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The CYC8 and TUP1 Proteins Involved in Glucose Repression in *Saccharomyces cerevisiae* Are Associated in a Protein Complex

FREDERICK E. WILLIAMS, USHASRI VARANASI, AND ROBERT J. TRUMBLY*

Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43699-0008

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Mutations of the yeast *CYC8* or *TUP1* genes greatly reduce the degree of glucose repression of many genes and affect other regulatory pathways, including mating type. The predicted CYC8 protein contains 10 copies of the 34-amino-acid tetratricopeptide repeat unit, and the predicted TUP1 protein has six repeated regions found in the β subunit of heterotrimeric G proteins. The absence of DNA-binding motifs and the presence of these repeated domains suggest that the CYC8 and TUP1 proteins function via protein-protein interaction with transcriptional regulatory proteins. We raised polyclonal antibodies against TrpE-CYC8 and TrpE-TUP1 fusion proteins expressed in *Escherichia coli*. The CYC8 and TUP1 proteins from yeast cells were detected as closely spaced doublets on Western immunoblots of sodium dodecyl sulfate-polyacrylamide gels. Western blots of nondenaturing gels revealed that both proteins are associated in a high-molecular-weight complex with an apparent size of 1,200 kDa. In extracts from Δ*cyc8* strains, the size of the complex is reduced to 830 kDa. The CYC8 and TUP1 proteins were coprecipitated by either antiserum, further supporting the conclusion that they are associated with each other. The complex could be reconstituted in vitro by mixing extracts from strains with complementary mutations in the *CYC8* and *TUP1* genes.

Carbon catabolite repression is a widespread phenomenon among microorganisms whereby the synthesis of enzymes required for the utilization of alternate carbon sources is inhibited in the presence of the preferred carbon source. In the yeast *Saccharomyces cerevisiae*, glucose or fructose are the preferred carbon sources and the process is usually referred to as glucose repression. Yeast cells grown in the presence of glucose repress the synthesis of many classes of enzymes, including those required for metabolism of other carbon sources, enzymes involved in gluconeogenesis and respiration, and vacuolar hydrolases such as proteases. In all cases which have been examined, regulation occurs at the level of transcription.

Our laboratory isolated mutations in two genes, *tup1* and *cyc8*, which abolish glucose repression of *SUC2*, which encodes invertase (37). Mutations in the *tup1* and *cyc8* genes had been isolated previously for their effects on phenotypes other than glucose repression. The *tup1* (thymidine uptake) mutants were first isolated for their ability to take up dTMP from the growth medium (41). Mutations in the same gene were subsequently isolated and given various names according to the phenotype of interest: *umr7*, *flk1*, *amm1*, and *cyc9*. The *umr7* mutants were resistant to UV-induced mutation of *CAN1* to *can1* (19, 20). *flk1* mutants were extremely flocculent or "flaky" and were insensitive to catabolite repression of maltase, invertase, and α-methylglucosidase (28, 35). *amm1* mutants stabilized plasmids containing a defective *ARS* element (36). A selection protocol for increased expression of iso-2-cytochrome *c* yielded *cyc9*, which is allelic to *tup1*, and a new mutant, *cyc8* (24). Mutations in *CYC8* were later isolated as suppressors of a *snf1* block on the expression of *SUC2* and were referred to as *SSN6* (suppressor of *snf1*) (4). *tup1* and *cyc8* mutants share many phenotypes, including calcium-dependent flocculation, mating-type defects in MATα cells, nonsporulation of homozygous diploids, and constitutive expression of many genes that are normally under glucose repression. These diverse phenotypes may reflect the involvement of *TUP1* and *CYC8* in multiple pathways or the interactions of these pathways with glucose repression.

Mutations which prevent expression of the *SUC2* gene are located in six different *SNF* (sucrose-nonfermenting) genes (22). *SNF1* encodes a protein kinase which is necessary for derepression of glucose-repressible genes (4). Double *snf1 cyc8* mutants are constitutive for invertase synthesis, implying that *CYC8* acts at a later step in the regulatory pathway than *SNF1* (5). It has been postulated that CYC8 is a negative regulator and that the role of the SNF1 protein kinase is to antagonize CYC8 function, possibly by directly phosphorylating the CYC8 protein (5). However, recent biochemical evidence argues against direct phosphorylation of CYC8 by SNF1 (30). We have shown that a *tup1* deletion can also suppress the *snf1* block on invertase synthesis (42).

The nucleotide sequence of the *CYC8* (*SSN6*) gene reveals an open reading frame capable of encoding a protein of 107 kDa (29, 37). The predicted CYC8 protein has long stretches of tandem glutamine residues, 31 in the C-terminal region and 16 near the N terminus. Immediately preceding the 31 glutamines are 30 repeats of alternating glutamine-alanine pairs. Similar stretches of polyglutamine have been found in many regulatory proteins from yeast and *Drosophila* cells (38, 42). Recently it was reported that a 34-amino-acid motif repeated several times in the yeast CDC16 and CDC23 proteins is repeated 10 times near the N terminus of the predicted CYC8 protein (32). These same repeats were found in the *nuc2* gene product of *Schizosaccharomyces pombe*, which is associated with the nuclear scaffold (15). Models of the 34-amino-acid repeat predict an amphipathic α helix which may mediate protein-protein interactions (15, 32).

We have recently characterized and sequenced the *TUP1* gene (42). The gene is capable of encoding a protein of 78 kDa which contains long stretches of glutamine but has no other similarities with the predicted CYC8 protein. At the C terminus of the TUP1 protein are six repeats of about 43

* Corresponding author.

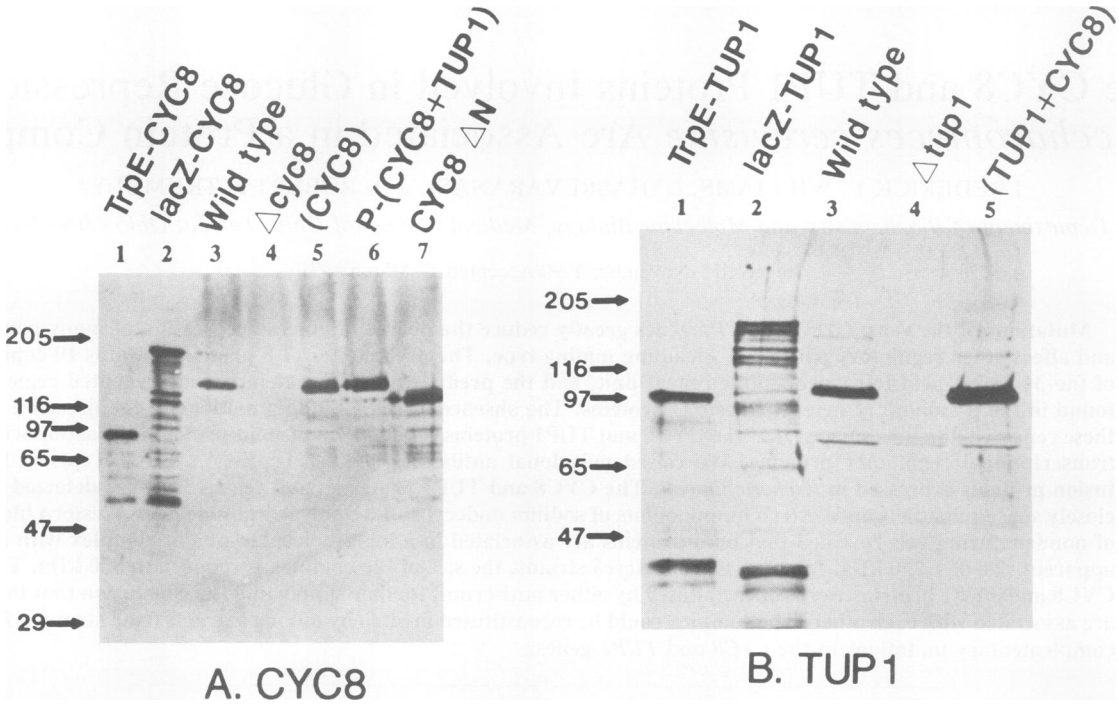

FIG. 1. Western blots of fusion proteins and yeast extracts. Protein extracts from bacteria expressing the fusion proteins (lanes 1 and 2, 100 ng of protein per lane) or yeast extracts (other lanes, 20 μg of protein per lane) were resolved on 7.5% acrylamide gels containing SDS. The samples were electroblotted to nitrocellulose and probed with either CYC8 antiserum (A) or TUP1 antibodies (B). Size standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (65 kDa), ovalbumin (47 kDa), and carbonic anhydrase (29 kDa). (A) Lanes: 1, TrpE-CYC8 expressed from pRT104 in bacteria; 2, LacZ-CYC8 expressed from pRT107 in bacteria; 3, RTY235 (wild type); 4, RTY363 (Δ*cyc8*); 5, RTY363(pRT81), CYC8 overproducer; 6, BJ2168(pTXL63) (protease-deficient strain overproducing CYC8 and TUP1); 7, RTY363(pRT131) (CYC8 N-terminal truncation). (B) Lanes: 1, TrpE-TUP1 expressed from pFW38 in bacteria; 2, LacZ-TUP1 expressed from pFW39 in bacteria; 3, RTY235 (wild type); 4, RTY418 (Δ*tup1*); 5, RTY363(pTXL63) (TUP1 and CYC8 overproducer).

amino acids each, which share conserved amino acid residues with repeated domains in proteins related to the β subunit of G proteins involved in signal transduction. This family includes β-transducin (11); the yeast proteins CDC4 (43), MSI1 (25), PRP4 (7), and STE4 (40); and the *Drosophila* protein encoded by *Enhancer of split* (14) involved in neurogenesis. Although the precise functional significance of these repeated structures is unknown, its conservation among these proteins suggests a role in signal transduction mediated through protein-protein interactions.

We describe here the identification and characterization of the CYC8 and TUP1 proteins by using polyclonal antibodies raised against fusion proteins. Under native conditions the two proteins are associated in a complex estimated to be 1,200 kDa by polyacrylamide gel electrophoresis. The association of the two proteins was confirmed by immunoprecipitation and also by reconstitution in vitro.

MATERIALS AND METHODS

Strains and media. All yeast strains were of the S288c genetic background. Strains RTY235 (MATα *his4-519 leu2-3 leu2-112 trp1-289 ura3-52*), RTY363 (MATa *his4-519 leu2-3 leu2-112 ura3-52 cyc8-Δ1::LEU2*), and RTY418 (MATα *his4-519 leu2-3 leu2-112 trp1-289 ura3-52 tup1-Δ1::TRP1*) were described previously (42). Strain BJ2168 (MATa *leu2 trp1 ura3-52 prc1-407 prb1-1122 pep4-3*), which is deficient in vacuolar proteases, was provided by Rob Preston. RTY493 (*cyc8-Δ1::LEU2*) was created by transformation of BJ2168 by pDSB (38). RTY535 (*tup1-Δ1::TRP1*) was derived from BJ2168 by transformation with pFW36 (42). Plasmids were amplified in *Escherichia coli* XL1-Blue (Stratagene). Bacteria expressing TrpE fusion proteins from pATH plasmids were grown in M9 medium (27) with 1% Casamino Acids and 50 μg of ampicillin per ml (M9CA+amp) or M9 medium with 1% Casamino Acids, 20 μg of tryptophan per ml, and 50 μg of ampicillin per ml (M9CAW+amp). Yeast strains bearing plasmids were grown in SD medium with the appropriate supplements (31), and other yeast strains were grown in YEPD medium (31). Yeast cells were transformed by the lithium acetate method (16).

Plasmids. The following plasmids used for overexpression of the CYC8 and TUP1 proteins in yeast cells were all derived from the 2μm vector YEp24 and were described previously: pRT22 and pRT81 containing the *CYC8* gene (38), pFW28 containing the *TUP1* gene (42), and pTXL63 carrying both the *CYC8* and *TUP1* genes (42). pRT131 encodes a CYC8 protein truncated by 66 amino acids at the N terminus expressed from the *ADH1* promoter and was constructed by subcloning a 3.1-kb *Hae*III-*Xba*I fragment consisting of nucleotides 1784 to 4862 of the *CYC8* sequence (38) into the *Pvu*II-*Xba*I sites of the expression vector pVT100-U (39). To create plasmids for the expression of CYC8 fusion proteins, the 1.5-kb *Pvu*II-*Hin*dIII fragment encoding the C-terminal 370 amino acids of CYC8 was first subcloned from pRT32 (38) into the *Hin*cII-*Hin*dIII sites of pUC19 to make pRT102. This sequence was then retrieved

as a *Bam*HI-*Hin*dIII fragment and ligated into the *Bam*HI-*Hin*dIII sites of pATH3 (13, 34) to yield pRT104 for expression of the TrpE-CYC8 fusion protein and into pUR288 (26) to create pRT107 for expression of the LacZ-CYC8 protein. The 1.7-kb *Bam*HI-*Hin*dIII fragment encoding the C-terminal 461 amino acids of TUP1 was subcloned from pFW1-1 (42) into the *Bam*HI-*Hin*dIII sites of pATH3 to make pFW38 for expression of the TrpE-TUP1 fusion protein and into the *Bam*HI-*Hin*dIII sites of pUR288 to yield pFW39 for expression of the LacZ-TUP1 fusion protein.

Fusion proteins. For expression of the fusion proteins, the plasmids were transformed into *E. coli* XL1-Blue. Cells containing the pATH vectors for expression of TrpE fusion proteins were grown overnight in M9CAW+amp, diluted 1:10 in M9CA+amp, and grown for 1 h with aeration at 30°C. Indoleacrylic acid was added to a final concentration of 5 μg/ml, and the culture was grown at 30°C for 2 additional hours. Strains transformed with the pUR288-derived plasmids for expression of LacZ fusion proteins were grown to mid-log phase in LB (27) plus 50 μg of ampicillin per ml and induced by the addition of isopropyl-β-D-thiogalactopyranoside to 1 mM. Cells were harvested by centrifugation and lysed by the addition of sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.00125% bromphenol blue) and boiling for 5 min. The fusion proteins were purified by loading 10 to 20 mg of protein onto a large 6% acrylamide gel containing SDS (17). After electrophoresis the gel was stained with 0.1% Coomassie blue in 40% methanol and 10% acetic acid for 30 min at 37°C and destained for 2 to 3 h in 40% methanol and 10% acetic acid. The protein band of interest was excised from the gel and cut into small pieces. The fusion protein was electroeluted from the gel in a Bio-Rad MiniProtean II chamber at constant current for 3 h (10 mA per tube) in a buffer containing 0.025 M Tris-HCl, 0.192 M glycine, pH 8.3, and 0.1% SDS. The protein was precipitated by the addition of 5 volumes of acetone at −20°C and centrifugation at 10,000 × *g* for 30 min. The pellet was dried and suspended in a mixture containing 50 mM Tris-HCl, pH 8, 0.1 mM EDTA, 1 mM dithiothreitol and 0.1% SDS.

Antibodies. For preparation of antibodies, bands corresponding to the fusion proteins were excised from SDS-polyacrylamide gels and homogenized with an equal volume of Freund's adjuvant. The protein-gel mixture was injected subcutaneously into rabbits in several places along their backs. The initial injections contained 100 to 200 μg of the fusion proteins. Boosters of approximately 50 μg of the fusion protein were administered every 2 weeks as needed. Serum was collected and tested 2 weeks after each booster, and the final bleeding was performed approximately 14 days after the final booster. The antibodies were purified by binding to immobilized fusion proteins and subsequent elution (33). The concentrations and specificities of the antisera and purified antibodies were determined by the grid blot method (18).

Yeast protein extracts. Yeast protein extracts were prepared by harvesting cells in the logarithmic phase (A_{600} = 0.5 to 1.5). The cells were washed twice in extraction buffer (200 mM Tris-HCl, pH 8, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol) (23), and the pellet was resuspended in 0.5 ml of extraction buffer with 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, and 1 μg of pepstatin per ml. The mixture was transferred to a 1.5-ml microcentrifuge tube. An equal volume of glass beads was added to the tube, and the cells were broken by vortexing in three 1-min bursts followed by cooling on ice. The vortexed mixture was centrifuged for 15 min in an Eppendorf centrifuge at maximum speed at 4°C. The protein extract was collected with a micropipettor, and the protein was quantitated by the method of Bradford (3).

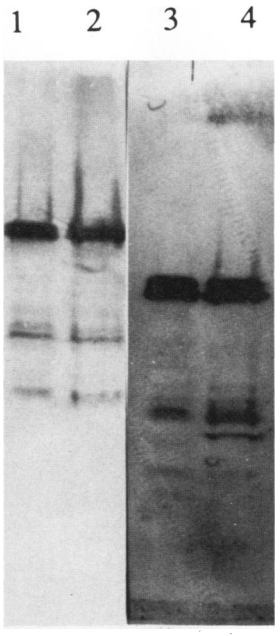

FIG. 2. Effect of glucose on CYC8 and TUP1 proteins. Yeast strain RTY363 (Δ*cyc8*) transformed with pTXL63, which contains both CYC8 and TUP1 genes, was grown overnight in SD medium without uracil. The culture was diluted 1:50 into either YEPD (2% glucose) or into YEP plus 0.05% glucose and grown for 6 h. Extracts were prepared, and samples containing 40 μg of protein were run on a SDS–7.5% acrylamide gel. The CYC8 and TUP1 proteins were detected by Western blotting. Lanes 1 and 2 were probed with CYC8 antiserum, and lanes 3 and 4 were probed with TUP1 antibody. Samples in lanes 1 and 3 were from YEPD medium, and those in lanes 2 and 4 were from low-glucose medium.

Western immunoblotting. The nondenaturing gels consisted of a 4% acrylamide stacking gel in 0.125 M Tris-HCl, pH 6.8, and the separation gels were of various acrylamide concentrations (4 to 6.5%) in 0.375 M Tris-HCl, pH 8.8. The reservoir buffer was 0.025 M Tris-HCl–0.192 M glycine, pH 8.3. The denaturing gels containing SDS were prepared according to the method of Laemmli (17) with 4% acrylamide stacking gels and 7.5% acrylamide separation gels.

Proteins were transferred from polyacrylamide gels to nitrocellulose filters by electroblotting (8). The filter was then incubated with antiserum or antibodies diluted in Tris-buffered saline plus 1% nonfat milk for 1 h at room temperature. The specific bands were detected with goat anti-rabbit immunoglobulin G–alkaline phosphatase (8).

RESULTS

Identification of the *CYC8* and *TUP1* gene products. The *CYC8* (= *SSN6*) and *TUP1* genes have been implicated in the control of glucose repression in yeast cells by genetic analysis. In order to identify and characterize these proteins, we raised polyclonal antisera by immunization of rabbits with TrpE-CYC8 and TrpE-TUP1 fusion proteins made in *E. coli*. The TrpE-CYC8 fusion protein contained the carboxyl-

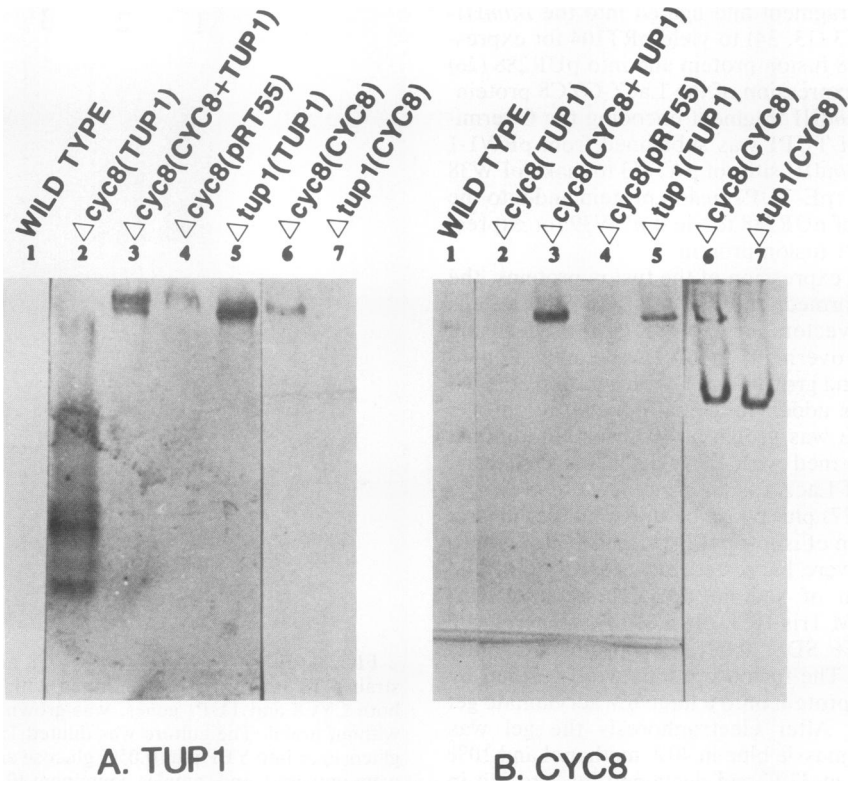

FIG. 3. Western blots of nondenaturing gels. Protein extracts from yeast cells overproducing CYC8 and/or TUP1 were separated on nondenaturing gels and analyzed by Western blotting. A total of 10 μg of yeast protein was loaded in each lane. (A) Blot treated with TUP1 antibodies; (B) blot treated with CYC8 antiserum. Lanes: 1, wild-type strain Y235; 2, RTY363(pFW28) (Δ*cyc8*, overproducing TUP1); 3, RTY363(pTXL63) (Δ*cyc8*, overproducing both CYC8 and TUP1); 4, RTY363(pRTY55) (Δ*cyc8*, overproducing a C-terminal truncation of CYC8); 5, RTY418(pFW28) (Δ*tup1*, overproducing TUP1); 6, RTY363(PRT81) (Δ*cyc8*, overproducing CYC8); 7, RTY418(pRT81) (Δ*tup1*, overproducing CYC8).

terminal 370 amino acids of CYC8, and the TrpE-TUP1 fusion protein had 461 C-terminal amino acids of the TUP1 protein.

The antisera raised against the TrpE-CYC8 and TrpE-TUP1 fusion proteins, hereafter referred to as CYC8 and TUP1 antisera, were used as probes of Western blots to detect the fusion proteins and the CYC8 and TUP1 proteins synthesized in yeast cells (Fig. 1). The CYC8 antiserum recognized the TrpE-CYC8 fusion protein (Fig. 1A, lane 1) as well as the LacZ-CYC8 protein (lane 2) produced in *E. coli*. In extracts prepared from wild-type yeast cells (lane 3), two closely spaced bands of apparent sizes 145 and 150 kDa were recognized by the CYC8 antiserum. The CYC8 and TUP1 doublets are not well resolved in Fig. 1 but are clearly seen in Fig. 2. These estimates are considerably larger than the 107 kDa predicted from the *CYC8* DNA sequence (38). These bands were absent in a *cyc8* deletion strain (Fig. 1A, lane 4) and amplified in strains transformed with multicopy plasmids bearing the *CYC8* gene (Fig. 1A, lanes 5 and 6). A strain with the chromosomal copy of *CYC8* deleted, transformed with pRT131, which encodes a CYC8 protein truncated at the N terminus, produces a doublet with apparent sizes 129 and 133 kDa. This is a further confirmation that these bands represent the *CYC8* gene product, since alterations in the *CYC8* coding sequence result in changes in the mobility of the bands. Since the species with the N-terminal truncations appear as doublets similar to the wild-type CYC8 protein, it seems unlikely that the doublet is a result of alternate processing of the 5′ terminus of the *CYC8* mRNA or N terminus of the CYC8 protein.

The identification of the TUP1 protein is shown in Fig. 1B. Because the unpurified TUP1 antiserum produced a high background on Western blots, affinity-purified TUP1 antibodies were used in all experiments. The TUP1 antibodies recognized both the TrpE-TUP1 and LacZ-TUP1 fusion proteins (Fig. 1B, lanes 1 and 2). In wild-type yeast cells, two bands with apparent sizes of 99 and 103 kDa were seen (lane 3), compared with the predicted size of 78 kDa. These bands were absent in a *TUP1* deletion strain (lane 4) and much stronger in a strain carrying the *TUP1* gene on a multicopy plasmid (lane 5).

Effect of glucose on the CYC8 and TUP1 proteins. Since glucose regulation could be mediated by controlling the amounts or activities of elements of the regulatory pathway, we examined the effect of glucose on the amounts and electrophoretic mobilities of the CYC8 and TUP1 proteins. A yeast strain overproducing both the CYC8 and TUP1 proteins was grown on media containing either 2 or 0.05% glucose. Extracts were prepared, and the CYC8 and TUP1 proteins were detected on Western blots. As shown in Fig. 3, both CYC8 and TUP1 protein doublets are present in similar amounts in high- and low-glucose media. Therefore, glucose repression is not mediated by gross changes in the amounts of the CYC8 or TUP1 proteins nor in the proportions of the two bands in the doublets.

Identification of the CYC8-TUP1 complex. The fact that

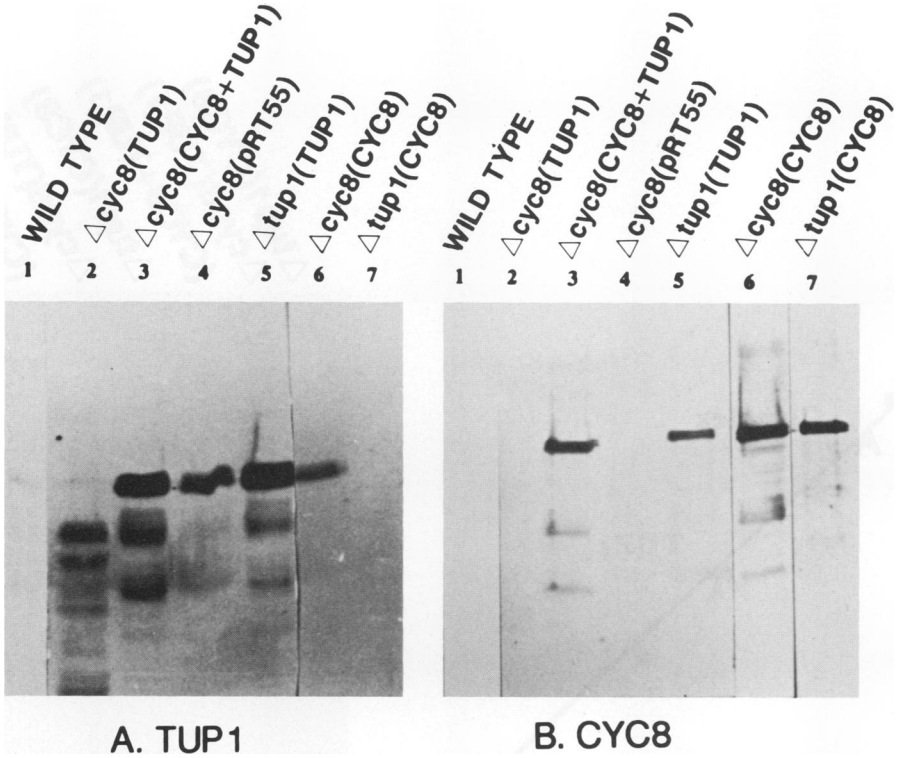

FIG. 4. Western blots of SDS gels. The methods and samples were the same as in Fig. 4, except the samples were separated by SDS-polyacrylamide gel electrophoresis. (A) Blot probed with TUP1 antibody; (B) blot probed with CYC8 antiserum.

cyc8 and *tup1* mutants share an identical range of phenotypes suggested that the two gene products may act together or might even be physically associated in the yeast cell. To test this idea, protein extracts from yeast cells were separated on nondenaturing polyacrylamide gels, and the CYC8 and TUP1 proteins were detected by Western blotting (Fig. 3). The same samples were analyzed by Western blotting of SDS gels as a control (Fig. 4). As shown in Fig. 3, the CYC8 and TUP1 antisera recognized a band of similar mobility on nondenaturing gels. This band was present at low levels in wild-type cells (Fig. 3, lanes 1) and was amplified in strains overproducing CYC8 and TUP1 (Fig. 3, lanes 3). Since evidence to be presented suggests that this band represents a high-molecular-weight complex which contains both CYC8 and TUP1 proteins, we will refer to it as the CYC8-TUP1 complex.

Mutations in either *CYC8* or *TUP1* affect the presence of the complex. The CYC8 protein had a much higher mobility in extracts from strain RTY418, which contains a *TUP1* deletion and carries *CYC8* on a multicopy plasmid (Fig. 3B, lane 7). This band does not represent a degradation product, since intact CYC8 protein was seen on SDS gels (Fig. 4B, lane 7). A strain which overproduced CYC8 in the presence of TUP1 showed the normal CYC8-TUP1 complex (Fig. 3, lanes 6) as well as the high-mobility CYC8 species (Fig. 3B, lane 6). When TUP1 was overproduced in a Δ*cyc8* strain, the complex was not seen, and most of the TUP1 protein was proteolyzed, as evident on nondenaturing gels (Fig. 3A, lane 2) and SDS gels (Fig. 4A, lane 2). In subsequent experiments using a protease-deficient strain (see below), the TUP1 protein was not degraded in the absence of CYC8 but was part of a complex with mobility greater than that of the CYC8-TUP1 complex.

Overproduction of TUP1 in a $CYC8^+$ strain (Fig. 3A, lane 5) resulted in two closely spaced bands recognized by the TUP1 antiserum. The lower-mobility band was also recognized by CYC8 antiserum and corresponds to the TUP1-CYC8 complex. The faster band corresponds in mobility with the complex without CYC8. Two bands of similar mobility are seen in a strain overproducing both CYC8 and TUP1 (Fig. 3, lanes 3), but the relative amounts of the two bands are reversed.

In a Δ*cyc8* strain harboring plasmid pRT55, which encodes a CYC8 protein lacking the C-terminal third of the protein, the complex is seen with a slightly slower mobility than the normal complex (Fig. 3A, lane 4). Plasmid pRT55 complements *cyc8* mutations (38), and these results indicate that the C-terminal third of CYC8 is not required for association in the complex. This truncated version of CYC8 is not visible on Western blots (Fig. 3B, lane 4), since the CYC8 antiserum was raised against the C-terminal third of CYC8.

We have consistently observed that overexpression of either the CYC8 or the TUP1 protein results in increased levels of the other protein compared with levels in wild-type cells (Fig. 3 and 4, lanes 4 to 6). A possible explanation is that assembly into the complex stabilizes the proteins by protecting them from proteolytic degradation.

Molecular size estimations. The size of the CYC8-TUP1 complex was determined by electrophoresis of extracts on a nondenaturing acrylamide gradient gel (2, 6). By this method the size of the complex containing both CYC8 and TUP1 was estimated to be 1,200 kDa (Fig. 5). As mentioned previously, in the absence of CYC8 the TUP1 protein was recovered mostly in a degraded form. However, in a Δ*cyc8* strain that was protease deficient and overproducing TUP1, a band of greater mobility than the complete complex was consistently

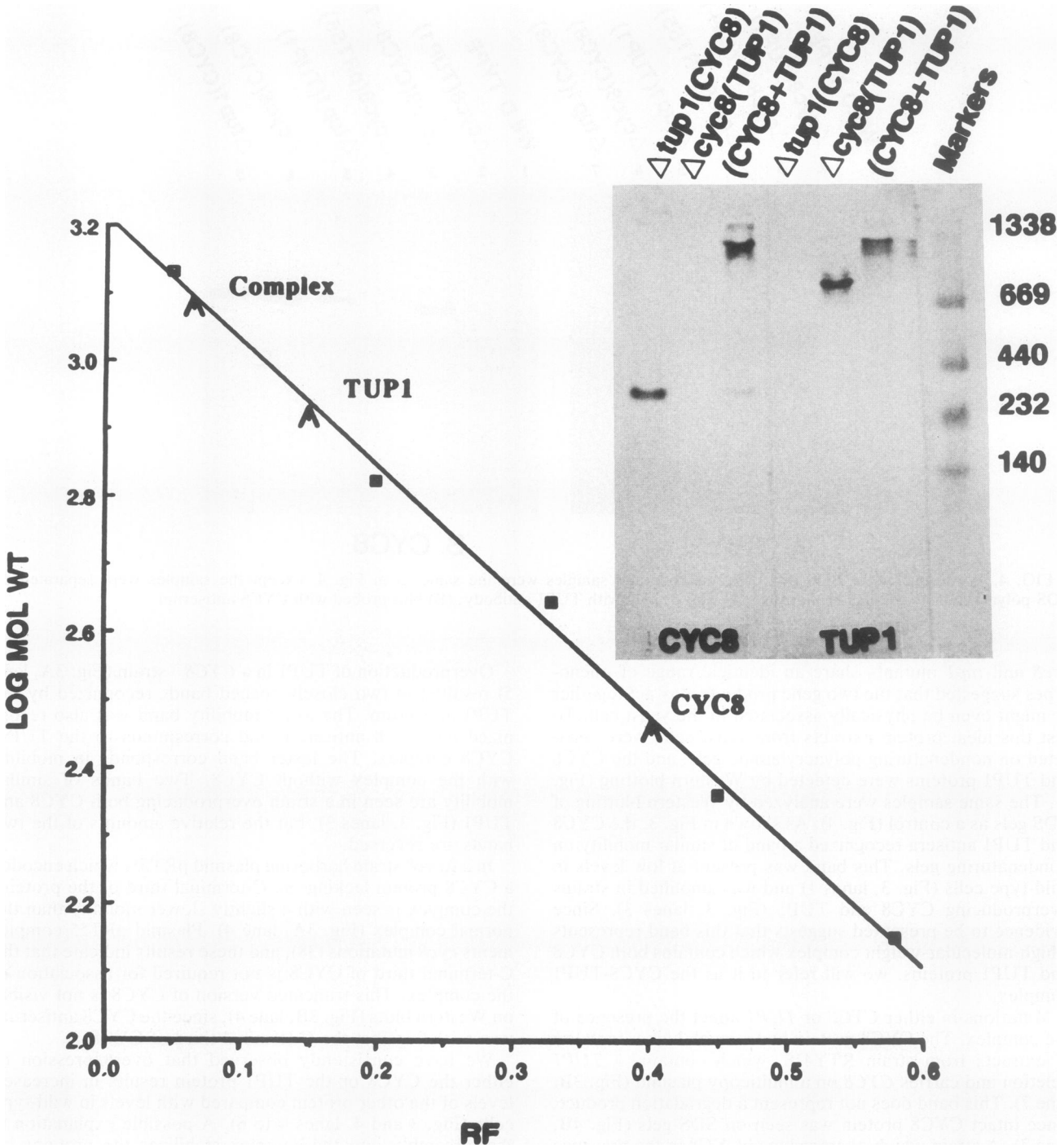

FIG. 5. Molecular weight estimations of the native CYC8 and TUP1 proteins. Protein extracts prepared from yeast cells were separated on a nondenaturing 4 to 20% acrylamide gradient gel (6) at 4°C for 24 h at 15 V/cm. The CYC8 and TUP1 proteins were detected by Western blotting. High-molecular-weight size standards (Pharmacia no. 17-0445-01) run on the gradient gel were transferred to nitrocellulose and stained with 0.5% Ponceau S in 1% acetic acid. Size standards: thyroglobulin tetramer (1,338 kDa), thyroglobulin dimer (669 kDa), ferritin (440 kDa), catalase (232 kDa), and lactate dehydrogenase (140 kDa). The molecular weights of the complete complex, TUP1 in the absence of CYC8, and CYC8 in the absence of TUP1 were estimated by regression analysis of the mobility relative to the bromphenol blue dye front (RF) versus the molecular weights of the standards. Strains: RTY535(pRT81) (Δ*tup1* overproducing CYC8), RTY493(pFW28) (Δ*cyc8* overproducing TUP1), and BJ2168(pTXL63) (overproducing CYC8 and TUP1).

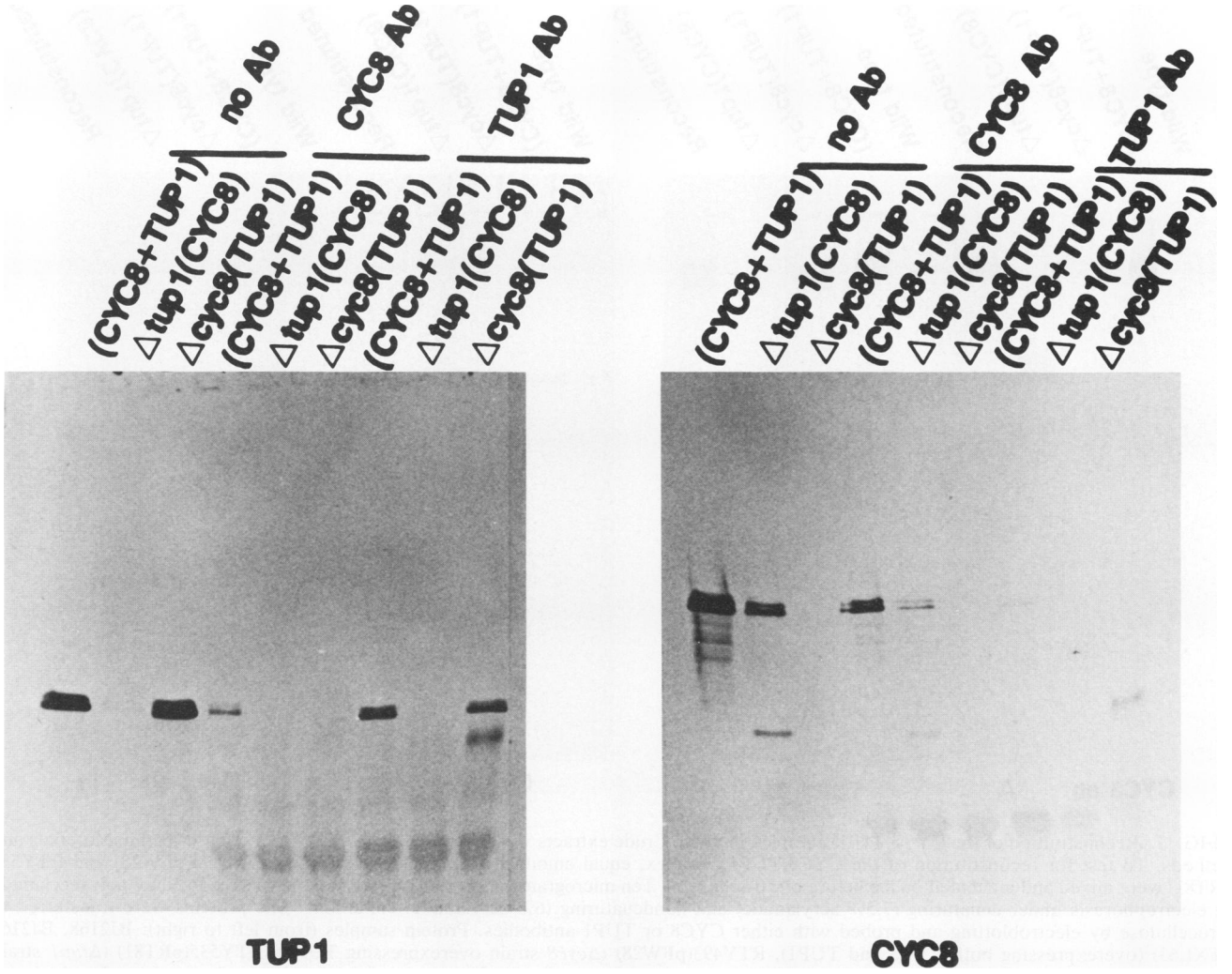

FIG. 6. Immunoprecipitation of CYC8 and TUP1. Cell extracts from yeast cells containing 200 μg of protein were diluted to 400 μl in immunoprecipitation buffer (250 mM NaCl, 25 mM Tris, pH 7.5, 5 mM EDTA, 0.05% Nonidet P-40). Immunoprecipitations were performed basically as described previously (10). The extracts were incubated with a 1:50 dilution of CYC8 or TUP1 antiderum for 1 h on ice, and immune complexes were absorbed with *Staphylococcus aureus* cells (Pansorbin; Calbiochem) for 30 min on ice. The cells were pelleted through a 1 M sucrose cushion, washed twice in immunoprecipitation buffer, and washed once with immunoprecipitation buffer without Nonidet P-40. The pellets were boiled in 50 μl of SDS sample buffer, and half of each sample was applied to duplicate SDS–7.5% acrylamide gels, which were blotted to nitrocellulose and probed with affinity-purified CYC8 or TUP1 antibodies. The labels at the top of the figure indicate the antiserum used in the immunoprecipitation or extracts before immunoprecipitation (no Ab). The labels at the bottom indicate the antibodies used to probe the Western blots. The yeast strains are the same as in Fig. 5.

observed [Fig. 5, Δ*cyc8* (TUP1)], and the TUP1 protein was not degraded. The size of this band was estimated to be 830 kDa. The CYC8 protein was present in a species of 300 kDa in the absence of the TUP1 protein. These numbers suggest that the complete complex is composed of a combination of the 830- and 300-kDa species. These results do not specify the number of CYC8 and TUP1 subunits present in the complex, nor do they rule out the presence of components other than CYC8 or TUP1.

Immunoprecipitations. To provide additional evidence for the association of the CYC8 and TUP1 proteins, we determined whether the two proteins could be coprecipitated by the CYC8 and TUP1 antibodies. Protein extracts were prepared from Δ*cyc8* and Δ*tup1* strains bearing plasmids containing *CYC8*, *TUP1*, or both genes. Immunoprecipitations were performed with either CYC8 or TUP1 antiserum, and the CYC8 and TUP1 proteins in the immunoprecipitates were detected on Western blots (Fig. 6).

The amounts of the CYC8 and TUP1 proteins in the extracts prior to immunoprecipitation are shown in the lanes labeled "no Ab." The CYC8 and TUP1 proteins from strains overproducing one or both proteins were effectively precipitated by their own antisera. A significant proportion of the TUP1 protein is precipitated by the CYC8 antiserum from the strain overproducing both proteins but not from the strain overproducing only TUP1. A small amount of CYC8 protein was precipitated by the TUP1 antiserum from the strain overproducing both proteins but not from the strain overproducing only CYC8. The finding that each protein can be precipitated by antiserum specific for the other protein

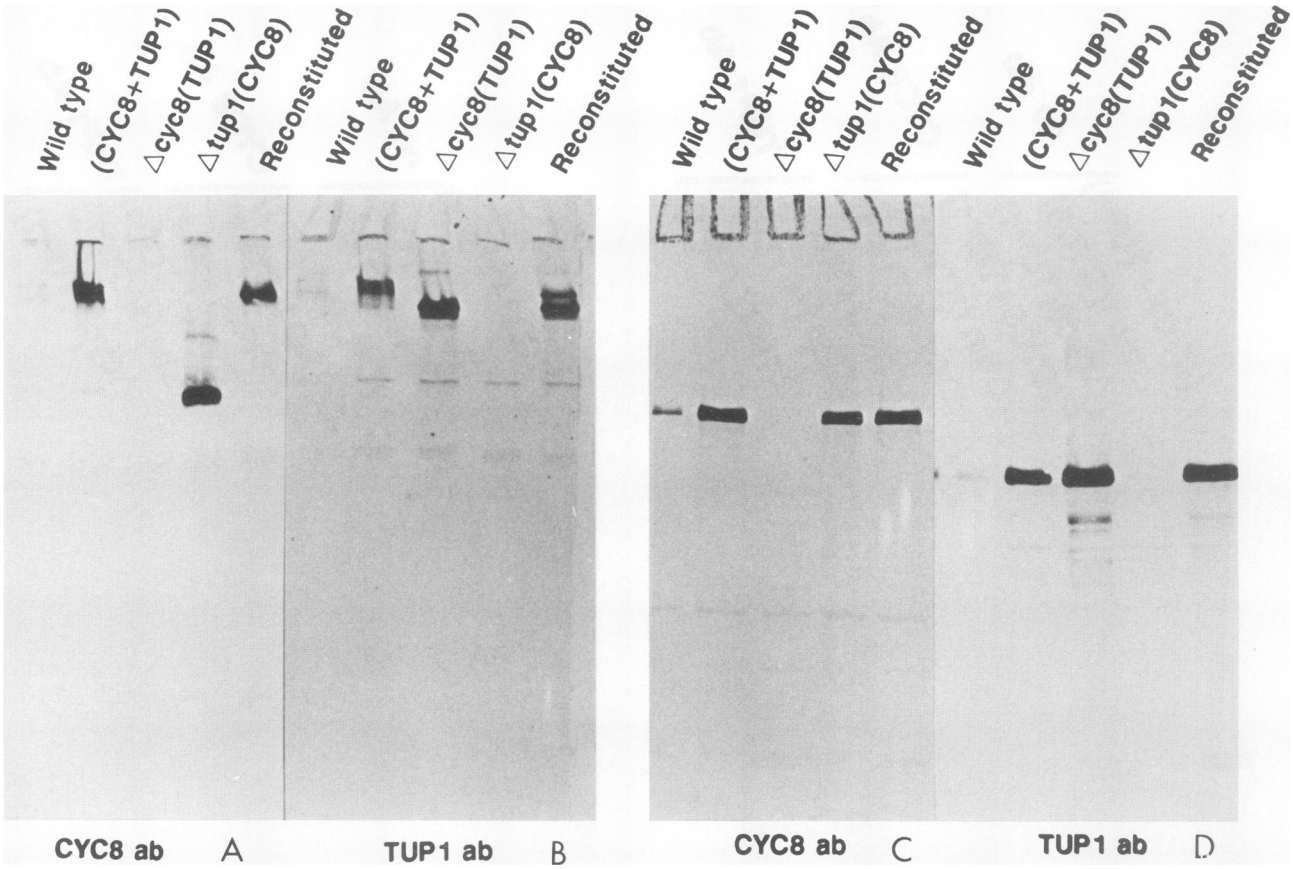

FIG. 7. Reconstitution of the CYC8-TUP1 complex in vitro. Crude extracts were prepared from yeast cells as described in Materials and Methods. To test for reconstitution of the CYC8-TUP1 complex, equal amounts of extracts from strains RTY493(pFW28) and RTY535 (pRT81) were mixed and incubated on ice before electrophoresis. Ten micrograms of protein of each sample was resolved by polyacrylamide gel electrophoresis under denaturing (7.5% acrylamide) and nondenaturing (6% acrylamide) conditions. The proteins were transferred to nitrocellulose by electroblotting and probed with either CYC8 or TUP1 antibodies. Protein samples (from left to right): BJ2168, BJ2168 (pTXL63) (overexpressing both CYC8 and TUP1), RTY493(pFW28) (Δ*cyc8* strain overexpressing TUP1), RTY535(pRT81) (Δ*tup1* strain overexpressing CYC8), and RTY493(pFW28) and RTY535(pRT81) extracts mixed before electrophoresis. (A) Nondenaturing gel probed with CYC8 antibodies; (B) nondenaturing gel probed with TUP1 antibodies; (C) SDS gel probed with CYC8 antibodies; (D) SDS gel probed with TUP1 antibodies.

only when both proteins are present together in the cell further supports the conclusion that the two proteins are associated in a complex.

Reconstitution of the CYC8-TUP1 complex in vitro. In order to further examine the nature of the association of CYC8 and TUP1, we asked whether complex formation could occur in vitro. Yeast cells overexpressing the TUP1 protein in a Δ*cyc8* background exhibit the 830-kDa species detected by TUP1 antibodies (Fig. 7). The CYC8 protein expressed in Δ*tup1* cells migrates as a 300-kDa band on nondenaturing gels. When these two extracts were mixed and then resolved by electrophoresis, the 1,200-kDa complex is formed in vitro. In this experiment, the two extracts were mixed and incubated on ice, and samples were taken at 0, 30, and 60 min to follow the time course of complex formation. Only the 0-min time point is shown in Fig. 7, since all of the time points had identical results. This result does not imply that complex formation is complete at that time, since the proteins could associate after being loaded on the gel.

Several other unusual aspects of the complex formation were revealed by the reconstitution experiment. The complex from cells overexpressing both CYC8 and TUP1 is resolved under optimal conditions into three closely spaced bands. These same bands are present but barely detectable in wild-type cells (Fig. 7). In the reconstituted samples, only the highest-mobility band is observed. Another unexplained feature of the complex formation is that all of the CYC8 protein entered into the complex, while only a fraction of the TUP1 protein associated with the larger complex. The same fraction of the TUP1 protein entered the complex even when greater amounts of the CYC8 extract were used (data not shown). The incomplete reconstitution of the TUP1 protein may be due to interaction with other components in the crude extracts and may be resolved when purer preparations are tested.

DISCUSSION

The CYC8 and TUP1 antisera identified the authentic CYC8 and TUP1 proteins on Western blots by several criteria. However, there was a considerable discrepancy between the sizes of the proteins predicted by the DNA sequence and the apparent sizes on SDS gels. The apparent

size of CYC8 is about 40 kDa larger than expected, and the CYC8 fusion proteins synthesized in *E. coli* have apparent sizes about 30 kDa greater than predicted. In the case of CYC8, errors in the DNA sequence are highly unlikely, since two laboratories independently determined the identical sequence for the CYC8 coding region (29, 38). Schultz et al. (30) recently reported that the SSN6 (= CYC8) protein had an apparent size on SDS gels of 135 kDa. A possible explanation for the increased size of the proteins is a posttranslational modification, such as the addition of carbohydrate, occurring in yeast cells. However, since the CYC8 fusion proteins made in *E. coli* also show a large discrepancy, it seems likely that most of the difference is due to the primary sequence of the protein. Similar examples of anomalous migration of proteins on SDS gels have been reported elsewhere (9, 21).

The major conclusion of this paper is that the CYC8 and TUP1 proteins are associated in a high-molecular-weight complex with an apparent size of 1,200 kDa. The primary evidence supporting this conclusion is the comigration of the CYC8 and TUP1 proteins detected on Western blots of nondenaturing polyacrylamide gels. Several results substantiate the conclusion that this comigration represents an actual association of the two proteins and is not simply fortuitous. In strains deleted for the *CYC8* gene, most of the TUP1 protein is recovered as proteolytic fragments, suggesting that a protease-sensitive site in TUP1 is normally shielded from proteases by association with the CYC8 protein. However, since the TUP1 protein when overexpressed is present in a complex devoid of CYC8 (Fig. 3A, lane 5), there may be other reasons besides a lack of association with CYC8 for the degradation of TUP1 in *cyc8* mutants. In a Δ*cyc8* protease-deficient strain, the TUP1 protein is not degraded but is present in a complex of 830 kDa. Although we have presented the apparent sizes of the native proteins as molecular masses according to common practice, migration on gradient gels is actually a function of the Stokes radius (2). Preliminary results from gel filtration and sucrose gradient centrifugation suggest that the true molecular masses of the complex and its constituents are considerably smaller than the estimates of apparent molecular mass from gel electrophoresis and that the complex has an extended rather than globular shape (unpublished results).

The association of the CYC8 and TUP1 proteins in a large complex suggests possible mechanisms for regulation of glucose repression in light of the repeated domains present in the two proteins. The repeated domains of CYC8 are found also in the nuc2 protein of *Schizosaccharomyces pombe*. The nuc2 protein is tightly associated with the nuclear scaffold and requires harsh conditions such as 8 M urea for solubilization. In contrast, we have found the CYC8 protein to be completely soluble. No difference in the recovery of CYC8 from yeast cells was noted between extraction with a low-salt buffer and boiling in SDS sample buffer (unpublished results). Therefore, the CYC8 protein is not associated with the nuclear scaffold or associates very weakly or under special conditions. In spite of this apparently negative result, the possible association of CYC8 or other members of the CYC8-TUP1 complex with the nuclear scaffold warrants further investigation. Transcriptionally active domains of chromosomes have been shown to be associated with the nuclear scaffold (12), providing a potential mechanism for controlling transcription of a large set of genes. The association of ARS and CEN elements with the nuclear scaffold was recently demonstrated in *S. cerevisiae* (1). Both CYC8 (30) and TUP1 (38a) proteins have been localized to the nucleus by immunofluorescence.

A crucial question is whether there are proteins besides CYC8 and TUP1 present in the complex. The determination of the composition of the complex will require its purification and characterization. The discovery of the other associated proteins, if any, will lead to answers regarding the association of the TUP1 and CYC8 proteins and the mechanism of their regulation of transcription by glucose repression.

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