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COT1, a Gene Involved in Cobalt Accumulation in *Saccharomyces cerevisiae*

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The *COT1* gene of *Saccharomyces cerevisiae* has been isolated as a dosage-dependent suppressor of cobalt toxicity. Overexpression of the *COT1* gene confers increased tolerance to cobalt and rhodium ions but not other divalent cations. Strains containing null alleles of *COT1* are viable yet more sensitive to cobalt than are wild-type strains. Transcription of *COT1* responds minimally to the extracellular cobalt concentration. Addition of cobalt ions to growth media results in a twofold increase in *COT1* mRNA abundance. The gene encodes a 48-kDa protein which is found in mitochondrial membrane fractions of cells. The protein contains six possible membrane-spanning domains and several potential metal-binding amino acid residues. The *COT1* protein shares 60% identity with the *ZRC1* gene product, which confers resistance to zinc and cadmium ions. Cobalt transport studies indicate that the *COT1* product is involved in the uptake of cobalt ions yet is not solely responsible for it. The increased tolerance of strains containing multiple copies of the *COT1* gene is probably due to increased compartmentalization or sequestration of the ion within mitochondria.

Trace metals play a major role as coenzymes or cofactors in many cellular processes. Several transition metals, including manganese, iron, nickel, cobalt, and copper, as well as zinc are required as catalysts in a variety of enzymatic reactions (18). Since these metals are normally found at relatively low concentrations in the environment, cells must accumulate them by using active transport processes (23). Concentrations of trace metals in excess of normal physiological levels, however, can be toxic. Toxicity results from the nonspecific combination of metal ions with cellular components and the disruption of the normal metabolism of other metals (16).

Many of the molecular mechanisms that are responsible for the transport and detoxification of a number of trace metals in prokaryotes are now known (24, 54). Ion transporters, reductases, and small proteins and compounds involved in metal sequestration enable bacteria to grow in environments containing high levels of toxic metals (54). In eukaryotes, work has centered on the function of the glutathione-related cadystins and the cysteine-rich metallothionein proteins in the detoxification and storage of metals (40).

In the yeast *Saccharomyces cerevisiae*, although the genes encoding transporters of H^+ (50), K^+ (15), and Ca^{2+} (48) have been identified, the study of trace metal transport is less well developed. Several trace metals are accumulated by an energy-dependent, low-specificity uptake system. The divalent cation transport system described by Fuhrmann and Rothstein (14) is responsible for the accumulation of Co^{2+}, Ni^{2+}, and Zn^{2+} ions. Competition experiments suggest that other metals, such as Mg^{2+}, Ca^{2+}, and Mn^{2+}, are also accumulated. Although the molecular components of this system are not yet known, it is likely that several types of proteins are involved. The driving force for metal uptake, for example, is likely to be the electrical potential established by the plasma membrane ATPase (for a review, see reference 3). Although H^+ ions do not seem to be cotransported with divalent cations (14), several lines of evidence suggest that the electrical potential developed by the ATPase provides the energy for the transport of divalent metals into the cytoplasm (3, 46). The protein or proteins that are required to facilitate the diffusion of trace metals across the membrane have not been identified. The plasma membrane mechanosensitive ion channel, which passes divalent cations, including Co^{2+} and Ca^{2+} (17), might allow trace elements to enter the cytoplasm.

In contrast to bacterial systems, in which toxic metals are pumped out of cells, toxic trace metals are often stored within yeast cells following transport across the plasma membrane (16). Excess metals that have gained access to the cytoplasm are sequestered by metal-binding proteins (40) or are compartmentalized within intracellular organelles such as the vacuole (45, 61, 62). The molecular biology of trace metal storage in yeast cells is best understood in the storage and detoxification of copper ions by the *CUP1* gene product. The *CUP1* gene encodes a small cysteine-rich metallothioneinlike protein that binds several metals (64). Although *CUP1* is normally expressed at low levels, an increase in gene copy number as well as copper-stimulated transcription of the *CUP1* gene results in elevated levels of the CUP1 protein. These increases in expression allow strains to tolerate millimolar concentrations of copper ions in the medium (10, 13, 27). Although the CUP1 protein has been shown to bind several metals (64), increased expression of the *CUP1* gene confers tolerance only to copper and cadmium (27, 30).

We describe the isolation of *COT1*, a gene that when overexpressed enables wild-type strains of *S. cerevisae* to grow in media containing as much as 20 mM $CoCl_2$. Deletion of the *COT1* gene makes cells more sensitive to cobalt than are wild-type strains. Since *COT1* also affects cobalt transport in a dosage-dependent manner, *COT1* appears to be involved in both the accumulation and detoxification of Co^{2+} ions.

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TABLE 1. Yeast strains

Strain	Genotype	Source or reference
CYX 118-5C	*ura3-52 leu1 HOL1 MAT***a**	This study
CYX 367-2C	*ura3-52 leu2-3,112 trp1-Δ1 MAT***a**	This study
CYP 510	*COT1* [YEp352] *ura3-52 leu2-3,112 trp1-Δ1 MAT***a**	This study
CYP 511	*COT1 [YEpCOT12] ura3-52 leu2-3,112 trp1-Δ1 MAT***a**	This study
CYP 514	*cot1-Δ1::URA3 ura3-52 leu2-3,112 trp1-Δ1 MAT***a**	This study
BJ2168	*COT1 leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2 MAT***a**	28
CYP 575	*COT1* [YEpCOT13] *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2 MAT***a**	This study
CYP 577	*COT1* [YEpCOT13T] *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2 MAT***a**	This study

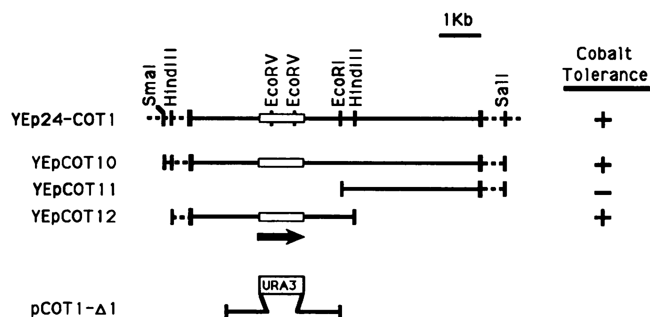

FIG. 1. Restriction maps and cobalt tolerance-conferring ability of plasmids containing the *COT1* gene and subclones. Plasmid constructions are described in Materials and Methods. CYX 367-2C transformants that carried the subclones were tested for growth on 5 mM $CoCl_2$ plates. Solid lines represent yeast DNA sequence; dashed lines represent YEp24 vector sequence; the heavy arrow and open boxes indicate the position of the proposed *COT1* open reading frame.

MATERIALS AND METHODS

Genetic techniques. Yeast strains used in this study are listed in Table 1. Yeast transformations were performed by the method of Ito et al. (25). *Escherichia coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiD* (*lac proAB*)] and MC1066a (*leuB600 trpC9830 pyrF74*::Tn*5* Kan^r *ara* r_K^- m_K^- *recA13*) (49) were used for construction of plasmids. JM109 was used for transfection with mp18-based phages by the method of Messing (41). Standard yeast genetic techniques were as described by Sherman et al. (51).

Media. Standard YPD was prepared as described previously (52). Synthetic low-sulfate and phosphate (LSP) medium contains 2% glucose, 80 mM NH_4Cl, 0.5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 mM NaCl, 10 mM KCl, vitamins, and trace elements as described previously (51). Divalent cations were added from sterile stocks to medium cooled to 65°C. Monovalent cations were added to growth media prior to autoclaving. Metal salts were added to media at the following concentrations: $CdCl_2$, 75 and 100 μM; $MnCl_2$, 15 and 20 mM; $NiCl_2$, 2.5 and 5 mM; $CuSO_4$, 0.3 and 1 mM; $ZnSO_4$, 25 and 40 mM; Rh(acetate)$_2$, 5 and 10 mM; CsCl, 50 and 100 mM; LiCl, 100 and 200 mM; NaCl, 0.8 and 1.2 M; and KCl, 2.0 and 2.5 M. Most metals were added to YPD. Zinc, copper, and rhodium were insoluble in YPD and were added to LSP plates.

Cloning and sequencing techniques. Yeast plasmid rescue was carried out by the glass bead method of Hoffman and Winston (22). Cloning techniques were as described by Maniatis et al. (39). Nested deletions were constructed by the method of Henikoff (21). Exonuclease III and exonuclease VII were purchased from Bethesda Research Laboratories, Bethesda, Md. DNA sequence analysis was performed by using Sequenase (United States Biochemical Corp.) and α-^{35}S-ATP (Amersham). Single-stranded phage were purified according to Messing (41). Additional sequence was obtained by using a primer synthesized by the University of Wisconsin Biotechnology Center (5'-GGCAACATTTACGG CCC-3') and double-stranded DNA sequencing (8).

Hybridizations. Nucleic acid hybridizations were carried out by the method of Southern as described by Maniatis et al. (39). The glass bead method of Hoffman and Winston (22) was used to purify yeast genomic DNA. Total yeast RNA was purified by the glass bead method of Lindquist (36). Probes were purified following agarose gel electrophoresis, using a Gene Clean kit (Bio 101), and labeled with [α-^{32}P]dCTP, using a random oligonucleotide priming kit (Pharmacia). The amount of probe hybridized to mRNA bands was quantitated by using a Betascope blot analyzer (Betagen, Waltham, Mass.).

Plasmid constructions. YEpCOT10 (Fig. 1) was constructed by digesting YEp24-COT1 with *Sal*I and *Sma*I. The *Sal*I end was made flush with Klenow fragment and deoxynucleoside triphosphates (dNTPs). The 7.3-kb yeast DNA fragment containing the *COT1* gene was ligated into the *Sma*I site of YEp352 (19). YEpCOT11 was made by deleting the 3.4-kb *Eco*RI fragment from YEpCOT10. YEpCOT12 was constructed by cloning the 3.6-kb *Hin*dIII fragment of YEpCOT10 into the *Hin*dIII site of YEp352 such that the *Eco*RI site of the insert was near the *Hin*dIII site of the multiple cloning site in the vector. YEpCOT13 contained the same insert as did YEpCOT12 but in the opposite orientation.

The *COT1* gene was disrupted by inserting the yeast *URA3* gene between the *Eco*RV sites of the *COT1* coding sequence. To avoid cleaving at *Eco*RV sites in YEp352 or YEp24, the *COT1* gene was subcloned to pUC19. For this, the insert from deletion phage H1-105 was used. This phage was generated during the production of nested deletions and contained approximately 0.9 kb of *COT1* 5' flanking sequence. The deletions were constructed such that an intact *Hin*dIII site remained at the deletion endpoint of each phage. Thus, H1-105 contained a *Hin*dIII site 1.0 kb upstream of the start codon and the *Eco*RI site found 0.3 kb downstream of the *COT1* stop codon. This 2.5-kb *Hin*dIII-*Eco*RI fragment containing the *COT1* open reading frame was removed from H1-105 replicative-form DNA and inserted at the *Hin*dIII and *Eco*RI sites of pUC19. This plasmid was cleaved with *Eco*RV and ligated to a fragment that carried the *URA3* gene. The *URA3* gene was contained on a 1.1-kb *Hin*dIII fragment that had been made blunt with Klenow fragment and dNTPs. The resulting plasmid, named pCOT1-Δ1, was cleaved with *Eco*RI and *Hin*dIII prior to transplacement.

Addition of the hemagglutinin epitope to COT1. The influenza virus hemagglutinin epitope that is recognized by the 12CA5 antibody (63) was added to the carboxy terminus of the COT1 protein by using the inverse polymerase chain

reaction (PCR) mutagenesis method of Hemsley et al. (20). To allow for efficient amplification during PCR, the carboxy-terminal coding region of *COT1* was contained on as small a plasmid as possible. For this, the 3.7-kb plasmid pCOT1-3 was constructed by removing the *Ssp*I fragment from YEp COT13 (see Fig. 8). This removed the 2-μm circle and *URA3* sequences, the first 906 bases of the coding region, as well as all upstream flanking *COT1* sequence. Plasmid pCOT1-3 contains the 3′ 411 bases of *COT1* coding sequence, about 1 kb of 3′ noncoding sequence, and YEp352 sequence containing the ampicillin resistance and bacterial origin of replication sequences.

Two oligonucleotide primers were synthesized such that following inverse PCR, sequence encoding the hemagglutinin 12CA5 epitope would be added to the carboxy terminus of the *COT1* coding sequence following a proline-glycine-glycine spacer. The primers were EA1 (5′-GTATGGGTA ACCACCAGGATGATCCTCTAAGCAATC-3′) and EA2 (5′-GACGTCCCAGACTACGCTTAAAAGGACTTTATA ACG-3′). PCR was carried out with a COY temperature cycler and *Taq*1 polymerase (Cetus). Clones containing the epitope sequence were identified by screening for the existence of a new *Bst*EII site, which is found in the oligonucleotide primers in the sequence coding for the proline-glycine-glycine spacer. The construction was confirmed by double-stranded DNA sequence analysis of the mutagenized region, using an oligonucleotide primer that corresponds to positions 1226 to 1244 of the *COT1* sequence. YEpCOT13TAG was constructed by replacing the *Msc*I-*Kpn*I fragment of YEpCOT13 with the *Msc*I-*Kpn*I fragment of the mutagenized version of pCOT1-3. YCpCOT13TAG was constructed by cloning the 3.6-kb *Eco*RI-*Hin*dIII fragment of YEpCOT 13TAG into YCp315 (53).

Subcellular fractionation and immunoblot analysis. Total cellular protein extracts were prepared as described by Bostian et al. (4). Soluble, plasma membrane, microsomal membrane, and mitochondrial membrane cellular fractions were isolated as described by Malpartida and Serrano (38). Vacuolar membranes were prepared by the method of Ohsumi and Anraku (44). Proteins were electrophoresed on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels (35) before being transferred to nitrocellulose (57). Monoclonal antibody 12CA5 (Berkeley Antibody), which recognizes an influenza virus hemagglutinin epitope, was used as a primary antibody. Detection of the primary antibody was carried out by using an anti-mouse immunoglobulin G-alkaline phosphatase conjugate kit from Promega.

Cobalt uptake. Cobalt uptake was measured by using a rapid filtration technique similar to that of Fuhrmann and Rothstein (14). Cells were grown to mid-logarithmic phase in LSP synthetic medium lacking uracil. The cells were harvested, washed twice, and resuspended in 50 mM Tris-succinate (pH 5.5). Suspensions of 10^8 cells per ml were preincubated with or without 100 mM glucose for 20 min at room temperature. Approximately 1 μCi of $^{57}Co^{2+}$ (Amersham) was then added with cold $CoCl_2$ to bring the final concentration of cobalt to 100 μM. This concentration is 10-fold lower than what is tolerated by the most sensitive strain tested and was found to have no detrimental effects on the viability of any of the strains throughout the assay. At each time point, 150 μl of sample was filtered onto a Millipore 0.45-μm-pore-size filter and washed three times with 15 ml of ice-cold wash buffer (50 mM Tris-succinate [pH 5.5], 20 mM $MgSO_4$, and 1 mM $CoCl_2$ with or without 100 mM glucose). $^{57}Co^{2+}$ contained on the filters was determined by counting in a Packard gamma ray counter on a ^{57}Co channel. As judged by controls in which glucose or cells were omitted, nonspecific binding of ^{57}Co to the external cell surface or filter was negligible.

Nucleotide sequence accession number. The GenBank accession number for the *COT1* gene sequence is M88252.

RESULTS

Cloning a cobalt tolerance gene from *S. cerevisiae*. Yeast strain CYX 118-5C grows in YPD containing at most 2 mM $CoCl_2$. CYX 118-5C was transformed with DNA from a YEp24-based yeast genomic library (7). Approximately 15,000 Ura^+ transformants were tested for cobalt tolerance by replica plating to YPD plates containing 10 mM $CoCl_2$. After incubating for 3 days at 30°C, seven colonies had grown on the 10 mM $CoCl_2$ YPD plates. All seven exhibited mitotic cosegregation of cobalt tolerance and uracil prototrophy, indicating that the cobalt tolerance was due to a sequence contained on the plasmids. Each plasmid was rescued from yeast cells and subjected to restriction analysis. Comparison of restriction site maps showed that all seven plasmids contained identical inserts of approximately 6.7 kb.

The region conferring cobalt tolerance was further defined by constructing subclones of the 6.7-kb insert of YEpCOT1 in the vector YEp352. The resulting plasmids were transformed into the cobalt-intolerant yeast strain CYX 367-2C. As shown in Fig. 1, the sequence conferring cobalt tolerance was determined to reside on a 3.3-kb *Hin*dIII fragment. DNA from this region was subsequently transferred to mp18 and sequenced.

Strains that carried plasmid YEpCOT12 tolerated as much as 20 mM $CoCl_2$ in the growth media (see Fig. 6). The growth rate was, however, significantly reduced by cobalt ions. To determine whether the increase in cobalt tolerance was due to a general increase in the resistance to toxic substances, the ability of YEpCOT12 to confer resistance to other metals was tested. Media containing different amounts of several metals were prepared (see Materials and Methods for details). Growth tests with CYX 367-2C carrying either YEp352 or YEpCOT12 showed that the plasmid conferring cobalt tolerance also conferred resistance to rhodium ions (data not shown). Increased tolerance to copper, cadmium, magnesium, manganese, nickel, zinc, lanthanum, cesium, potassium, sodium, or lithium was not observed, even though several of these ions are similar to cobalt in both charge and ionic radius. These results indicate that the tolerance is not a general increase in resistance to all metals. Instead, the plasmid confers specific tolerance for Co^{2+} and Rh^{2+} ions, which have similar chemistries.

Sequence of the gene conferring cobalt tolerance. The 3.3-kb *Hin*dIII insert of YEpCOT12 was subcloned into the *Hin*dIII site of mp18 in both orientations. Nested deletions were prepared and sequenced. Analysis of the sequence revealed a single large open reading frame of 1,317 bp (Fig. 2). We believe this to be the gene responsible for the observed cobalt tolerance and have named it *COT1*, for increased cobalt tolerance. The *COT1* open reading frame contains upstream TATA-like elements at positions −44 and −133. A proposed transcription termination sequence (65) is located at 1409 to 1422 (TAGT...TTT), 89 bases beyond the termination codon.

The sequence predicts an unmodified 48,200-kDa protein of 439 amino acids, assuming that translation initiates at the first ATG in the open reading frame. A codon bias index (1) of 0.19 indicates that codon usage in *COT1* is similar to that

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-1002 GGACTTTCACCGCGCTTAGATCAAGAGGGCATCATGAGGCTACCTACAATTGACATACTAAGTCAAGAAAAATAATAGTGTATCGAATAC -913

-912 GCGTGGTAGCTACATTAAATACTAAAGTATGGTTTTTTTTTTAAGTAGGTTTACTCTTCATATTCTTCATTTTCATTTTTCCTTTTCTT -823

-822 CCCGTTCTTCATTTTTATTCCTAGAAAAAAAAATAAATATGTCTATCGTTAACCTAAACATAAACAAAGTTTAGCCCGCTTATTTAAATG -733

-732 ATGCCCAGTACTTTTTTTCAAAACTCATATAATTATATATATCAAGAATGCTTTCCAACCACCGCTTGTATTAAGGTGTAGAAAGGTGGT -643

-642 TTTTGTATTCAAAAAGAGTACAAGCCCATTATAGCTAACTTAACAGTCCACCCTACTATATCCCACGCATATCTGGTTTTAACTTGTGGC -553

-552 GGGCGATATGGAACGAATTTTTCAAAGTTAACCATAGAAAAAAGCATGTGATCAAGGCAGAGTGAACGAGGGAAAAAGAAAGTGTGGAGA -463

-462 AGGCCACCTTTTGAAAAGTTTACCAATCGATCGGTCTCTCAGCACTTTCTACATTTAATCTCTTTATAGTGAACATCGCTGGCTCATGCT -373

-372 GGTTCCCATGCATAACTCACCCACGGCCGCCAACGGCCTTATCACTTACTGTCGCCAGCTCCGGTCTTAGAAAGGGTAAAAAAAACCGGG -283

-282 TGTATACTATACACTCATACATCCGCTCACCCGTTTCCAGTAGTGAATTTTCATTTTCTGTACGAAGGCAATATAAGCTGACGATCCGCA -193

-192 TAAACGAGAAGACTCATTTGTAATCGAGAATATCGTACAATAAAGGAATTTATTGGTCTTATATATTCTACGTGGGAGCTCGAAAAGCAT -103

-102 TTCTGGTTCTATTTAGTAACCAATTATTTTCACTTTGCTCAGAATAGTTCTGCATAGCTATAGAAAGAAAGTTAACACAAAGTACGGAAA -13

-12 GATTGAGTAAATATGAAACTCGGAAGCAAACAGGTAAAAATTATATCCTTGTTGCTGCTAGACACAGTGTTCTTCGGGATCGAGATAACT 78
-4 MetLysLeuGlySerLysGlnValLysIleIleSerLeuLeuLeuLeuAspThrValPhePheGlyIleGluIleThr 26
1

79 ACCGGGTACTTGTCTCACTCTTTGGCTCTAATCGCGGACTCATTCCATATGCTAAACGATATAATTTCTCTTGTGGTTGCACTTTGGGCC 168
27 ThrGlyTyrLeuSerHisSerLeuAlaLeuIleAlaAspSerPheHisMetLeuAsnAspIleIleSerLeuValValAlaLeuTrpAla 56
2

169 GTAAATGTTGCCAAAAACAGAAATCCGGATTCAACGTACACTTATGGTTGGAAAAGGGCGGAGATTTTGGGTGCTCTGATTAACGCCGTC 258
57 ValAsnValAlaLysAsnArgAsnProAspSerThrTyrThrTyrGlyTrpLysArgAlaGluIleLeuGlyAlaLeuIleAsnAlaVal 86

259 TTTTTGATTGCCTTATGTGTCTCAATTTTGATAGAAGCGCTACAAAGAATTATTGCTCCCCCCGTGATTGAAAATCCTAAGTTTGTGTTG 348
87 PheLeuIleAlaLeuCysValSerIleLeuIleGluAlaLeuGlnArgIleIleAlaProProValIleGluAsnProLysPheValLeu 116
3

349 TATGTGGGTGTCGCAGGGTTGATATCGAACACCGTTGGACTTTTCTTATTTCACGACAATGATCAAGAGCATGGACATGGACACGGACAT 438
117 TyrValGlyValAlaGlyLeuIleSerAsnThrValGlyLeuPheLeuPheHisAspAsnAspGlnGluHisGlyHisGlyHisGlyHis 146
4 EcoRV

439 TCCCATGGCGGTATCTTTGCCGACCATGAGATGCATATGCCATCATCCCACACACATACACATGCCCATGTTGATGGAATAGAGAATACT 528
147 SerHisGlyGlyIlePheAlaAspHisGluMetHisMetProSerSerHisThrHisThrHisAlaHisValAspGlyIleGluAsnThr 176

529 ACACCAATGGATAGTACGGATAACATTAGTGAGATTATGCCTAATGCTATAGTAGATAGTTTTATGAACGAAAATACTAGATTATTGACA 618
177 ThrProMetAspSerThrAspAsnIleSerGluIleMetProAsnAlaIleValAspSerPheMetAsnGluAsnThrArgLeuLeuThr 206

619 CCGGAAAATGCATCCAAGACGCCATCATACTCAACGTCAAGCCATACGATTGCCAGCGGCGAAAATTACACAGAACACAACAAGCGCAAG 708
207 ProGluAsnAlaSerLysThrProSerTyrSerThrSerSerHisThrIleAlaSerGlyGluAsnTyrThrGluHisAsnLysArgLys 236

709 AGATCTTTAAATATGCATGGTGTGTTTCTTCACGTTTTGGGCGATGCTCTTGGCAACATCGGCGTTATGTTGTCTGCATTTTTCATTTGG 798
237 ArgSerLeuAsnMetHisGlyValPheLeuHisValLeuGlyAspAlaLeuGlyAsnIleGlyValMetLeuSerAlaPhePheIleTrp 266
5

799 AAGACCGACTATTCTTGGAAGTATTATACAGATCCGCTTGTCTCATTGATAATTACCGGTATAATTTTTTCCTCTGCGCTTCCTCTATCG 888
267 LysThrAspTyrSerTrpLysTyrTyrThrAspProLeuValSerLeuIleIleThrGlyIleIlePheSerSerAlaLeuProLeuSer 296
6

889 TGCAAGGCTTCCAAAATATTGTTACAAGCGACACCTTCCACTTTATCCGGCGATCAAGTAGAAGGTGATCTTTTGAAAATACCAGGAATA 978
297 CysLysAlaSerLysIleLeuLeuGlnAlaThrProSerThrLeuSerGlyAspGlnValGluGlyAspLeuLeuLysIleProGlyIle 326

979 ATAGCTATTCATGATTTCCGTGTTTGGAATTTAACAGAGTCTATTTTTATTGCATCTTTGCATATTCAACTAGATATCAGCCCGGAACAA 1068
327 IleAlaIleHisAspPheArgValTrpAsnLeuThrGluSerIlePheIleAlaSerLeuHisIleGlnLeuAspIleSerProGluGln 356
EcoRV
1069 TTTACTGACCTGGCCAAAATAGTTAGATCAAAACTTCACCGCTATGGCATTCACTCCGCTACTTTGCAACCTGAATTTATTACCAGAGAG 1158
357 PheThrAspLeuAlaLysIleValArgSerLysLeuHisArgTyrGlyIleHisSerAlaThrLeuGlnProGluPheIleThrArgGlu 386
MscI
1159 GTTACTTCAACCGAAAGAGCCGGAGACTCCCAAGGTGATCATCTACAAAATGACCCGCTTTCATTAAGGCCAAAGACATATGGTACTGGC 1248
387 ValThrSerThrGluArgAlaGlyAspSerGlnGlyAspHisLeuGlnAsnAspProLeuSerLeuArgProLysThrTyrGlyThrGly 416

1249 ATTTCAGGTTCCACTTGTCTTGTCGACGACGCTGCCAACTGCAACACAGCTGATTGCTTAGAGGATCATTAAAAGGACTTTATAACGAAA 1338
417 IleSerGlySerThrCysLeuValAspAspAlaAlaAsnCysAsnThrAlaAspCysLeuGluAspHisEnd 446

1339 AAGCGTGAAGAATTAAATACTTTTAAAAATCGTTATACGGTACATATACATACACACACACACATATATATAGTCGTCTATAATGTTTTT 1428

1429 ATACAATGTTTATCATAAATTTTGTTACAGTAAGAAGTATAGGAGATAATATAAAAAAAGAGGAAAAGCGAAAACAGGTGATAATAATGA 1518

1519 TGATCATAATAATAATATAATAGCAATAGTCTTGCAAAACAACAACTTGAGGAAGGAAAATAGTAATAATGGTGTTTAGCTTTTTTTAGA 1608

1609 TTACTCCAGCTTCTAAAAAAGAGTACAATACTTAGCACAATAATGCTGTTATCTTTTTTCATTTA 1673
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FIG. 2. DNA sequence of the *COT1* gene. The deduced protein sequence is shown below. Positions of the six proposed transmembrane domains are boxed. Staggered histidine residues are indicated by arrowheads. Putative TATAA-like sequences are underlined. Proposed transcription termination sequences are doubly underlined. Positions of restriction sites used in the construction of the *cot1-Δ1* and epitope-tagged alleles are also shown.

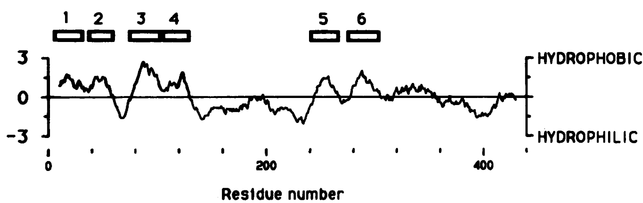

FIG. 3. Hydropathy plot of the *COT1* gene product generated by using the Genetics Computer Group PEPPLOT sequence analysis program (9), using a window size of 19 amino acid residues. *y*-axis values refer to the hydropathy values of Kyte and Doolittle (34). Residue numbers are given below the plot; open bars above indicate potential membrane-spanning domains.

found for genes that are not highly expressed. A hydropathy plot of the COT1 protein was generated by using the residue specific hydrophobicity index of Kyte and Doolittle (34) and a window size of 19. As shown in Fig. 3, this plot revealed six predominantly hydrophobic regions that could participate in membrane-spanning domains (32). This result may indicate that the *COT1* gene product is a membrane protein. Since signal peptide, stop transfer, and signal peptidase sequences are not found near the amino terminus of the COT1 sequence, this protein does not closely resemble proteins that are inserted into a secretory pathway membrane.

In contrast to most metal-sequestering proteins, the *COT1* product has relatively few cysteine residues (5 of 439). The sequence between membrane-spanning domains 4 and 5 (amino acid residues 134 to 238) is rich in histidine (13.3%), serine (12.4%), and threonine (10.5%) residues. The significance, if any, of the serine and threonine residues is not known. Nine of the fourteen histidine residues in this region are found in two sequence blocks of alternating histidine residues at positions 140 to 148 and 163 to 169 (arrowheads in Fig. 2). This sequence may be of interest, since several metalloproteins use the indole group of histidine side chains to coordinate metal ions (18).

COT1 is structurally related to ZRC1. A search of the Swiss-Prot protein sequence library identified the product of the *ZRC1* gene of *S. cerevisiae* as having significant sequence similarity to the COT1 protein. The *ZRC1* gene was isolated as a high-copy zinc and cadmium toxicity suppressor of unknown function (29). The deduced sequences of COT1 and ZRC1 are similar in both size (ZRC1, 442 amino acids; COT1, 439 amino acids) and predicted topology (six putative transmembrane domains each). A comparison of the amino acid sequences of COT1 and ZRC1 by using the Genetics Computer Group BESTFIT program (9) shows that the two proteins share 60% amino acid identity (78% with conservative substitutions; Fig. 4). Significant stretches of identity between the two proteins are found throughout the sequences. These regions of identity lie predominantly within or near the proposed membrane-spanning domains of each protein (boxed regions). The residues found within the membrane-spanning regions of the two proteins are 86% identical (93% with conservative substitutions). This identity is not due simply to a preponderance of hydrophobic residues within the membrane-spanning domains. There is much less identity in the hydrophilic loop between proposed membrane-spanning domains 4 and 5 of the two proteins (19%). This region of ZRC1 sequence is, however, also rich in serine (19%) and histidine (13%) residues. As in COT1, the histidine residues within the ZRC1 deduced hydrophilic loop sequence are found staggered in blocks. These blocks occur at positions 141 to 145, 163 to 167, and 216 to 220 of the ZRC1 sequence. Searches of the NBRF, GenBank, and EMBL protein sequence libraries failed to find any other sequences with significant similarity to the COT1 protein sequence.

Expression of the *COT1* gene. RNA blot analysis was performed to investigate the relationship between *COT1* gene dosage and *COT1* mRNA levels. The 3.3-kb *Hin*dIII fragment of YEpCOT12 was used to probe total RNA purified from strains carrying different numbers of *COT1* gene copies. As shown in Fig. 5, *COT1* is expressed in wild-type log-phase cells as a 1.5-kb RNA. The size of this mRNA agrees well with that of the 1.3-kb *COT1* open reading frame. That this was the only transcript recognized by the 3.3-kb *Hin*dIII fragment is further evidence that the *COT1* open reading frame is indeed responsible for conferring increased cobalt tolerance. This transcript is overexpressed when carried on an episomal vector. In a strain

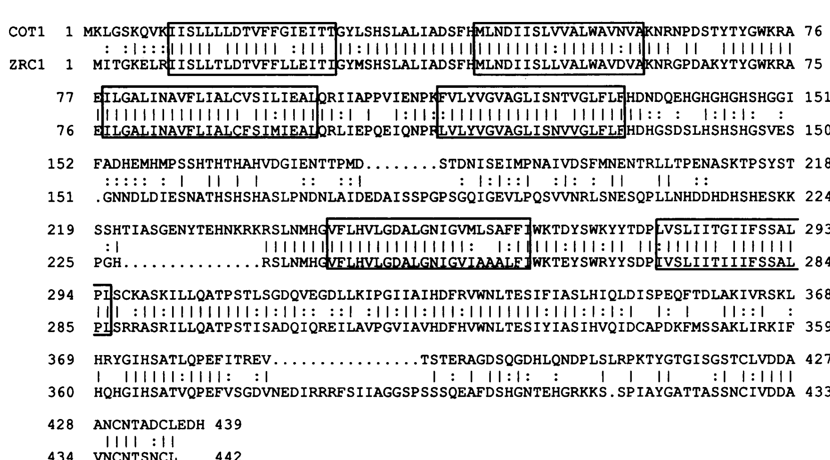

FIG. 4. Sequence comparison of COT1 and ZRC1 proteins generated by using the Genetics Computer Group BESTFIT sequence analysis program (9). Bars indicate identical residues; colons indicate conservative substitutions. Conservative amino acid substitutions are derived from the following groups of amino acids: (L,I,V,M), (A,G,P,S,T), (R,K,H), (Q,D,E,N), and (F,Y,W). Boxed regions correspond to possible membrane-spanning domains of the proteins.

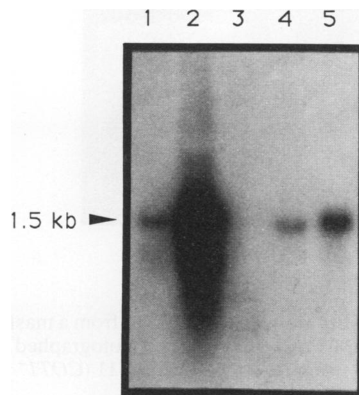

FIG. 5. RNA blot analysis of *COT1* mRNA. A ^{32}P-labeled *Hin*dIII fragment of plasmid YEpCOT12 was used to probe total RNA from strains carrying different copy numbers of *COT1*. Lanes 1, CYP510 (*COT1*$^+$); 2, CYP 511 (*COT1*$^+$ [YEpCOT12]), 3, CYP 514 (*cot1-Δ1*); 4, CYX 367-2C (*COT1*$^+$) grown in YPD; 5, CYX 367-2C (*COT1*$^+$) grown in YPD plus 2 mM $CoCl_2$.

containing the 2 μm-based episomal plasmid YEpCOT12, which is present in many copies in the cell, the *COT1* transcript is approximately 20 times more abundant than in a wild-type strain (Fig. 5, lane 2).

Since transcription of the *CUP1* gene increases dramatically when cells are stressed with exogenous copper ions (5, 30), we tested the ability of cobalt to induce expression of *COT1*. Wild-type strain CYX 367-2C was grown overnight in YPD medium containing 0 or 2 mM $CoCl_2$. Equal amounts of total RNA were electrophoresed and probed with the 3.3-kb *Hin*dIII fragment of YEpCOT12. As shown in Fig. 5, lanes 4 and 5, the level of *COT1* mRNA in strains grown in the presence of cobalt ions appears to be approximately twice that of the control. To ensure that this increase was not due to the presence of spontaneous *COT1*-overexpressing mutants that may have been selected during growth in YPD containing 2 mM $CoCl_2$, we examined the level of *COT1* message taken at time points shortly after the addition of cobalt to the medium. The average induction of *COT1* mRNA by cobalt in five trials was 2.2 ± 0.8-fold 40 min after the addition of 2 mM cobalt to the culture. This approximately twofold induction of *COT1* by cobalt ions is 10 times smaller than the induction of *CUP1* expression by copper ions (5, 30).

In many cases, resistance to toxic substances is dependent on not only the transcriptional regulation of genes encoding detoxifying proteins but also the number of copies of these genes (31, 43, 59). In addition to transcriptional regulation, yeast strains capable of growth in high levels of exogenous copper contain tandem copies of the *CUP1* gene at the *CUP1* locus (5, 30). Since *COT1* was isolated as conferring increased cobalt tolerance to cells when present at high gene copy number, we tested several cobalt-resistant mutants for the presence of duplicated copies of *COT1*. Southern analysis was carried out on 25 cobalt-resistant strains. The cobalt-resistant strains were isolated in a two-step selection as being capable of growth on 5 mM $CoCl_2$ YPD. First, 33 independent mutants that grew on 3 mM $CoCl_2$ YPD were isolated. These strains were patched and subsequently transferred to 5 mM $CoCl_2$ YPD plates by replica plating. From the original 33 strains, 25 independent strains that were capable of growth on 5 mM $CoCl_2$ YPD plates were isolated. Although the nature of the mutations is unknown, all of the strains appeared to carry a single copy of the *COT1* gene at the *COT1* locus (data not shown). This result suggests that tandem duplication of the *COT1* gene is not a common mechanism for acquiring cobalt tolerance in yeast strains.

COT1 is not essential for growth. COT1 is constitutively expressed in media containing only trace amounts of cobalt. This may mean that the primary function of COT1 is one that is not directly involved with Co^{2+} metabolism. In order to explore the possibility that the *COT1* gene product plays some other role in yeast biology, a strain containing a null allele of *COT1* was constructed. For this, the *COT1* open reading frame was disrupted by replacing the 682-bp *Eco*RV fragment with the *URA3* gene (see Materials and Methods). This construction removed amino acids residues 124 to 351 from the *COT1* coding sequence (Fig. 1). *Eco*RI- and *Hin*dIII-cleaved pCOT1-Δ1 was transformed into CYX 367-2C by one-step gene transplacement (47). Southern analysis showed that viable Ura$^+$ transformants contained a single disrupted chromosomal copy of the *COT1* gene (data not shown). We believe that this eliminates *COT1* function, since the *COT1* message was not observed on Northern (RNA) blots of RNA prepared from this strain (Fig. 5, lane 3). The results indicate that COT1 is not essential for growth.

The growth of CYP 514 (*cot1-Δ1*) relative to that of CYP 510 (*COT1*$^+$) was tested under several conditions. Temperature (24 and 37°C), osmolarity (1.5 M sorbitol), and calcium concentration (100 μM, 100 mM, 500 mM, and 750 mM $CaCl_2$) were varied in growth tests on YEPD plates. Under none of these conditions did the disrupted *COT1* allele confer a growth disadvantage on strains that carried it. Similarly, no new auxotrophies, as has been observed in the case of mercury resistance (55), were observed in strains that carried a disruption of the *COT1* gene.

A difference in growth was observed, however, on cobalt-containing media. Strains that carry a single copy of *COT1* grow on media containing at most 2 mM $CoCl_2$. When strains were tested on YEPD plates containing a range of $CoCl_2$ concentrations, it was found that a strain that carried a disrupted copy of *COT1* failed to grow on plates containing 2 mM $CoCl_2$ and grew on at most 1 mM $CoCl_2$ (Fig. 6). Increased sensitivity to other metals was not observed. This result, coupled with the increased tolerance conferred by overexpression of *COT1*, indicates that the level of cobalt tolerance in yeast depends on the amount of *COT1* gene product in the cell.

Cobalt transport is dependent on the copy number of the *COT1* gene. A common feature of several metal detoxification systems is the reduction of the intracellular free toxic metal concentration (40). In many cases, this reduction results from increased efflux from the cytoplasm (54). Metals that are pumped out of the cytoplasm can be either transported out of the cell or compartmentalized into an internal membrane-bounded organelle. The dosage-dependent resistance conferred by the *COT1* gene and the primary structure of the gene product led us to believe that the *COT1* product might be involved in the transport of cobalt ions. To test this possibility, we measured cobalt transport by using a $^{57}Co^{2+}$ uptake assay. For the assays, the genetically closely related strains CYP 510, CYP 511, and CYP 514 (Table 1) were constructed. As shown in Fig. 7, wild-type strain CYP 510, which contains a single genomic copy of *COT1*, accumulates cobalt when incubated with glucose. This uptake is dependent on both glucose and temperature and represents active transport similar to that previously described by others (14, 42). Strain CYP 511, in which the *COT1* gene is overexpressed, accumulates more cobalt than does the wild type.

FIG. 6. Growth phenotypes of strains carrying different numbers of *COT1* gene copies. Strains were transferred from a master plate to LSP plates containing different concentrations of $CoCl_2$ by replica plating. The 0, 2, and 5 mM $CoCl_2$ plates were photographed after 2 days of growth; the plate containing 20 mM $CoCl_2$ was photographed after 5 days of growth. The strains used were CYP 511 (*COT1*$^+$ [YEpCOT12]), CYP510 (*COT1*$^+$), and CYP 514 (*cot1-Δ1*).

The rate of uptake during the first 40 min is about 150% of that of the wild type. CYP 514, which contains a null allele of the *COT1* gene, accumulates less cobalt than does the wild type. The uptake rate is about 55% of the rate of the wild type. From this result, it appears that cobalt transport activity is affected by the amount of *COT1* product present in cells but is not completely dependent on it. Although the cobalt uptake activity of the *cot1-Δ1* strain was less than the wild-type activity, it was not abolished. This result suggests that *COT1*-independent cobalt accumulation mechanisms must exist.

The rate of cobalt transport correlates with the extent of cobalt tolerance of the strain. A strain that has increased tolerance to cobalt due to overexpression of the *COT1* gene has greater net Co^{2+} uptake; a strain that has decreased tolerance has decreased net Co^{2+} uptake. This probably means that in addition to an increase in the uptake, there is an increase in either the compartmentalization or sequestration of cobalt ions within cells that leads to the observed tolerance. Since this assay measured only net uptake, however, the observed difference could be due to decreased efflux of Co^{2+} ions in the case of the overexpressing strains and increased efflux in the case of the null mutants. These possibilities were ruled out by preloading cells with $^{57}Co^{2+}$ and finding that the net cobalt efflux from both the wild type and mutants was negligible (data not shown).

FIG. 7. $^{57}Co^{2+}$ accumulation by strains with different copy numbers of the *COT1* gene. The strains used were CYP 511 (*COT1*$^+$ [YEpCOT12]), CYP 510 (COT1$^+$), and CYP 514 (*cot1Δ-1*). Wild-type uptake at 60 min was chosen as 100%. The points represent averages of four experiments in each case; error bars indicate standard deviation. Values for cobalt accumulated after 60 min of incubation were as follows: CYP 510, 40 nmol/10^8 cells; CYP 511, 52 nmol/10^8 cells; and CYP 514, 22 nmol/10^8 cells.

The *COT1* product is located in the mitochondrial membrane. The cobalt-tolerant phenotypes and dosage-dependent cobalt transport of strains harboring extra copies of *COT1* imply that the *COT1* product may be involved in either the sequestration or compartmentalization of cobalt ions. We sought to discover the intracellular location of the *COT1* product. To do this, the *COT1* gene was tagged (33) with a sequence coding for an influenza virus hemagglutinin epitope (63) and introduced into cells on both episomal and centromeric vectors. The construction was such that following transcription and translation of the tagged gene, the hemagglutinin 12CA5 epitope would be positioned at the carboxy terminus of the *COT1* product (Fig. 8). To ensure that the activity of the tagged product was retained, we tested the constructs for the ability to confer cobalt tolerance. The

FIG. 8. Construction of plasmids carrying the epitope-tagged version of the *COT1* gene. Cross-hatched region represents the added epitope. See Materials and Methods for details. The deduced sequences of both untagged and tagged versions of the COT1 protein are shown below the diagram.

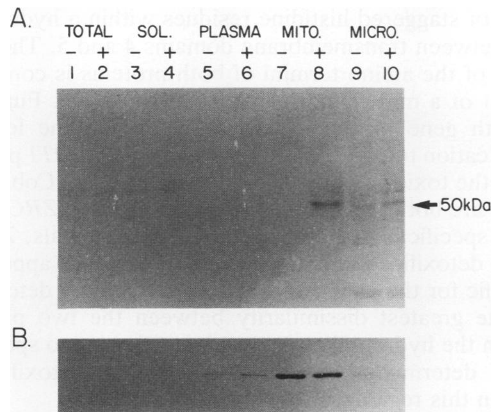

FIG. 9. Immunoblot analysis of epitope-tagged COT1 protein. Each lane contained 20 μg of protein. Lanes: 1 and 2, total protein; 3 and 4, soluble fraction; 5 and 6, plasma membrane fraction; 7 and 8, mitochondrial membrane fraction; 9 and 10, microsomal membrane fraction. Fractions in odd-numbered lanes were prepared from strain CPY 575, which contains YEpCOT13 (untagged). Fractions in even-numbered lanes were prepared from strain CPY 577, which contains YEpCOT13T (tagged). The blot in panel A was probed with 12CA5 antibody; in panel B, 5-μg aliquots of the same fractions were loaded per lane and probed with anti-F_1 ATPase β-subunit antibody.

ability of the tagged gene to confer increased tolerance to cobalt ions was indistinguishable from that of a wild-type gene. The tagged gene carried on an episomal vector conferred tolerance to 20 mM $CoCl_2$ when transformed into a wild-type recipient. The tagged gene carried on a centromeric vector complemented the cobalt-hypersensitive phenotype of the *cot1-Δ1* mutation. This result indicates that the addition of the 12CA5 epitope did not significantly alter the function of the COT1 protein.

Subcellular fractions of multiply protease-deficient (28) cells harboring YEpCOT13T were then examined for the presence of the tagged *COT1* product by immunoblotting with the 12CA5 antibody. Although we could not consistently detect a cross-reacting band in whole cell extracts (Fig. 9, lane 2), a 50-kDa band that is recognized by the antibody was detected in the mitochondrial membrane fraction (lane 8). Strains transformed with the untagged version of the *COT1* gene on an episomal vector (YEpCOT13) did not produce a cross-reacting protein in this fraction (lane 7). This result indicates that the band observed in lane 8 corresponds to the tagged COT1 protein. The size of this band compares well with the expected molecular weight of the tagged protein, which is 49,510 (48,200 due to the *COT1* open reading frame plus 1,310 due to the tag). This finding suggests that the tagged version of the COT1 protein is not extensively modified. Nonspecific cross-reacting bands of approximately 55 and 45 kDa were seen in both soluble and microsomal cellular fractions. Since these bands were also observed in fractions prepared from cells carrying the untagged control, however, they could not be attributed to the tagged version of the COT1 protein. Tagged product was undetectable in extracts prepared from strains that carried the centromeric vector YCpCOT13T (data not shown). The COT1 protein was not observed in soluble (lane 4), plasma membrane (lane 6), microsomal membrane (lane 8), or vacuolar membrane (data not shown) fractions.

The identification of the *COT1*-containing fraction as the mitochondrial fraction was confirmed by probing a similar blot with a polyclonal antibody directed against the β subunit of the F_1 ATPase of the inner mitochondrial membrane (26). As shown in Fig. 9B, signal due to the F_1 ATPase β subunit was enriched in those fractions expected to contain mitochondrial membranes. Although some signal was observed in all membrane fractions, the mitochondrial fractions were found to have approximately seven times more F_1 ATPase β-subunit signal than did either plasma or microsomal membrane fractions, as measured by densitometric scanning of the immunoblot. These results indicate that the COT1 protein cofractionates with and is probably associated with mitochondrial membranes. The possibility exists, however, that COT1 is associated with another, unidentified membrane that has been enriched in the mitochondrial membrane fractions.

DISCUSSION

The *COT1* gene was isolated as a dosage-dependent suppressor of cobalt toxicity. Overexpression of the *COT1* gene confers increased tolerance to cobalt. Deletion of the *COT1* gene leads to hypersensitivity to cobalt, which indicates that the level of tolerance to high concentrations of cobalt is dependent in part on the amount of *COT1* gene product in the cell. Whether COT1 is involved in the tolerance of low concentrations of cobalt is not known. Although cobalt is considered an essential element (18, 23, 24), it is not routinely added to yeast growth media. We could not demonstrate a requirement for additional cobalt in the growth media for strains that overexpress or are depleted of COT1 (data not shown), which suggests that COT1 is not required for growth in media containing contaminating amounts of cobalt ions. This is not surprising, however, since other mechanisms for the accumulation of cobalt exist in yeast cells (Fig. 7).

Although the basis of the inhibition of yeast cell growth by cobalt is not understood, it is clear that the COT1 protein does not mediate the toxic effects of Co^{2+} ions on cells. Co^{2+} ions do not inhibit a function of the *COT1* product that is essential for growth, since the *COT1* product does not carry out a function essential to yeast growth. A toxic interaction between the COT1 protein and Co^{2+} ions is not likely either, since the *COT1* product is not required for the inhibition of growth by cobalt ions. In fact, strains that produce no *COT1* product are more sensitive to Co^{2+} ions than is the wild type. It appears that the COT1 protein is not a target of the toxic effects of Co^{2+} ions within cells. Instead, COT1 appears to act in a dosage-dependent manner to counteract the adverse effects of cobalt ions on cells. In this way, COT1 is similar to both the CUP1 and ZRC1 proteins of *S. cerevisiae*.

The levels of both cobalt tolerance and cobalt uptake in yeast cells roughly correlate with the level of expression of the *COT1* gene. Strains that overexpress *COT1* are tolerant to cobalt and exhibit a rate of uptake of the toxic ion that is approximately twice that of wild-type strains. In this respect, these strains are similar to the manganese-resistant *MNR1* mutants of *S. cerevisiae*, which also accumulate increased levels of the metal to which they are resistant (2). In the case of the *MNR1* mutants, the increased tolerance results from increased uptake by the vacuole. This is believed to lead to a decrease in the cytoplasmic concentration of toxic Mn^{2+} ions. Similarly, the increased tolerance of the high-copy-number *COT1* strains may result from the increased sequestration or compartmentalization of cobalt within the cell. The uptake assay results indicate, however,

that the *COT1* gene product is not solely responsible for the cobalt uptake activity of yeast cells. Although increased dosage of the *COT1* gene apparently increases the amount of cobalt uptake, deleting the gene decreases the uptake rate by only 45% (Fig. 7). This finding suggests that other proteins may be involved in the transport of cobalt. A discrepancy exists in the correlation of gene dosage with uptake rate in that a 20-fold increase in *COT1* mRNA leads to a less than twofold increase in the rate of Co^{2+} uptake. This difference might result from another factor being rate limiting for Co^{2+} uptake. Alternatively, a 20-fold increase in mRNA abundance due to overexpression of the *COT1* gene may not lead to a 20-fold increase in the amount of COT1 protein.

Transcription of the *COT1* gene is consistent with the existence of a direct role for the COT1 protein in Co^{2+} metabolism. *COT1* appears to be expressed constitutively at low (contaminating) concentrations of cobalt ions in the media. This basal expression of *COT1* suggests that the COT1 protein may participate in the regulation of cobalt levels under normal physiological conditions. It may, for example, be important in the supply of metal that is required for metalloenzyme or cofactor synthesis. Upon incubation of *COT1*$^{+}$ strains at an extracellular cobalt concentration of 1 mM, a two- to threefold increase in the level of *COT1* mRNA relative to the level of *ACT1* mRNA occurs. The increase in the level of *COT1* mRNA is much more modest than the 20-fold increase in *CUP1* message upon induction with Cu^{2+} ions. Nonetheless, it appears that additional *COT1* product may be produced in response to environmental cobalt stress. The mechanism responsible for the increase in *COT1* mRNA is not known. A *trans*-acting factor(s) similar to the ACE1/CUP2 protein which activates *CUP1* transcription could be involved (56, 60). *COT1* does not contain the *CUP1* upstream activation elements (11), and it is therefore unlikely that its transcription would be regulated by *ACE1* itself.

The sequence of the *COT1* gene might give some clues to the role that the COT1 protein plays in conferring increased tolerance to cobalt. The presence of extended runs of hydrophobic residues (Fig. 2 and 3) within the deduced protein sequence suggest that the *COT1* gene product is an integral membrane protein. Since the epitope-tagged *COT1* gene product is found in mitochondrial membrane fractions of cells but not soluble fractions, it seems likely that at least one of the six proposed transmembrane domains actually does cross the membrane. Many proteins that are imported into the mitochondria contain an amino-terminal targeting sequence that is composed of between 12 and 26 residues that are capable of forming an amphiphilic α helix (58). The first 19 amino acid residues of the COT1 protein can be modeled into such a helix.

Also of note are the staggered histidine residues in the hydrophilic loop between putative transmembrane domains 4 and 5. The imidazole ring of histidine is an important metal-coordinating group in a number of metalloenzymes (18, 23). In several metal-binding proteins, the B-X-B motif, where B is a metal-binding residue, is often found at coordination sites (18). The sequences H-G-H-G-H-G-H-S-H, found at amino acid positions 140 to 148, and H-T-H-T-H-A-H, found at amino acid positions 163 to 169 (Fig. 2), might be involved in the coordination of cobalt ions.

The sequence similarities between the *COT1* and *ZRC1* gene products suggest that the two proteins perform similar functions but differ in the ions with which they interact. Sixty percent of the amino acid residues in the deduced amino acid sequences of the two proteins are identical. Both proteins contain six putative transmembrane domains and clusters of staggered histidine residues within a hydrophilic region between transmembrane domains 4 and 5. The composition of the amino termini of both proteins is consistent with that of a mitochondrial targeting sequence. Functionally, both gene products help to determine the level of divalent cation tolerance in *S. cerevisiae*. The *COT1* product reduces the toxicity of cobalt and rhodium ions. Cobalt and rhodium are both group 9 transition metals. The *ZRC1* gene product specifically detoxifies the group 12 metals, Zn and Cd. The detoxification mechanism in both cases appears to be specific for the chemistry of the ions that are detoxified. Since the greatest dissimilarity between the two proteins occurs in the hydrophilic regions, it is tempting to speculate that the determinant for the specificity of detoxification resides in this region.

On the basis of the salient features of the deduced sequence, we envision two possible functions of the COT1 protein in mediating cobalt tolerance. Since the sequences of the *COT1* and *ZRC1* gene products are so similar, the proposed COT1 functional models can be applied to the ZRC1 protein except that the ZRC1 protein is expected to interact with Zn^{2+} or Cd^{2+} ions instead of Co^{2+}. The existence of putative membrane-spanning domains, coupled with the dosage-dependent uptake phenotypes, suggests that the *COT1* product may function as a Co^{2+} transporter. If this is the case, the detoxification of cobalt could result from increased compartmentalization of the ions within mitochondria. The sequence of the *COT1* gene suggests that it is not likely to encode an ion-translocating pump. The sequence contains no consensus ATP binding sites, nor is it similar to any of the known ion pumps. The driving force for cobalt uptake, however, might be due to the mitochondrial membrane potential. The electrochemical potential of H^{+} across the inner mitochondrial membrane is believed to be responsible for the active transport of Ca^{2+} into the mitochondrial matrix (6). The transport of ions across this membrane can occur by facilitated diffusion and does not require pumping. In this model, the histidine residues of the hydrophilic loop might bind cobalt ions reversibly. They could be involved in discriminating between cobalt and other ions, providing the ion specificity of the transporter.

A second possible role for the COT1 protein is that of a cobalt-immobilizing protein. Proteins and small molecules that bind metals tightly function in metal detoxification by presumably reducing the cytoplasmic free toxic ion concentration (40). The *COT1* product may function in a similar way. Cobalt ions could be removed from solution in the cytoplasm by being bound to the COT1 protein. The histidine residues in the hydrophilic loop between membrane-spanning domains 4 and 5 are good candidates for the site of metal coordination. Increased uptake could result from the increased binding capacity of cells carrying *COT1* on a high-copy-number vector. In this case, the *COT1* product would be similar to the metallothioneins in function. Structurally, however, the *COT1* product is unlike the known metallothioneins. Whereas the metallothioneins are small, soluble proteins that utilize the thiol group of cysteine to bind metals, the *COT1* product is larger, is found in the mitochondrial membrane, and would probably use histidine residues to bind metal ions. Where the ions would be stored in this model is being resolved by determining in which mitochondrial membrane COT1 is found and on what face of that membrane the histidine residues reside. It is clear that several other possibilities for the actual function of the *COT1* product (and by analogy the *ZRC1* product) exist. For

example, these proteins may function as metalloenzymes, reductases, or regulators of ion storage or transport proteins.

Regardless of whether the *COT1* product is involved in transport or sequestration of cobalt ions, the localization of the COT1 protein to the mitochondria suggests that mitochondria could be a major site of Co^{2+} storage within yeast cells. Mitochondria are known to sequester large amounts of other divalent cations such as Ca^{2+} and Mg^{2+} in other cell types (6, 12). Although the vacuole is generally considered to be the site of internal ion storage in yeast cells (45, 61, 62), the fact that we could not detect the *COT1* product in the vacuolar cell fractions suggests that the *COT1* product is not involved in vacuolar Co^{2+} storage. This does not preclude the storage of cobalt ions in the vacuole by some other mechanism. It appears that COT1 is responsible for only about 45% of the cobalt uptake observed in wild-type cells. The remaining 55% could be accounted for by COT1-independent transport into the vacuole. We did not, however, observe any phenotypes that suggested that COT1 played a role in mitochondrial energy metabolism. It does not appear that COT1 is required for respiratory function of the mitochondria, as either loss of *COT1* function or overexpression of *COT1* does not alter the ability of a strain to grow on nonfermentable carbon sources. Similarly, the cobalt-related phenotypes of either *cot1-Δ1* or *COT1*-overexpressing strains were not affected by the presence of *rho* mutations (data not shown).

Deletion of the *COT1* gene results in the partial loss of cobalt uptake activity in an assay that is essentially the same as that of Fuhrmann and Rothstein (14). This result indicates that the COT1 protein is a component of the divalent cation transport system but that other components that are responsible for cobalt transport must exist. Moreover, since the COT1 protein, which probably functions in the compartmentalization or sequestration of cobalt, affects the cobalt transport rate, the divalent cation transport system rate may be a composite of the rate of transport of ions across the plasma membrane as well as the rate of their subsequent compartmentalization or sequestration. The genetic dissection of metal transport may uncover additional trace element transport activities and aid in the isolation of the gene products that are responsible for them.

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