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Sulfur Availability and the *SAC1* Gene Control Adenosine Triphosphate Sulfurylase Gene Expression in *Chlamydomonas reinhardtii*[1]

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A *Chlamydomonas reinhardtii* adenosine triphosphate (ATP) sulfurylase cDNA clone (pATS1) was selected by complementing a mutation in the ATP sulfurylase gene (*cysD*) of *Escherichia coli. E. coli cysD* strains harboring pATS1 grow on medium containing sulfate as the sole sulfur source and exhibit ATP sulfurylase activity. The amino acid sequence of the *C. reinhardtii* ATP sulfurylase, derived from the nucleotide sequence of the complementing gene (*ATS1*), is 25 to 40% identical to that of ATP sulfurylases in other eukaryotic organisms and has a putative transit peptide at its amino terminus. ATP sulfurylase mRNA was present when cells were grown in sulfur-replete medium, but accumulated to higher levels when the cells were exposed to sulfur-limiting conditions. Furthermore, sulfur-stress-induced accumulation of the *ATS1* transcript was reduced in a strain defective in *SAC1*, a gene that is critical for acclimation to sulfur-limited growth.

Sulfate must be activated prior to being metabolized. Initially, sulfate is combined with ATP in a reaction catalyzed by ATP sulfurylase to form APS. APS may then be phosphoryalated by APS kinase to form PAPS. Both APS and PAPS are subsequently used in the synthesis of sulfur-containing molecules.

ATP sulfurylases are widely distributed in nature. These enzymes (Levi and Wolf, 1969; Shaw and Anderson, 1972; Shoyab et al., 1972; Osslund et al., 1982; Burnell, 1984; Yu et al., 1989; Schmutz, 1990; Li et al., 1991; Renosto et al., 1991) and the genes that code for them (Foster et al., 1994; Klonus et al., 1994; Leustek et al., 1994; Borges-Walmsley et al., 1995; Chen and Leustek, 1995; Li et al., 1995; Murillo and Leustek, 1995; Rosenthal and Leustek, 1995) have been isolated from several organisms. All prokaryotic ATP sulfurylases identified so far are multimeric proteins and similar in primary sequence. In *Escherichia coli*, the ATP sulfurylase has two subunits of 27 and 62 kD that are encoded by *cysD* and *cysN*, respectively (Leyh et al., 1988). The enzyme from the cyanobacterium *Synechococcus* 6301 is also composed of two subunits, which have molecular masses of 41 and 44 kD (Mishra and Schmidt, 1992). *Rhizobium meliloti* contains three copies of two genes that produce a bifunctional product having both ATP sulfurylase and APS kinase activities (Schwedock and Long, 1990, 1992). One set of these genes, located on the chromosome, is thought to be involved in Met and Cys biosynthesis, whereas the other genes, located on the pSym megaplasmids, are important for synthesis of nodulation factors (Schwedock and Long, 1992).

The eukaryotic form of ATP sulfurylase shows no similarity in primary sequence to the prokaryotic forms. The molecular mass of ATP sulfurylase activity in eukaryotic organisms varies from 42 (corn roots) to 800 kD (rat liver enzyme), and the enzyme can be either multimeric or monomeric (Onajobi et al., 1973; Osslund et al., 1982; Yu et al., 1989). The enzyme from *Penicillium chrysogenum* is an oligomer of 420 kD that is composed of identical or very similar subunits, each with a molecular mass of 69 kD (Martin et al., 1989; Renosto et al., 1990; Foster et al., 1994). In cabbage (*Brassica capitata*) the enzyme is a dimer composed of two subunits of 57 kD each (Osslund et al., 1982). *Euglena gracilis* contains monomeric forms of ATP sulfurylase in both the mitochondria and the cytoplasm (Li et al., 1991), which have molecular masses of 52.3 and 55 kD, respectively.

Recently, genes encoding ATP sulfurylase have been characterized from a number of eukaryotic organisms (Cherest et al., 1987; Foster et al., 1994; Borges-Walmsley et al., 1995). As in prokaryotes, some of these genes encode only ATP sulfurylase activity, whereas others encode both ATP sulfurylase and APS kinase activities. The genes of fungi (Cherest et al., 1987; Foster et al., 1994; Borges-Walmsley et al., 1995) and plants (Klonus et al., 1994, 1995; Leustek et al., 1994; Murillo and Leustek, 1995) encode only ATP sulfurylase activity. It is interesting that the ATP sulfurylases of the filamentous fungi *Aspergillus nidulans* and *Penicillium chrysogenum* have C-terminal extensions similar in sequence to APS kinase. This C-terminal domain may bind PAPS and be important for controlling ATP sulfurylase activity, but it has no APS kinase activity (Renosto et al., 1990). The ATP sulfurylase genes of the euchu-

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Abbreviations: APS, adenosine 5′-phosphosulfate; LB, Luria broth; PAPS, phosphoadenosine 5′-phosphosulfate; TAP, Tris-acetate-phosphate medium; TAP-S, Tris-acetate-phosphate medium lacking in sulfur.

roid worm *Urechis caupo* encodes a protein that is bifunctional, containing both ATP sulfurylase and APS kinase activities (Rosenthal and Leustek, 1995). A fused sulfurylase-kinase protein is also thought to be present in mice (Li et al., 1995).

We have been examining the acquisition and utilization of sulfur by *Chlamydomonas reinhardtii* and the acclimation of this organism to sulfur-limited growth. *C. reinhardtii* is a unicellular green alga that adjusts to sulfur limitation both by altering metabolic processes and by increasing its capacity for scavenging sulfur from its environment. Arylsulfatase, a periplasmic protein specifically synthesized by *C. reinhardtii* during sulfur limitation (de Hostos et al., 1988, 1989), catalyzes the hydrolysis of arylsulfate esters, releasing sulfate for transport into the cell. In addition, sulfur limitation of *C. reinhardtii* triggers the synthesis of a new sulfate transport system with an elevated affinity for sulfate (approximately 5-fold) and an increased capacity for transport (approximately 10-fold) (Yildiz et al., 1994), the production of several periplasmic proteins with unknown functions (de Hostos et al., 1988), and the down-regulation of photosynthetic electron transport (Davies et al., 1996). These responses are all under the control of the *SAC1* (sulfur acclimation) gene, which has recently been isolated and characterized (Davies et al., 1996).

To further analyze the acclimation of *C. reinhardtii* to sulfur-limited growth, we screened a *C. reinhardtii* cDNA expression library for functional complementation of an *E. coli* mutant lacking ATP sulfurylase. A complementing clone was isolated and shown to contain a gene (designated *ATS1*) for a *C. reinhardtii* ATP sulfurylase. Based on the nucleotide sequence, the *C. reinhardtii* enzyme is similar to ATP sulfurylases in other eukaryotes, but exhibits no similarity to prokaryotic ATP sulfurylase genes. The level of *ATS1* mRNA is elevated during sulfur-limited growth and this increase in mRNA accumulation appears to be at least partially under the control of the *SAC1* gene.

MATERIALS AND METHODS

Strains and Culture Conditions

Escherichia coli strain TSL3 (*cysD*91) carries a mutation in the *cysD* gene (Leyh et al., 1988). *E. coli* cells were grown in either LB or M9 minimal medium (Maniatis et al., 1982) supplemented with thiamine (20 μg/mL) and, when appropriate, Cys (40 μg/mL). Ampicillin was added to the medium to a concentration of 100 μg/mL.

Chlamydomonas reinhardtii strain CC125 was obtained from the Genetics Stock Center (Duke University, Durham, NC). The *sac1* strain, defective in sulfur acclimation responses, has been previously described (Davies et al., 1994, 1996). The cells were grown in TAP or TAP-S (Davies et al., 1992) medium; for the latter, chloride salts were substituted for sulfate salts. Cells were grown at 27°C with constant shaking and illuminated with cool-white fluorescent light at 150 μmol photons m^{-2} s^{-1}.

Recombinant Library and Isolation of ATP Sulfurylase cDNA

A cDNA library was constructed using poly(A) mRNA isolated from *C. reinhardtii* strain CC125 grown in TAP medium. Poly(A) RNA was isolated by oligo(dT) cellulose affinity chromatography using the protocol described by the manufacturer (BRL). The cDNA, prepared using a kit (Stratagene), was ligated into the vector Lambda ZAPII (Stratagene). The recombinant library, which contained 5 $\times$ 10^5 primary recombinant phages, was amplified by standard procedures (Maniatis et al., 1982). Approximately 1 $\times$ 10^6 Lambda ZAPII recombinant phages were used for mass excision of pBluescriptII SK− (Stratagene) phagemid. The excision was performed with ExAssist helper phage and SOLR cells, according to the manufacturer's instructions (Stratagene).

Competent, auxotrophic TSL3 cells, prepared using rubidium chloride according to the method of Hanahan (1983), were transformed with 10 to 100 ng of DNA from the excision library. Transformants were plated on M9 Glc minimal medium containing 100 μg/mL ampicillin to select for prototrophic transformants. When pBluescript was transformed into TSL3 no colonies able to grow on M9 medium were obtained.

ATP Sulfurylase Assay

Protein extracts were prepared from cells grown in LB medium. Cells from 100 mL of culture were pelleted by centrifugation (5,000*g* for 5 min), washed once with 50 mM Tris-HCl (pH 8), and then resuspended in 0.5 mL of 20 mM Tris-HCl (pH 8.0), 30 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 10% glycerol. The cells were lysed by passage through a French pressure cell (16,000 p.s.i.), cell debris and membranes were pelleted by centrifugation at 12,000 rpm (SS34 rotor, Beckman) for 15 min at 4°C, and ATP sulfurylase activity was assayed in the supernatant (Leyh et al., 1988). The assay included 25 mM ATP, 5 mM GTP, 1 unit/μL pyrophosphatase, and 2.5 μCi/μL $^{35}SO_4$. The reactions were incubated for 1 h at 30°C, boiled for 1 min, and centrifuged for 2 min in a microfuge at full speed to remove cell debris. Aliquots of the reaction mixture were applied to a cellulose TLC, PEI-F plate (J.T. Baker, Phillipsburg, NJ), resolved in 0.9 M LiCl and analyzed by autoradiography. In control reactions baker's yeast ATP sulfurylase (Sigma) was added to a final concentration of 0.1 unit/μL.

DNA Sequencing

The sequence of the cDNA insert in a plasmid that complemented the *cysD* strain (the plasmid was designated pATS1) was determined using the dideoxy-chain termination method (United States Biochemical). A series of plasmids containing nested deletions of the insert DNA, generated using a kit (Erase-a-Base, Promega), were used as sequencing templates. Sequence analysis and database searches were performed using PileUp software (Genetics Computer Group, Madison, WI).

Nucleic Acid Isolation and Manipulation

Isolation of nucleic acids and hybridization of the labeled DNA fragments to restriction digests of genomic DNA and total RNA were performed as described previously (Davies et al., 1992).

RESULTS

Isolation of a *C. reinhardtii* ATP Sulfurylase cDNA Clone

The *E. coli cysD* strain TSL3 contains a mutation in the ATP sulfurylase gene and is a Cys auxotroph; it is unable to grow on M9 medium containing sulfate as the sole source of sulfur. A *C. reinhardtii* expression library in pBluescript was transformed into TSL3, and colonies able to grow on M9 medium were selected. The plasmid pATS1, isolated from one such transformant, was sufficient to suppress the Cys auxotrophic phenotype of TSL3. Figure 1A shows that although both TSL3/pBS and the TSL3/pATS1 were able to grow on LB-ampicillin plates, only TSL3/pATS1 was able to grow on M9-ampicillin medium.

Cell extracts of TSL3 and TSL3/pATS1 were assayed for the presence of ATP sulfurylase activity. The formation of radiolabeled APS from $^{35}SO_4^{-2}$ and ATP was detected by TLC. As shown in Figure 1B, a reaction product with the same R_F value as APS (0.49) was synthesized when either purified ATP sulfurylase (lane 2) or extracts of TSL3 strains harboring pATS1 (lane 4) were included in the reaction mixture. Furthermore, a reaction product with an R_F of PAPS was also present when the assay was performed with extracts from TSL3/pATS1. Reaction mixtures that included either no protein extract (lane 1) or protein extract from untransformed TSL3 (lane 3) did not synthesize APS or PAPS. These results demonstrate that the pATS1 cDNA clone suppresses the Cys auxotrophy of TSL3 and restores to this strain the ability to synthesize ATP sulfurylase. Hence, pATS1 likely encodes a *C. reinhardtii* ATP sulfurylase.

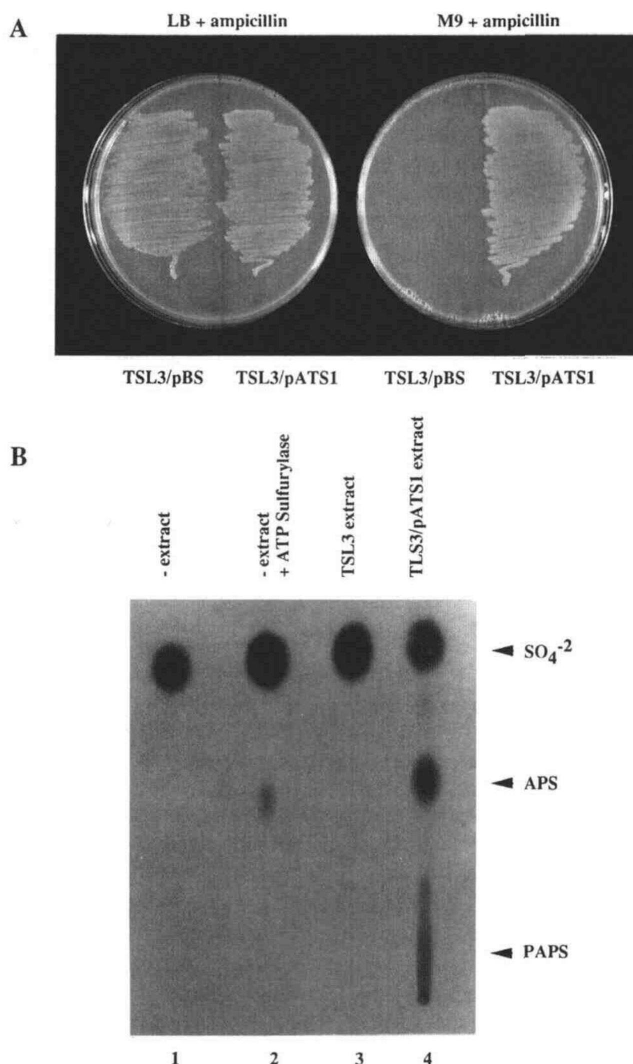

Figure 1. A, Complementation of *E. coli* strain TSL3, which is defective in *cysD*. TSL3 harboring pBluescript (pBS) or the plasmid pATS1 were grown on either LB or M9 medium. B, ATP sulfurylase assay of *E. coli* TSL3 or TSL3 harboring pATS1. Extracts from the strains were incubated with $^{35}SO_4$ and ATP, and reaction products were separated by TLC (Schwedock and Long, 1990) and analyzed by autoradiography. Lane 1, Reaction mixture minus cell extract; lane 2, reaction mixture with purified baker's yeast ATP sulfurylase (Sigma) instead of cell extract; lane 3, reaction mixture with cell extract from TSL3; lane 4, reaction mixture with cell extract from TSL3 harboring pATS1.

Sequence Analysis of *C. reinhardtii* ATP Sulfurylase cDNA

The 1.9-kb cDNA insert of pATS1 was sequenced and shown to contain an open reading frame for a polypeptide of 437 amino acids (Fig. 2) with a molecular mass of 48,507 D. The *ATS1* transcript has a putative 5′ untranslated region of 92 bases and a putative 3′ untranslated region of 507 bases. As shown in Figure 2, the predicted amino acid sequence of the protein encoded by pATS1 is similar to ATP sulfurylases from other eukaryotic organisms. The identity of the *C. reinhardtii* sequence with ATP sulfurylases from *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Arabidopsis thaliana* is 36, 33, and 25%, respectively (75, 72, and 66% similar, respectively). No significant similarity to the *E. coli* or the *Rhizobium meliloti* ATP sulfurylases was observed. Like the *A. thaliana* ATP sulfurylase, the *C. reinhardtii* ATP sulfurylase has an N-terminal extension relative to fungal ATP sulfurylases. This extension has the characteristics of a transit peptide (see "Discussion"), which functions in the transport of proteins into the chloroplast (Keegstra, 1989).

Hybridization to Genomic DNA

The pATS1 insert cDNA was hybridized to *C. reinhardtii* genomic DNA to test for the presence of additional *ATS* genes on the *C. reinhardtii* genome. Figure 3B shows an autoradiogram of *C. reinhardtii* genomic DNA digested with *Bam*HI, *Hin*dIII, *Eco*RI, and *Sal*I either singly or in combination and hybridized with the pATS1 insert (Fig. 3A). The results of this analysis indicate that *ATS1* hybrid-

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Chlamydomonas    1 MVPRAAAGPVARGVAARVRAAPAPVVAAKSSRRSSVVVRATAAPVAQVRTAEGLQVPHGP
Saccharomyces    1 .....................................................MPAPHGG
Aspergillus      1 ....................................................MANTPHGG
Arabidopsis      1 ....MASMAAVLSKTPFLSQPLTKSSPNSDLPFAAVSFPSKSLRRRVGSIRAGEIAPDGG

Chlamydomonas   61 AAKLVNLMAPASEHAALKAACNKRIELSDRT....CDVELLTVGAFSPLEGFHNKAEYDS
Saccharomyces    8 ILQDLIARDALKKNELLSEAQSSDILVWNLTPRQLCDIELILNGGFSPLTGFLNENDYSS
Aspergillus      9 VLKDLIARDAPRHDQLEAEAATLPSIV..LTERQLCDLELIMNGGFSPLEGFMNQKDYDG
Arabidopsis     57 KLVGLIVEEPKRREKKHEAADLPRVEL...TAIDLQWMHVLSEGWASPLGGFMRESEFLQ

Chlamydomonas  117 VV..ANMRMTNGLLFGLPIVLDTDSE.....DIVPGDKLLL..TYQGQDLAVMTVDSKFT
Saccharomyces   68 VV..TDSRLADGTLWTIPITLDVDEAFANQ..IKPDTRIALFQ.DDEIPIAILTVQDVYK
Aspergillus     67 VV..AESRLADGNLFSMPITLDASKAVIEQAGLKPGERVTLRDFRDDRNLAILTIDDIYR
Arabidopsis    114 TLHFNSLRLDDGSVVNMSVPIVLAIDDEQKARIGESTRVALFNS.DGNPVAILSDIEIYK

Chlamydomonas  168 PNKPLECLKCYGTSSLEHPAVQMVAMERGKYYIGGPIKGLALPTRVFPCAS....PADVR
Saccharomyces  123 PNKTIEAEKVFRGDP.EHPAISYLFNVAGDYYVGGSLEAIQLPQH.YDYPGLRKTPAQLR
Aspergillus    125 PDKEKEAKLVFGGDP.EHPAIKYLNTKVEEYYIGGKLEAVNKLNH.YDYVGLRYTPAELR
Arabidopsis    173 HPKEERIARTWGTTAPGLPYVDEAITNAGNWLIGGDLEVLEPVKYNDGLDRFRLSPAELR

Chlamydomonas  224 STLPANQ..DVLAFQCRNPIHKAHYELFIRALDRNVRN..PGAVCLVHPTCGPTQDDDIP
Saccharomyces  181 LEFQSRQWDRVVAFQTRNPMHRAHRELTVRA....ARE..ANAKVLIHPVVGLTKPGDID
Aspergillus    183 IHFDKLGWTRVVAFQTRNPMHRAHRELTVRA....ARA..RQANVLIHPVVGLTKPGDID
Arabidopsis    233 KELEKRNADAVFAFQLRNPVHNGHALLMTDTRRRLLEMGYQKPYSFASSVSGFTKADDVP

Chlamydomonas  280 GVVRFRTYEVLKEE..TKNPRLRWAYLPYSMHMAGPREAIQHMIIRKNYGCTHFIIGRDM
Saccharomyces  235 HHTRVRVYQEIIKR..YPNGIAFLSLLPLAMRMSGDREAVWHAIIRKNYGASHFIVGRDH
Aspergillus    237 HFTRVRAYQALLPR..YPNGMAALALLPLAMRMGGPREAVWHAIIRKNHGATHFIVGRDH
Arabidopsis    293 LDWRMKQHEKVLEDGVLDPETTVVSIFPSPMHYAGPTEVQWHAKARINAGANFYIVGRDP

Chlamydomonas  338 AGCKSSISGQDFYRAYDAQDLANKHAAELNMQTVASLNIAY.TEEKGYVTADIAKAENLH
Saccharomyces  293 AGPGKNSKGVDFYGPYDAQELVESYKHELDIEVVPFRMVTYLPDEDRYAPIDQIDTTKTR
Aspergillus    295 AGPGKNSKGQEFYGPYDAQHAVEKYRAELGIEVVEFQQVTYLPDTDEYMPKDEVPAG.TK
Arabidopsis    353 AGMGHPVEKRDLYDADHGKKVLSMAPGLERLNILPFRVAAYDKTQGKMAFFDPSRPQDF.

Chlamydomonas  397 VLNLSGTKFRQMLRAGDDIPEWFAFKSVVAVLR...EQIQSEAN*...............
Saccharomyces  353 TLNISGTELRRRLRVGGEIPEWFSYPEVVKILR...ESNPPRPKQGFSIVLGNSLTVSRE
Aspergillus    354 TLDISGTELRNRLRTGAHIPEWFSYPEVVKILR...ESSPPRALQGFTIFLTGYMNSGKD
Arabidopsis    412 .LFISGTKMRTLAKNNENPPDGFMCPGGWKVLVDYYESLTPAGNGRLPEVVPV*......

Chlamydomonas      ............................................................
Saccharomyces  410 QLSIALLSTFLQFGGGRYYKIFEHNNKTEL.................LSLIQDFIGSGSG
Aspergillus    411 AIARALQVTLNQQGGRSVTLLLGDTVRHELSSELGFSREDRHTNVQRIAFVAGELTRAGA
Arabidopsis        ............................................................

Chlamydomonas      ............................................................
Saccharomyces  453 LIIPNQ.......WEDDKDSVVGKQNVYLL.................DTSSSADIQ.L
Aspergillus    471 AVIAAPIAPYEHSRKAAREAVQSTGGSFFLVHVNTPLEYCEATDKRGIYAKARRGEIKGF
Arabidopsis        ............................................................

Chlamydomonas      .............................................
Saccharomyces  486 ESADEPFHTLYKK...LSYSWKTMAFLYFNRHKMLPSQK*.....
Aspergillus    531 TGVDDPYEAPTNANLVVDVSKQSVRSIVHEIILMLESEGYFERL*
Arabidopsis        .............................................
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Figure 2. Comparison of the deduced amino acid sequence of the *C. reinhardtii ATS1* with *S. cerevisiae MET3* (Cherest et al., 1987), *A. nidulans sC* (Borges-Walmsley et al., 1995), and *A. thaliana APS1* (Leustek et al., 1994). The sequences were aligned using the PileUp program (Genetics Computer Group). Identical (black boxes) and similar (gray boxes) amino acids are designated.

izes to a 10-kb region of the *C. reinhardtii* genome between the *Bam*HI and *Hin*dIII sites shown in Figure 3E. However, these data do not determine how many *ATS* genes are present in this area. To determine if more than one *ATS* gene is present in the 10-kb *Bam*HI/*Hin*dIII genomic fragment, radiolabeled DNA fragments specific to the 5′ and 3′ regions of the *ATS* cDNA were hybridized to total genomic DNA digested with *Bam*HI/*Hin*dIII and *Bam*HI/*Hin*dIII/*Sca*I (Fig. 3, C and D). Both the 5′- and 3′-specific probes hybridized to the 10-kb *Bam*HI/*Hin*dIII fragment. This piece of DNA is cut by *Sca*I into a 7-kb fragment that hybridizes specifically to the 5′ *ATS1* probe (Fig. 3C) and a 3-kb fragment that hybridizes specifically to the 3′ *ATS1* probe (Fig. 3D). These data demonstrate the presence of a single *ATS* gene within the 10-kb *Bam*HI/*Hin*dIII fragment, but do not exclude the possibility of the existence of additional ATP sulfurylase genes that are not similar enough to *ATS1* to be detected in this analysis.

Expression Analysis of ATP Sulfurylase Gene

To determine if expression of *ATS1* is regulated by sulfur availability, total RNA was isolated from cells grown in sulfur-replete and sulfur-deficient medium for 1, 2, 4, 6, 8, and 10 h. The RNA was resolved by denaturing agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with the 1.9-kb insert of pATS1. A transcript of about 2 kb was detected in cells grown on sulfur-replete medium. The size of the transcript suggests that the insert of pATS1 is full-length or nearly so. The ATP sulfurylase mRNA increases in abundance during sulfur-limited growth (Fig. 4A, left). As presented in Figure 4B, quantitation of the mRNA levels normalized to 18S rRNA (which does not change during sulfur starvation) demonstrates that sulfur limitation leads to a 5- to 6-fold increase in the

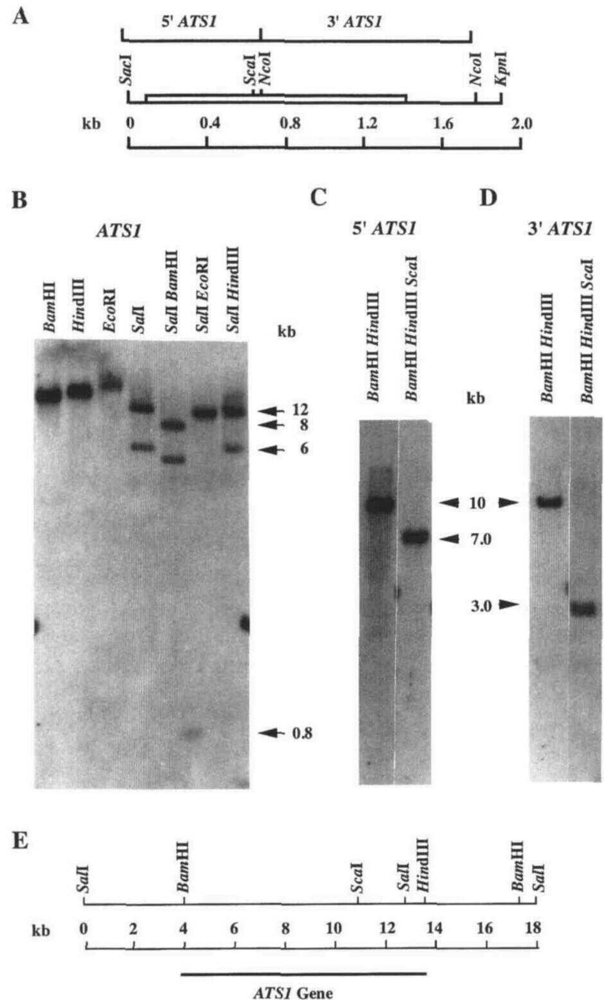

Figure 3. Hybridization of the *ATS1* gene to *C. reinhardtii* genomic DNA. A, Restriction map of the *ATS1* cDNA. The open box indicates the coding region of the *ATS1* gene. The two DNA fragments, 5′*ATS1* and 3′*ATS1*, which are used in hybridization experiments presented in B, C, and D, are shown above the restriction map. B, Genomic DNA was digested with *Bam*HI, *Hin*dIII, *Eco*RI, *Sal*I, *Sal*I/*Bam*HI, *Sal*I/*Eco*RI, and *Sal*I/*Hin*dIII. The restriction fragments were separated on 0.8% agarose gels by electrophoresis, transferred to nitrocellulose membranes, and hybridized with the 1.9-kb insert of pATS1 excised by cutting with *Sac*I and *Kpn*I. C and D, Total genomic DNA digested with *Bam*HI/*Hin*dIII and *Bam*HI/*Hin*dIII/*Sca*I, and the restriction fragments separated by electrophoresis, transferred to nitrocellulose membranes, and hybridized with the 0.7-kb 5′*ATS1* (C) and the 1.1-kb 3′*ATS1* (D) fragments. E, Restriction map of the genomic *ATS1* gene. The length of the region with homology is represented by a thickened line below the restriction map. For B, C, and D, the numbers contiguous with the autoradiograms indicate the estimated sizes (in kb) of the hybridizing fragments.

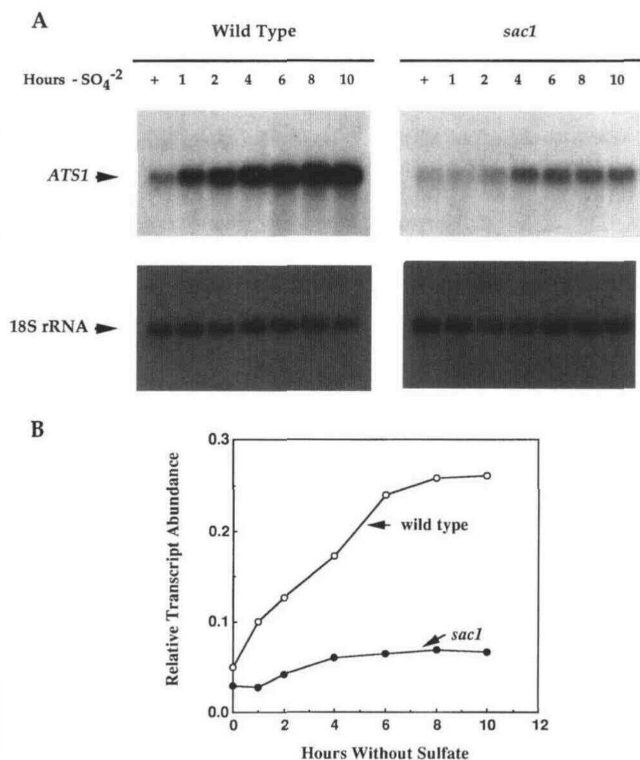

Figure 4. Accumulation of *ATS1* mRNA during sulfur starvation in wild-type cells and the *sac*1 mutant. Total RNA was isolated from cells grown in TAP (sulfur-replete) and from those subjected to sulfur deprivation (TAP-SO_4) for various times. Ten micrograms of RNA was resolved on a 1% agarose gel by electrophoresis, transferred to nitrocellulose membranes, and hybridized to the 1.9-kb *ATS1* gene. Hybridization of *ATS1* to total RNA isolated from CC125 (the wild-type strain) (left) and the *sac1* mutant (right) at 0, 1, 2, 4, 6, 8, and 10 h after sulfur deprivation. B, Quantitation of the signals observed in A was with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To standardize the hybridization signals, the membranes were stripped and rehybridized to a fragment of 18S rDNA.

accumulation of the *ATS1* transcript, with maximum levels of transcript accumulation at 4 h after the elimination of sulfur from the medium.

ATP Sulfurylase Expression in the *sac1* Mutant

We previously isolated regulatory mutants of *C. reinhardtii* that are aberrant in their responses to sulfur limitation (Davies et al., 1994). The *sac1* mutant is aberrant in all of the responses to sulfur limitation that we have examined (Davies et al., 1996). To investigate whether the expression of *ATS1* is regulated by the same factors that control acclimation to sulfur limitation, the accumulation of *ATS1* mRNA was examined in the *sac1* mutant grown under sulfur-sufficient conditions and after exposure to sulfur limitation for 1 to 10 h. As shown in Figure 4A (right), the *ATS1* transcript increases to a small extent in the *sac1* mutant during sulfur limitation; quantitation of transcript levels, shown in Figure 4B, demonstrates that *ATS1* mRNA increases less than 2-fold in the *sac1* mutant. This experiment was repeated three times and gave essentially identical results, indicating that increased accumulation of *ATS1* mRNA during sulfur-limited growth is influenced by *SAC1*.

DISCUSSION

A cDNA clone from *C. reinhardtii* encoding ATP sulfurylase was isolated by functional complementation of an *E. coli* Cys auxotroph (strain TSL3), which is devoid of ATP sulfurylase activity. The complemented strain was selected for its ability to grow on M9 minimal medium with inorganic sulfate as the sole sulfur source. The sequence of the cDNA in plasmid pATS1 that was isolated from complemented TSL3 and the presence of ATP sulfurylase activity in extracts of TSL3 harboring pATS1 confirmed that the selected clone encoded an ATP sulfurylase.

The deduced amino acid sequence of *C. reinhardtii ATS1* exhibited similarity to ATP sulfurylases from a number of fungi and vascular plants. The similarity to the fungal ATP sulfurylases extends over the entire coding region of the *C. reinhardtii* protein, except for an extension of the *C. reinhardtii* sequence at the N terminus of about 50 amino acids. The *A. nidulans*, *P. chrysogenum*, and *S. cerevisiae* enzymes have C-terminal extensions of over 100 amino acids that are not present on the *C. reinhardtii* protein. The C-terminal extensions on the *A. nidulans* and *P. chrysogenum* ATP sulfurylases exhibit similarity to APS kinases (Borges-Walmsley et al., 1995) and probably function in allosteric control of enzyme activity by PAPS (the product of the APS kinase reaction). It has been shown that the ATP sulfurylase from these filamentous fungi are strongly inhibited by PAPS (Renosto et al., 1990). Since the *C. reinhardtii* ATP sulfurylase has no similarity to APS kinase, it may not be susceptible to allosteric control by PAPS. Similarly, there is no C-terminal extension on the vascular plant ATP sulfurylases (Klonus et al., 1994; Murillo and Leustek, 1995).

The N-terminal extension present on the *C. reinhardtii* ATP sulfurylase is probably a transit peptide involved in the transport of proteins into the chloroplast. This is suggested by the fact that sulfate is reduced in the chloroplast by the photosynthetic electron transport chain, and that the majority of the ATP sulfurylase in plants is localized to plastids (Lunn et al., 1990; Renosto et al., 1993; Klonus et al., 1994; Leustek et al., 1994). The putative presequence on *ATS1* exhibits several characteristics that support the idea that it functions in chloroplast targeting. Although there is little sequence similarity among transit peptides, they are generally enriched in hydroxylated and small hydrophobic amino acids, have a net positive charge, and lack acidic amino acids (von Heijne et al., 1989); the putative ATS1 presequence shows all of these characteristics. The ATP sulfurylases of both *A. thaliana* and *Solanum tuberosum* (Klonus et al., 1994; Murillo and Leustek, 1995) also have presequences that are similar to transit peptides. In the case of *A. thaliana*, an in vitro import assay was employed to show that the primary translation product of the ATP sulfurylase (mature protein plus transit peptide) was able to pass through the double envelope membrane of the chloroplast (Leustek et al., 1994).

A single region of the *C. reinhardtii* genome shows strong hybridization to *ATS1*. This region spans a maximum of 10 kb. Using 5′ and 3′ regions of the *ATS1*, we were able to demonstrate that this region contains a single ATP sulfurylase gene. If other ATP sulfurylase genes are present on the *C. reinhardtii* genome they do not hybridize to *ATS1* under the conditions of hybridization used in these studies. Some plants have been shown to have multiple isozymes for ATP sulfurylase, which are localized to various subcellular compartments (Brunold and Suter, 1989; Lunn et al., 1990; Renosto et al., 1993). Multiple isozymes may reflect posttranslational polypeptide modifications and/or multiple ATP sulfurylase genes. In *S. tuberosum*, two distinct cDNA clones for the ATP sulfurylase have been isolated (Klonus et al., 1994). One of these genes encodes a protein with a putative transit peptide, whereas the other encodes a protein lacking a transit peptide; the latter protein may be localized in the cytosol of the cell. In contrast, the three cDNA clones that have been isolated from *A. thaliana* all appear to be chloroplast-localized (Murillo and Leustek, 1995).

The level of the *ATS1* transcript appears to be strongly regulated by the concentration of available sulfur in the environment. Low levels of the *ATS1* mRNA were observed under nutrient-replete conditions. Increases in *ATS1* mRNA in *C. reinhardtii* cultures were detected within 1 h of depleting the medium of sulfur. By 6 h of sulfur starvation the mRNA levels increased to between 5- and 6-fold. Although still not tested, it is likely that this increase in *ATS1* mRNA is transcriptionally controlled, similar to what is observed for the *ARS1* gene (de Hostos et al., 1989). Increases in enzymes involved in sulfate assimilation have been observed in microorganisms, fungi, and plants that are grown in sulfur-deficient medium (Cherest et al., 1985; Chen and Leustek, 1995). The accumulation of ATP sulfurylase mRNA in *S. cerevisiae* has been shown to increase when there is no organic sulfur source available to the organism (Cherest et al., 1985). Sulfur starvation has also been shown to result in increased accumulation of both ATP sulfurylase and APS kinase in *Brassica juncea* (Chen and Leustek, 1995).

Regulatory factors that control the responses of eukaryotic organisms to sulfur limitation have been defined to some extent in the fungi. The increased production of ATP sulfurylase in *S. cerevisiae* is likely to be mediated by the transcriptional activator MET4 (Thomas et al., 1989, 1992). The Met4 protein is a Leu-zipper-containing transcriptional activator of the bZIP type (Thomas et al., 1992; Kuras and Thomas, 1995). In *Neurospora crassa* the Cys3 protein, which is also a bZIP-type DNA-binding protein, serves as a positive activator of sulfur-regulated genes (Marzluf, 1968; Paietta et al., 1987; Paietta, 1992).

We have isolated and characterized a number of mutants of *C. reinhardtii* that are unable to acclimate to sulfur limitation (Davies et al., 1994). The *sac1* mutant does not produce arylsulfatase in response to sulfur limitation and exhibits aberrations in the induction of sulfate transport capacity and the production of sulfur stress-specific periplasmic proteins. Furthermore, the *sac1* mutant is unable to effect a decrease in photosynthetic electron transport, which appears to be either directly or indirectly responsible for a marked loss in the ability of this strain to survive sulfur limitation (Davies et al., 1996). The SacI protein may be involved in sensing the sulfur status of the cell, or it may serve as an activator of transcription, similar to Met4 and Cys3 (although it does not show homology to either). Like all of the other acclimation responses listed above, *SAC1* appears to be involved in controlling the increase in *ATS1* mRNA that is observed during sulfur-limited growth.

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