Sulfate Transport in *Neurospora crassa*: Regulation, Turnover, and Cellular Localization of the CYS-14 Protein†

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ABSTRACT: Uptake of inorganic sulfate in *Neurospora crassa* is governed by the sulfur regulatory circuit and is under the control of positively and negatively acting regulatory genes. Two genetically and biochemically distinct systems are responsible for the uptake of sulfate from the environment. One of these, sulfate permease II, encoded by the *cys*-14 gene, functions primarily in mycelia. A defined region of the CYS-14 protein was highly expressed in *Escherichia coli* and purified. Anti-CYS-14 antibody was produced and used to detect the CYS-14 protein in *N. crassa* extracts. The CYS-14 protein has an approximate molecular weight of 95K, in agreement with its calculated size based on its predicted amino acid sequence. The steady-state level of the CYS-14 protein is highly regulated in wild-type mycelia and constitutive in an *scon*-1 mutant, whereas no CYS-14 protein could be detected in a *cys*-3 mutant. Following the accumulation of the *cys*-14 mRNA, that reaches its maximum in about 6 h, the CYS-14 protein accumulates to a maximum level in about 8 h after derepression. During conditions of sulfur repression, the CYS-14 protein turns over with a half-life of approximately 2 h. The CYS-14 protein appears to be localized in the plasma membrane, suggesting that it functions as a sulfate ion transporter.

In *Neurospora crassa*, a number of enzymes which function in the uptake and early steps of assimilation of a variety of sulfur sources are subject to genetic and metabolic control (Marzluf & Metzenberg, 1968). The synthesis of an entire set of enzymes, including aryl sulfatase (Metzenberg & Ahlgren, 1970), a methionine permease (Pall, 1971), two sulfate permeases (Marzluf, 1970a,b), and an extracellular protease (Hanson & Marzluf, 1973), occurs in a concerted fashion when the cellular sulfur level becomes limited. If sulfur sources are available, a metabolite derived from methionine, perhaps cysteine (Jacobson & Metzenberg, 1977), acts to repress the synthesis of the enzymes in the sulfur circuit.

Three regulatory genes have been shown so far to control the sulfur regulatory circuit. The *cys*-3 gene serves to turn on the expression of the structural genes, evidenced by the pleiotropic loss of the entire set of sulfur-controlled enzymes in *cys*-3 mutants (Marzluf & Fu, 1989). Mutants of another unlinked gene, *scon*-1 (previously designated *scon*), show constitutive expression of the sulfur-controlled enzymes, indicating a negative control function for the *scon*-1 gene product (Burton & Metzenberg, 1972). More recently, another negative regulatory gene, *scon*-2, has been identified and cloned (Paietta, 1990). Northern blot analyses demonstrated that the expression of both *cys*-3 and *scon*-2 is regulated by *scon*-1 (Fu et al., 1989; Paietta, 1990) and that the *scon*-2 gene product regulates the expression of *cys*-3 (Paietta, 1990). Thus, a simple model for the operation of the sulfur circuit is that the *scon*-1 gene product senses the cellular level of a sulfur metabolite and accordingly controls the expression of *cys*-3. The *scon*-2 gene may be involved in the transmission of the signal between *scon*-1 and *cys*-3. The CYS-3 protein, in turn, controls the expression of the entire set of structural genes in the circuit.

One of the most extensively studied components of the sulfur regulatory circuit in *N. crassa* is the dual sulfur transport systems. Two unlinked genetic loci, *cys*-13 and *cys*-14, are involved in the uptake of sulfate and may encode distinct permease species (Marzluf, 1970b). In addition to their regulation by the sulfur control genes, both also appear to be regulated developmentally: *cys*-13 is expressed primarily in germinating conidia, whereas *cys*-14 expression is almost entirely limited to mycelia (Marzluf, 1970a). Not only are these two systems genetically distinct but also their biochemical characteristics are readily distinguishable (Marzluf, 1970a).

Although *cys*-13 and *cys*-14 mutants are each deficient in a distinct sulfate transport activity, it has remained a mystery whether these genes encode a membrane-bound transporter or contribute in some other fashion to sulfate uptake. The *cys*-14 gene has been cloned and characterized recently (Ketter & Marzluf, 1988; Ketter et al., 1991). It appears that *cys*-14 gene expression is controlled at the level of mRNA content. The nucleotide sequence of the gene revealed a long open reading frame that encodes a protein with a calculated molecular weight of 87K. The predicted amino acid sequence of the protein contains 12 highly hydrophobic, possible helical regions, suggesting that the CYS-14 protein might be associated with the plasma membrane.

The aim of the present study was to immunologically detect the CYS-14 protein in *N. crassa* and to investigate the regulation of its expression at the protein level. Furthermore, to address the question whether the function of the CYS-14 protein is regulated by de novo synthesis and turnover, a direct examination of its accumulation and turnover was carried out along with kinetic mRNA studies. Finally, we also report here the detection of the CYS-14 protein in the plasma membrane fraction which suggests that it may indeed function as a membrane-bound sulfate transporter.

Materials and Methods

Strains, Plasmids, and Growth Conditions. The *Neurospora crassa* wild-type strain 74OR23-1A (FGSC 987) and the *cys*-3 mutant (FGSC 1089) were obtained from the Fungal

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Genetics Stock Center, Kansas City, KS. The *scon* mutant (allele RLM 36-21) was kindly provided by R. L. Metzenberg. The temperature-sensitive revertant of *cys*-3 was described previously (Marzluf & Metzenberg, 1968) as was the *cys*-14 mutant (Marzluf, 1970b). Cultures were grown in Vogel's minimal medium that lacked the usual sulfur source but was supplemented with 0.25 mM L-methionine (derepressing conditions) or 5 mM L-methionine (repressing conditions) and with 1.5% sucrose as carbon source.

A defined region of the CYS-14 protein was expressed in the *Escherichia coli* strain BL21(DE3)pLysS (Studier & Moffatt, 1986) which carries the gene for T7 RNA polymerase in the chromosome under the control of the lac UV 5 promoter. This host strain enables IPTG-mediated induction of foreign genes that are put under the control of the T7 RNA polymerase promoter in the pET vector series (Rosenberg et al., 1987).

Isolation of the Expressed CYS-14 Protein Fragment from E. coli. A fresh colony of strain BL21(DE3)pLysS containing the recombinant plasmid pCYS14EXP (see Results) was grown overnight in 5 mL of 2×YT medium; 0.1–0.3 mL of the overnight culture was inoculated into 500 mL of the same medium and grown until the absorbance at 590 nm reached 0.6–0.7; then cells were induced with 1 mM IPTG and grown for an additional 2.5–3.0 h. Cells were harvested and resuspended in 2 mL of lysis buffer (100 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 2 mM DTT, and 1 mM PMSF) and lysed by sonication for six 30-s periods with cooling. The mixture was centrifuged (1000*g*, 10 min), and the pellet was washed several times with lysis buffer and then resuspended in 2 mL of lysis buffer containing 8 M urea in order to dissolve any inclusion bodies. Urea was removed by extensive dialysis against lysis buffer. After an ammonium sulfate precipitation (30%), a fairly enriched preparation of the expressed CYS-14 protein was obtained. Pure CYS-14 protein (single band in SDS–PAGE) was obtained by electroelution of the desired band from an SDS–polyacrylamide gel.

Antibody Production and Western Blot Analyses. Rabbit anti-CYS-14 polyclonal antibodies were raised in white New Zealand rabbits using two injections of approximately 100 μg of protein, separated by a 6-week interval.

For Western blots, discontinuous protein gels (Laemmli, 1970) were run and proteins electrotransferred to nitrocellulose as described (Sambrook et al., 1989). To detect CYS-14 protein on the filters, the ImmunoSelect kit (biotinylated goat anti-rabbit IgG, streptavidin–alkaline phosphatase system; BRL, Gaithersburg, MD) was used according to the manufacturer with some modifications to improve specificity and sensitivity. The most important of these modifications was that an absorption of the secondary antibody with *Neurospora crassa* whole cell extracts was essential for specificity, i.e., to remove nonspecific background.

Isolation of Subcellular Fractions of Neurospora crassa. Whole cell extracts were prepared by extensively grinding 1.5 g of frozen mycelia in a mortar and pestle with 1 g of sand and 1 mL of 100 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM PMSF. Debris was pelleted by centrifugation at 3000*g* for 10 min. The supernatant was stored in aliquots at −70 °C. Typically, 2–8 μL was loaded on protein gels for analyses.

For the isolation of subcellular fractions, the method of Bowman et al. (1981) was employed except for the following modification. In spite of several efforts, we were unable to detect the CYS-14 protein in any of the fractions when cell lysis was carried out with spheroplasting enzymes and a glass homogenizer possibly because components of the spheroplasting enzyme mixture degrade some membrane-spanning proteins during the preparation of the spheroplasts. Therefore, cell lysis was achieved by very gentle grinding of frozen mycelia. All additional steps were done as described.

Nucleic Acid Techniques. DNA was sequenced by the dideoxy chain termination method (Sanger et al., 1977) using the modified T7 bacteriophage DNA polymerase Sequenase (USB Corp., Cleveland, OH). *Neurospora crassa* total RNA was isolated by the method of Reinert et al. (1981) from wild-type cells grown on media in either 5 mM methionine (sulfur repression) or 0.25 mM methionine (sulfur derepression), and the poly(A+) RNA was then purified as described (Aviv & Leder, 1972). The blotting of the RNA samples to nitrocellulose membranes and subsequent hybridization were carried out according to Thomas (1980) and White and Bancroft (1982). All other techniques were done as described (Sambrook et al., 1989).

Results

Expression and Purification of a CYS-14 Protein Fragment and Production of Polyclonal Antibodies. The amino acid sequence of the CYS-14 protein was predicted on the basis of the nucleotide sequence of the *cys*-14 gene (Ketter et al., 1991). The CYS-14 protein is fairly large, consisting of 781 amino acids with a calculated molecular weight of 87K. For the purpose of antibody production, only a part of this protein was expressed because the expression of eukaryotic proteins of this size is usually not readily achieved in *E. coli*. Moreover, the predicted amino acid sequence of the CYS-14 protein contains several hydrophobic regions which could make its high-level expression and isolation difficult, and possibly even toxic to *E. coli*. Therefore, a short hydrophilic region of CYS-14 was chosen for expression.

A 1-kb *Bgl*II–*Eco*RI fragment of the *cys*-14 cDNA clone (Ketter et al., 1991) containing the region coding for the last 238 carboxy-terminal amino acids and a 130 bp long nontranslated 3′ region was subcloned into the pET3c expression vector so that the resulting expressed protein would contain 12 amino acids at its amino-terminal end coded for by vector sequences. This plasmid was designated pCYS14EXP (Figure 1); its nucleotide sequence around the fusion site was determined in order to prove that we obtained the desired in-frame fusion. pCYS14EXP was then transformed into the BL21-(DE3)pLysS host strain, and conditions for its expression were optimized (not shown) so that the CYS-14 protein fragment was the most abundant protein in induced *E. coli* cells (Figure 2). As the level of expression increased, the previously soluble CYS-14 protein fragment was found to be in inclusion bodies, and was purified as described under Materials and Methods. Pure protein, obtained by electroelution of the desired band from polyacrylamide gels (Figure 2), was used for polyclonal antibody production.

The specificity of the anti-CYS-14 antibody was first tested with extracts derived from *E. coli* cells which contained the expressed CYS-14 protein fragment. Whole cell extracts of induced (1 mM IPTG) or uninduced *E. coli* cells were run in discontinuous SDS–polyacrylamide gels in duplicate. One gel was then stained with Coomassie brillant blue for protein detection, whereas proteins resolved in the other gel were electrophoretically transfered to a nitrocellulose filter and the blot probed with the anti-CYS-14 antibody. As shown in Figure 2, the polyclonal antibody specifically recognized the expressed CYS-14 protein fragment.

Western Blot Analyses of the CYS-14 Protein in Wild-Type N. crassa. Wild-type cells were grown overnight under sul-

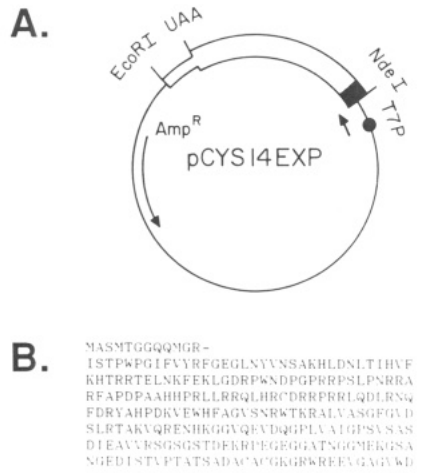

FIGURE 1: Map of pCYS14EXP. Panel A: A 1.0-kb *Bgl*II–*Eco*RI fragment of a *cys*-14 cDNA clone (open bar) was subcloned into pET3c that had been cut with *Bam*HI and *Eco*RI. The expressed CYS-14 protein fragment contains 12 amino acids at its amino-terminal end that are encoded by vector sequences (solid bar). The carboxy end of the *cys*-14 coding region is shown by the stop codon UAA. The dot indicates the position of the T7 RNA polymerase promoter that was used to express the CYS-14 protein fragment. Panel B: The amino acid sequence of the expressed protein is shown. The first 12 residues (first lane) are coded for by the vector.

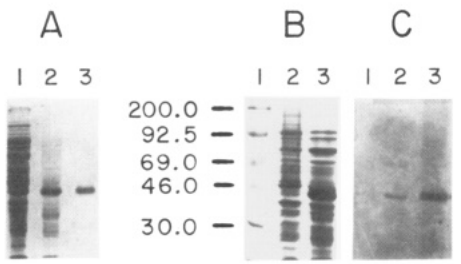

FIGURE 2: Expression of the CYS-14 protein in *E. coli*. Panel A: A crude *E. coli* extract containing the expressed CYS-14 protein (lane 1) was run in a 10% stacking polyacrylamide gel along with a highly enriched fraction (lane 2) that was obtained by the lysis of inclusion bodies and ammonium sulfate precipitation. Pure CYS-14 protein fragment was isolated by electroelution from a preparative polyacrylamide gel (lane 3). Panel B: *E. coli* cells containing pCYS14EXP were grown under uninduced (lane 2) or induced (lane 3) conditions and run in a similar gel as in panel A along with molecular weight ($\times 10^{-3}$) markers (lane 1). Panel C: An exact copy of the gel shown in panel B was run, and the resolved proteins were electroblotted onto a nitrocellulose filter and probed with anti-CYS-14 antibody. The antibody selectively recognized the expressed CYS-14 protein in *E. coli*.

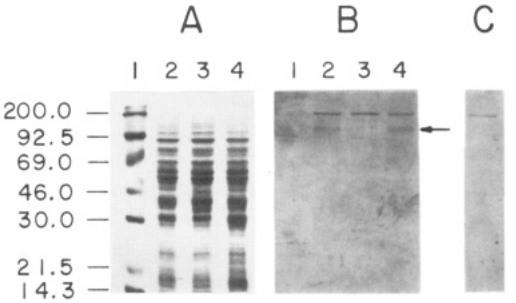

FIGURE 3: Identification of the native CYS-14 protein in extracts of wild-type *N. crassa*. Panel A: Wild-type cells were grown under repressing (lane 3) or derepressing (lanes 2 and 4) conditions, and whole cell extracts were prepared and electrophoresed as described in the legend to Figure 2; then the gel was stained. Lane 1 contains molecular weight markers ($\times 10^{-3}$). Panel B: Proteins in an exact copy of the gel shown in panel A were Western-blotted and probed with the anti-CYS-14 antibody. A band, shown by the arrow, with an approximate molecular weight of 95K was detected in derepressed but not in repressed cells. Panel C: Control; a duplicate of lane 2 in panel A was developed by omitting both the primary (anti-CYS-14) and the secondary (goat anti-rabbit IgG) antibodies. The 150-kDa protein was visualized by this procedure, indicating that it does not cross-react with the anti-CYS-14 antibody but rather that it binds streptavidin.

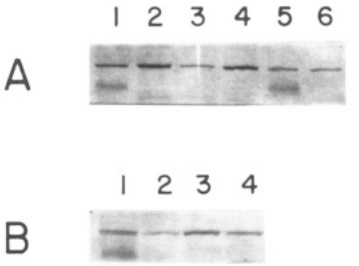

FIGURE 4: Western blot analyses of the expression of the CYS-14 protein in mutant *N. crassa* strains. Wild-type (panel A, lanes 1 and 2), *cys*-3 (panel A, lanes 3 and 4), *cys*-14 (panel A, lanes 5 and 6), and a *cys*-3 temperature-sensitive revertant (panel B, lanes 1 and 2 grown at 25 °C; lanes 3 and 4 grown at 37 °C) were grown under repressing (panel A, lanes 2, 4, and 6; panel B, lanes 2 and 4) or derepressing (panel A, lanes 1, 3, and 5; panel B, lanes 1 and 3) conditions, and then whole cell extracts were analyzed as described in the legend to Figure 2. The CYS-14 protein was present in derepressed wild-type, in derepressed *cys*-14, and in the derepressed *cys*-3 temperature-sensitive revertant at the permissive temperature, but missing in all other cases.

fur-repressing (high methionine) or sulfur-derepressing (low methionine) conditions. Crude whole cell extracts were prepared from the mycelia, and two identical gels were run. One was stained for proteins to demonstrate that approximately equal amounts of protein were loaded in each lane (Figure 3, panel A). Proteins resolved in the other gel were electroblotted and probed with the anti-CYS-14 antibody (Figure 3, panel B). A protein with an approximate molecular weight of 95K was detected in extracts of cells grown under derepressing conditions, whereas it was not visible in repressed cells. A second, constitutive band was detected in both extracts. However, control experiments (Figure 3, panel C) showed that this band corresponds to a protein that strongly binds streptavidin and is therefore visualized during the development procedure. This protein proved to be helpful as an internal standard. The specific reaction of the anti-CYS-14 antibody with a 95-kDa protein, the approximate size of the predicted CYS-14 protein, in sulfur-derepressed (but not repressed) cells, strongly suggested that we had indeed detected the *N. crassa* CYS-14 protein. The steady-state level of the protein appears to be highly regulated, as expected on the basis of previous functional (Marzluf, 1972) and mRNA (Ketter & Marzluf, 1988) analyses.

Regulation of cys-14 Expression in N. crassa Mutants. To examine the effect of the regulatory genes *cys-3* and *scon*-1 on *cys*-14 expression at the protein level, a set of immunoblot experiments was carried out using whole cell extracts prepared from mutant cells grown under repressing or derepressing conditions. The results are shown in Figure 4. No CYS-14 protein at all was detected in *cys*-3 mutant cells grown with either low or high methionine levels, as expected on the basis of the positive control of the *cys*-3 gene product on the expression of the *cys*-14 gene. Further proof was obtained by examining a *cys-3* temperature-sensitive revertant. The CYS-14 protein was present at the wild-type level in this mutant when grown with low sulfur at 25 °C, the permissive temperature. At 37 °C, however, no CYS-14 protein was found in cells grown under either condition.

N. crassa cells that are mutant for another sulfur regulatory gene, *scon*-1, are not sensitive to sulfur repression (Burton & Metzenberg, 1972). In agreement, the CYS-14 protein was

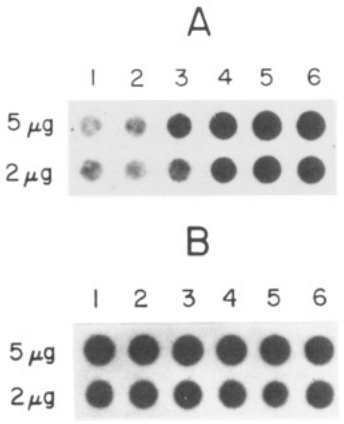

FIGURE 5: Dot blot analysis of *cys*-14 mRNA accumulation. RNA samples were blotted by using a Minifold apparatus and then hybridized with the *cys*-14 gene (panel A) or in control experiments with the α-tubulin gene (panel B) in order to show that similar amounts of mRNA were loaded for each time point. Five micrograms and 1 μg of poly(A+) mRNA were loaded in the first and second rows, respectively. The columns represent different time points after transfer to derepressing medium, as follows: 0 h (repressed cells, column 1), 1 h (column 2), 2 h (column 3), 4 h (column 4), 6 h (column 5), and 8 h (column 6).

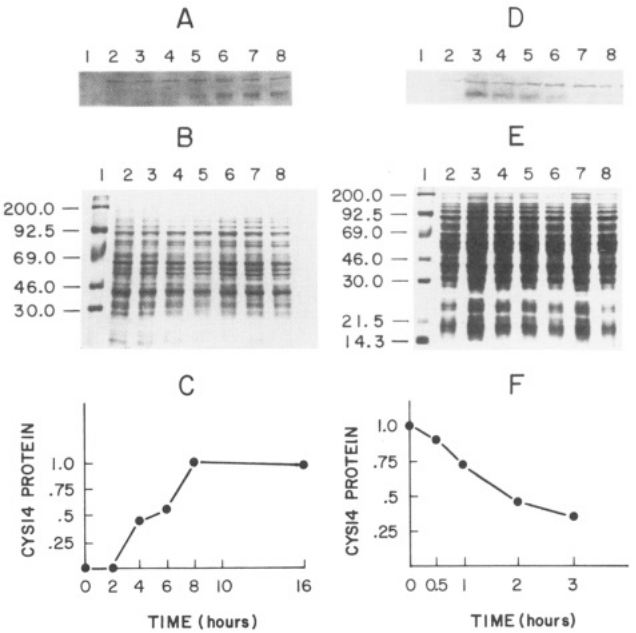

FIGURE 6: Accumulation and turnover of the CYS-14 protein. For the analysis of the CYS-14 protein accumulation after derepression (panels A–C), wild-type cells were grown overnight under sulfur-repressing conditions and then transferred to low-sulfur medium. Whole cell extracts were prepared at 0 (lane 2), 0.5 (lane 3), 1.0 (lane 4), 2.0 (lane 5), 4.0 (lane 6), 6.0 (lane 7), and 8.0 (lane 8) h after transfer and run in two gels, one for Western blot analysis (panel A) and one for staining (panel B). The Western blot was densitometrically scanned by using the constitutive 150-kDa protein as an internal standard, and results of this analysis were plotted (panel C). To examine the turnover of the CYS-14 protein (panels D–F), wild-type cells were grown overnight under sulfur-derepressing conditions and were then transferred into a high-sulfur medium. Whole cell extracts were prepared at 0 (lane 3), 0.5 (lane 4), 1.0 (lane 5), 2.0 (lane 6), 3.0 (lane 7), and 4.0 (lane 8) h after repression and analyzed as described for panels A–C. Lane 1 in panels A, B, D, and E contains molecular weight markers ($\times 10^{-3}$); lane 2 in panels D and E is a negative control, an extract of repressed mycelia.

found to be expressed in *scon*-1 cells grown under low- or high-sulfur conditions, although the protein level was significantly lower in both cases than in derepressed wild-type cells (data not shown). These results reflect the negative control function of the *scon*-1 gene product.

We also tested the *cys*-14 mutant for any cross-reacting material. It appears that this mutant contains a full-length (or nearly full-length) protein that is as highly regulated as that in wild-type cells (Figure 4). These data suggest that the mutation in the *cys*-14 gene is a missense mutation.

Turnover and Accumulation of the cys-14 mRNA and Protein. The expression of the *cys*-14 gene appears to be regulated at the level of mRNA content (Ketter & Marzluf, 1988). The turnover of the mRNA has recently been examined and found to be rapid, with a half-life of approximately 15 min (Ketter et al., 1991). In order to be able to compare the kinetics of mRNA and protein accumulation and turnover, we also examined the time course of *cys*-14 mRNA accumulation using dot blot analysis. Wild-type cells were grown overnight under sulfur-repressing conditions (high methionine) and then transferred to medium containing low methionine (sulfur derepression). Samples were quickly cooled and harvested at several time points after transfer for mRNA isolation. In preliminary experiments, we isolated mRNA from cells that had been derepressed for 0, 0.5, 1, and 2 h; however, hardly any *cys*-14 message could be detected even after 2 h. mRNA was then isolated from cells that were harvested at 4, 6, and 8 h after transfer. RNA samples were blotted and hybridized with the cDNA of *cys*-14 (Figure 5). After autoradiography, the nitrocellulose filter was analyzed in a β-scanner. The *cys*-14 mRNA appears to accumulate to maximum in approximately 6 h.

The time course of accumulation of the *cys*-14 message after derepression agrees well with data derived from sulfate uptake studies in which 8 h was required for maximum function (Marzluf, 1972). The half-life of *cys*-14 mRNA turnover, 15 min, is significantly shorter than the 2 h that was previously found for the functional half-life of the *cys*-14-encoded permease activity (Marzluf, 1972).

The anti-CYS-14 antibody allowed us to directly investigate the turnover and accumulation of the CYS-14 protein. Wild-type mycelia were grown overnight in low-sulfur medium and then were transferred into high-sulfur medium and harvested at 0, 0.5, 1, 2, 3, and 4 h after transfer. Western blots of the crude cell extracts and the data obtained by densitometrically scanning these blots are shown in Figure 6. The constitutive streptavidin binding band was used as an internal standard for the densitometric analyses. The half-life of the CYS-14 protein appears to be approximately 2 h, in good agreement with the functional half-life of the permease activity. This result clearly demonstrates that the function of the CYS-14 protein is not simply inhibited in repressed cells and that loss of transport activity upon sulfur repression is due to protein turnover.

To examine the accumulation of the CYS-14 protein after derepression, wild-type cells were grown overnight in high-sulfur medium and then transferred into medium containing low sulfur. Whole cell extracts prepared from mycelia that had been harvested at 0, 1, 2, 4, 6, 8, and 16 h after derepression were analyzed as before. No CYS-14 protein could be detected in cells up to 2–4 h after derepression; the CYS-14 protein level reached its maximum in about 8 h (Figure 6). This time course of protein accumulation agrees well with data from functional studies (Marzluf, 1972) and closely follows the accumulation of the *cys*-14 mRNA.

Cellular Localization of the CYS-14 Protein. It was previously demonstrated that the *cys*-14 gene product was involved in sulfate transport and that changes in membrane composition could cause loss of function of the sulfate transport system (Marzluf, 1973). Therefore, it was suggested that the *cys*-14

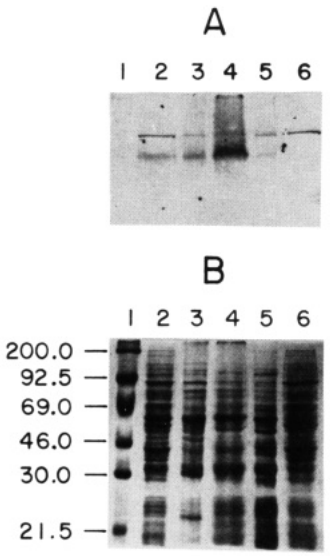

FIGURE 7: Cellular localization of the CYS-14 protein. Several subcellular fractions of wild-type *N. crassa* grown with low sulfur were isolated as described under Materials and Methods. Proteins of these fractions were run in two identical gels, one of which was stained (panel B) and the other Western-blotted and probed with the anti-CYS-14 antibody (panel A). Lanes: 1, molecular weight markers ($\times 10^{-3}$); 2, whole cell extract; 3, 12K pellet; 4, 40K pellet; 5, 100K pellet; 6, 100K supernatant. See text for details.

gene product is a membrane-bound sulfate permease; however, it has not previously been possible to determine whether the CYS-14 protein is actually found in the plasma membrane. We thus examined the cellular location of the CYS-14 protein.

Wild-type cells were grown overnight in low-sulfur medium, and different subcellular fractions were isolated as described under Materials and Methods. Approximately equal amounts of protein from these fractions were run in gels, blotted, and probed with the anti-CYS-14 antibody (Figure 7). Some CYS-14 protein could be detected in a 14K pellet fraction which is a mixed fraction containing mostly mitochondrial but some plasma membrane proteins (Bowman et al., 1981). Most of the CYS-14 protein, however, was found in the 40K pellet, which was shown to be a fairly pure plasma membrane fraction (Bowman et al., 1981). Only very little CYS-14 protein could be detected in a higher speed (100K) pellet and none in the final cytosolic fraction. It is apparent from these results that the CYS-14 protein is indeed localized in the plasma membrane.

Discussion

The *cys*-14 gene is one of the two structural genes that had been shown previously to be involved in sulfate transport in *N. crassa*. Here we report an immunoblot analysis of the expression, regulation, and cellular localization of the CYS-14 protein.

By use of nucleotide sequence data (Ketter et al., 1991), a hydrophilic region of the predicted CYS-14 protein was expressed in *E. coli* and used to raise anti-CYS-14 polyclonal antibody. This antibody proved to be specific when tested with *E. coli* and *N. crassa* extracts. In crude whole cell extracts prepared from derepressed wild-type *N. crassa* mycelia, an approximately 95-kDa protein was detected, whereas the same protein was missing from extracts of repressed mycelia. The size of the detected protein agrees well with its predicted molecular weight; therefore, we concluded that we detected the native CYS-14 protein. These experiments may represent the first case in which a protein has been detected in *Neurospora crassa* based entirely upon probes derived from the nucleotide sequence of its structural gene.

The CYS-14 protein was completely missing in a *cys*-3 mutant grown under either repressed or derepressed conditions, whereas it was expressed at the wild-type level in a temperature-sensitive *cys*-3 revertant only when grown under derepressed conditions at the permissive temperature. These data strongly support the view that the *cys*-3 gene is a positive regulator of *cys*-14 gene expression. Cells of *scon*-1, a constitutive mutant, contained the CYS-14 protein under both repressed and derepressed conditions, in accordance with the negative control function of the *scon* genes upon *cys*-14 expression.

We determined the time required for accumulation of the CYS-14 protein to a maximum level after derepression and the half-life for its turnover after repression. The values we obtained, 8 h for accumulation and 2 h for half-life, agree very well with data from previous sulfate uptake studies (Marzluf, 1972) indicating that the permease function is a direct reflection of the CYS-14 protein presence. Thus, it appears that the regulation of the CYS-14 protein function is mediated largely if not exclusively by its de novo synthesis and turnover.

These experiments are the first that directly examine protein turnover of a membrane transporter in a filamentous fungus. However, other indirect studies may be compared with our findings. It appears that the 2-h half-life of the CYS-14 protein corresponds to a moderately rapid turnover. Activity of a transport system for tryptophan in *Neurospora* turns over with a half-life of only 15 min (Wiley & Matchett, 1968). On the other hand, a methionine transport system proved to be very stable with only a slight decline even after 3 h (Pall, 1971), and a glucose transport system was found not to turn over but to be inhibited by glucose (Schneider & Wiley, 1971). It is also interesting that two enzymes which are controlled in parallel by the sulfur regulatory circuit have very different turnover characteristics. Arylsulfatase is completely stable, whereas the *cys*-14-encoded permease turns over relatively rapidly. It seems obvious that protein turnover provides an additional level of control in the operation of the sulfur regulatory circuit. This turnover mechanism affects the CYS-14 protein, but not arylsulfatase or other hydrolases, possibly because the transport system occupies a strategic point by controlling the uptake of sulfate into the cell.

The accumulation of the CYS-14 protein to a maximum level after derepression takes about 8 h and seems to directly respond to the accumulation of the *cys*-14 mRNA. This time course is significantly slower than the accumulation of nitrate reductase which appears to reach a maximum in less than 30 min (Okamoto and Marzluf, unpulished results). The synthesis of arylsulfatase required 12 h to reach a maximum level (Paietta, 1989) and thus occurs somewhat slower than that of CYS-14. Arylsulfatase enzyme activity parallels the increase of its mRNA (Paietta, 1989), indicating that arylsulfatase function is also regulated by de novo protein synthesis. This may well be a general feature of the whole sulfur circuit.

The relatively slow response to sulfur limitation at both transcriptional and translational levels in the sulfur circuit may indicate that an intracellular pool of sulfur metabolites is present at the onset of sulfur starvation. Alternatively, the induction of the regulatory cascade, possibly involving several de novo protein synthesis steps, may be time-consuming. Time course analyses of the accumulation of the *scon*-2 and *cys*-3 mRNAs and proteins could address this problem.

The *cys*-14 gene product was suggested to be a plasma membrane associated sulfate permease based on indirect evidence and predicted structural features (Marzluf, 1973; Ketter

et al., 1991). The results presented in this paper convincingly demonstrate that the CYS-14 protein is localized in the plasma membrane fraction, and therefore suggest that it very likely functions as a sulfate ion transporter.

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