybdenum such as *Saccharomyces*. In this work, we show that this transporter is encoded by *CrMoT2* gene.

That *CrMoT2* is a gene for a molybdate transporter is supported by different facts: (*i*) inhibition of *Mot2* expression by an antisense RNA strategy links its expression levels to molybdate transport activity; (*ii*) heterologous expression of Cr*Mot2* cDNA in *Saccharomyces* results in a molybdate transport activity similar to that provided by Cr*Mot1* cDNA, and with similar responses to sulfate and tungstate inhibition; (*iii*) the apparent Km for molybdate is low and would correspond to a high affinity system; (*iv*) its expression in *Chlamydomonas* responds to the molybdate availability signal; and (*v*) a human MOT2 analog expressed heterologously shows very similar properties to the *Chlamydomonas* MOT2 with respect to molybdate transport activity, apparent Km, and inhibition characteristics.

CrMOT2 allowed us to identify similar proteins in algae, plants, and animals, all of which carry conserved motifs in their sequence that would define a unique family of molybdate transporters. Interestingly, some organisms like plants and algae carry both types of molybdate transporters MOT1 and MOT2, like *Chlamydomonas*. So molybdenum homeostasis (20) will have to be reevaluated in these organisms under this view. CrMOT1 shows upregulation by nitrate, whereas CrMOT2 is upregulated by Mo deficiency. This upregulation does not seem to be strong enough by N deprivation because transcriptome analysis in *Chlamydomonas* following nitrogen deprivation activates expression of some Moco biosynthesis genes such as *Cnx1E*, *Cnx2*, and *Cnx6* but not *MoT1* (24). However, upregulation of CrMOT1 by nitrate is high enough (11), suggesting that once nitrate assimilation can occur, molybdate provision has to be ensured by cells. Transcriptome analysis performed in *Arabidopsis* has shown the relevance of molybdenum for plant biology because its deficiency has ample effects affecting expression of genes involved in sulfur and nitrogen metabolism, stress responses, signal transduction, and the levels of amino acids and many other C and N compounds (25). *CrMoT2* transcription is activated in conditions of low Mo availability and partially inhibited by the presence of ammonium as nitrogen source; however, the presence of nitrate does not have an effect in *CrMoT2* expression. Activation by low external molybdate concentration is also present in the bacterial molybdate transport system (10) and in *Arabidopsis MoT1* (12). Nevertheless *Chlamydomonas MoT1* shows a different transcription pattern that is activated by nitrate and insensitive to external Mo control (11). By EST population analyses *HsMoT2* transcript has been detected in a number of tissues and cell types, suggesting a higher transcript accumulation in cervix, stomach, nerve, and skin (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.654660).

Saccharomyces expressing either CrMOT2 or HsMOT2 exhibit a very similar Km of about 550 nM, which is comparable to Km values reported for molybdate transport in bacteria (50–100 nM) (10) and plants (7–20 nM) (20). This Km is also in the range to the values reported for high affinity transport of other micronutrients in humans such as copper (5 μM) (26), zinc (3 μM) (27), and selenium (279 nM) (28). The differential regulation of MOT1 and MOT2 in *Chlamydomonas*, together with the different affinity showed for both transporters, might point to

a division of the work, though it could be shared, to provide Mo depending on cell needs in response to environmental changes. While MOT1 seems to be connected to the nitrate assimilation demand, CrMOT2 might be responsible to ensure the molybdate provision in scarceness of this anion. Since animals do not assimilate nitrate, it is consistent the presence in these organisms of a molybdate transporter not related to nitrate metabolism.

Quantitatively nitrate assimilation is of the highest importance for *Chlamydomonas* cells, which have this compound as the main source of the macroelement N, so that under conditions of nitrate assimilation the provision of molybdenum for a functional nitrate reductase enzyme is the highest among all other molybdoenzymes because it is critical for cell viability. In fact, *Chlamydomonas* molybdenum cofactor biosynthesis mutants can grow normally in ammonium-containing media, but they die in nitrate as the only nitrogen source. Therefore, for *Chlamydomonas*, having different molybdate transporters responding to environmental changes such as nitrogen source or Mo availability is of a vital interest to maintain a right intracellular concentration of this metal in order to carry out its critical biological functions.

However, and in contrast to animals, sulfite detoxification is not so important for a single cell living in aquatic environments to which sulfite could be readily exported by transport systems.

Transport activities of CrMOT2 and HsMOT2 show the same inhibition pattern in the presence of analogous oxianions such as tungstate or sulfate. Activity of both transporters was inhibited by tungstate but practically insensitive to high concentrations of sulfate. The very scarce inhibition of sulfate on these two systems supports their high specificity toward molybdate. Molybdate transport inhibition by tungstate but not by sulfate has been reported in a number of specific molybdate transporters such as *Chlamydomonas* and *Arabidopsis* MOT1 (11, 12), the bacterial ABC system from *Escherichia coli* (10), and *Anabaena variabilis* (29). In addition, this inhibition pattern has also been reported for the sulfate transporter from *Stylosanthes hamata* SHST1 that mediates a nonspecific molybdate transport (30). Deficiency in the molybdate transport mediated by MOT2 results in resistance to high molybdate concentrations (50 mM) in *Chlamydomonas*. CrMOT2 activity deficiency has been related before with this high Mo tolerance in the *Chlamydomonas* strain 21gr (14). This strain is deficient in molybdate transport (14), lacks CrMOT2 activity, has a normal MOT1 transport activity (11), and tolerates high molybdate and tungstate concentrations (14). *MoT2* coding region in strain 21gr is not affected because its genomic DNA did not have any detected mutation, as found after its sequencing. This strain is affected in expression of this gen (Fig. S3). *MoT2* downregulation in strain 21gr may be related to the molybdate tolerance of this strain. In addition, these results suggest that CrMOT2 mediates Mo transport with a high capacity. However MOT1 deficiency does not lead to Mo tolerance, and it might mediate a molybdate transport process with high affinity but low capacity.

The four conserved motifs found in MOT2 proteins are the identity sign of a previously undescribed family of proteins probably related to molybdenum uptake in algae, plants, and animals. This family, together with MOT1 family, will be an essential tool to understand Mo homeostasis in eukaryotic cells and represents the starting point in the knowledge of molybdate transport in animal cells.

Materials and Methods

Algal Strains. Details of algal strains used in this work are listed in Table S1.

Plasmids. *Saccharomyces* expression vector PDR196 was kindly provided by Nicolaus von Wiren. *CrMoT2* and *HsMoT2* cDNA were cloned in PDR196 linearized with *Eco*RI and *Xho*I. pGENDP vector was kindly supplied by Jean-David Rochaix. pGENDP-Mot2as (Fig. S4) was obtained inserting a 270 pb *CrMoT2* cDNA antisense fragment in the *Eco*RI-*Pst*I sites of pGENDP vector. Antisense fragment was obtained by PCR amplification using the oligonucleotides 5′GCGCTTTCGGCTGTTTAGGA3′ as upper primer, and 5′ CGGGGGCTGTGCTTGGTG3′ as lower primer.

***Chlamydomonas* Transformation.** Wild-type strain 704 was efficiently transformed by the glass beads method (31) with some modifications: Polyethylene glycol 8000 was used at a final concentration of 2.5% (w/v), about 10^8 cells were shaken for only 8 s, and pGENDP-Mot2as plasmidic DNA concentration was from 0.1 to 0.3 μg/transformation reaction. Transformants were selected in ammonium-containing medium supplemented with 25 μg/mL paromomycin. Transformation efficiency was 1,000 transformants/μg DNA of pGENDP-Motas plasmid.

***Saccharomyces* Transformation.** Cells from an overnight culture were resuspended in 30 mL of YPAD medium and grown to a density of about 10^7 cells/mL. Cells were harvested by centrifugation and resuspended in 1 mL of sterile distilled water. Washed cells were harvested by centrifugation and resuspended in 1 mL of 100 mM lithium acetate and incubated for 5 min at 30 °C. Cells suspension was harvested and supernatant was removed with a micropipette. The following transformation mix was added on top of cell pellet: 240 μL polyethylene glycol 4000 (50% w/v), 36 μL lithium acetate (1 M), 10 μL salmon sperm DNA (10 mg/mL), 64 μL distilled water, and 200 ng of plasmid DNA. Cells were shaken by vortex for at least 1 min to resuspend the cell pellet in the transformation mix and incubated at 42 °C for 20 min. Cells were harvested and supernatant was removed using the micropipette. Then cells were gently resuspended the pellet in 200–400 μL of sterile distilled water by slowly pipetting up and down. Cell suspension was plated in the medium that selects for the presence of the plasmid. *Saccharomyces* colonies were visible in 2–4 d at 30 °C.

***Chlamydomonas* Total RNA Isolation.** Total RNA was isolated from *Chlamydomonas* cells grown in a Mo-free media with 8 mM ammonium till exponential growth, washed with nitrogen- and molybdate-free medium, and transferred to the indicated media and then induced for 1.5 h. Isolation of total RNA of *Chlamydomonas* was performed according to methods described previously (32).

Quantitative Real-Time PCR. Quantization of *CrMoT2* transcripts was performed by real-time PCR. Reverse transcription of 2 μg of total RNA was carried out using SuperScript™ II Reverse Transcriptase (Invitrogen) by following manufacturer instructions. Quantitative real-time polymerase chain reaction was performed on an iCycler iQreal-time PCR detection system (Bio-Rad) using SYBR Green I (Molecular Probes) as a fluorescent dye. RNA levels were normalized by using ubiquitin-ligase gene as an internal standard (33).

Primers sequences for *CrMoT2*: forward, 5′-GTGTGCGTGGTGCTGTACAAGGTCA-3′; reverse, 5′-CTTCCACGGCCGCTTAATCACCTC-3′.

Molybdate Determination. Molybdate transport was determined from molybdate depletion in the medium. Determinations of molybdate were carried out in 10 mL of cell-free growth medium by following the spectrophotometric method previously described by Cardenas and Mortenson (34). To avoid interferences in final measurements, samples were previously extracted with 0.8 mL of isoamyl acetate.

Molybdate Transport in *Chlamydomonas*. Cells were grown in ammonium-containing medium to exponential growth without added molybdate; then cells were washed with nitrogen- and molybdate-free medium and transferred to 8 mM ammonium and 700 nM molybdate medium.

Molybdate Transport in *Saccharomyces*. Yeast cells were grown in YNB-rich medium. Molybdate uptake was measured in a MES buffer (pH 5.8) containing 1% glucose and 700 nM molybdate.

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