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The Cyc8 (Ssn6)-Tup1 Corepressor Complex Is Composed of One Cyc8 and Four Tup1 Subunits

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The Cyc8 (Ssn6)-Tup1 corepressor complex is required for repression in several important regulatory systems in yeast cells, including glucose repression and mating type. Cyc8-Tup1 is recruited to target genes by interaction with diverse repressor proteins that bind directly to DNA. Since the complex has a large apparent molecular mass of 1,200 kDa on nondenaturing gels (F. E. Williams, U. Varanasi, and R. J. Trumbly, Mol. Cell. Biol. 11:3307–3316, 1991), we used a variety of approaches to determine its actual subunit composition. Immunoprecipitation of epitope-tagged complex and reconstitution of the complex from in vitro-translated proteins demonstrated that only the Cyc8 and Tup1 proteins were present in the complex. Hydrodynamic properties showed that these proteins have unusually large Stokes radii, low sedimentation coefficients, and high frictional ratios, all characteristic of asymmetry which partly accounts for the apparent high molecular weight. Calculation of native molecular weights from these properties indicated that the Cyc8-Tup1 complex is composed of one Cyc8 subunit and four Tup1 subunits. This composition was confirmed by reconstitution of the complex from Cyc8 and Tup1 expressed in vitro and analysis by one- and two-dimensional gel electrophoresis.

The Cyc8 (Ssn6) and Tup1 proteins are required for repression of transcription in several regulatory pathways in yeast cells, including glucose repression and mating type. The Cyc8 and Tup1 proteins constitute a corepressor complex, hereafter referred to as Cyc8-Tup1, that mediates repression after recruitment to promoter sites by interaction with specific DNA-binding repressor proteins (20, 48).

Corepressors have recently been identified in higher eukaryotic cells. The groucho (31) and extra sex combs (12) proteins appear to act as corepressors that function during *Drosophila* development. Both of these proteins possess the WD repeats found in Tup1 but no homology outside of this region. In mammalian cells, two unrelated corepressors that interact with the retinoic acid receptor were recently discovered by two different groups (5, 17).

Yeast strains bearing mutations in either *cyc8* or *tup1* have multiple mutant phenotypes, including constitutive expression of glucose-repressible genes, calcium-dependent flocculation, mating defects in *MAT*α cells, inability of homozygous diploids to sporulate, poor growth on alternate carbon sources such as glycerol, and temperature sensitivity (34, 42). These diverse phenotypes are believed to result from failure to repress different classes of genes. Both Cyc8 and Tup1, when fused to a LexA DNA-binding domain, can repress transcription from reporter genes with upstream LexA binding sites. Repression by LexA-Cyc8 is dependent on the presence of Tup1, but repression by LexA-Tup1 is independent of Cyc8, suggesting that repression is mediated directly by Tup1 (20, 44). Cyc8 is thought to be primarily involved in interactions with different repressor proteins that bind directly to DNA (45).

Cyc8-Tup1 appears to interact with a diverse group of repressor proteins for different families of genes: (i) the zinc finger Mig1 protein for glucose-repressible genes (29, 41); (ii) Rox1, related to high-mobility-group proteins, that represses genes normally expressed under anaerobic conditions (49); (iii) the Matα2 repressor, a homeobox protein, required for repression of **a**-specific genes (15, 20); (iv) repression of DNA damage-inducible genes by an unknown repressor (50); and (v) repression of the *HXT* genes encoding glucose transporters by the Rgt1 repressor (30).

The Cyc8 and Tup1 proteins contain long stretches of polyglutamine, which are found in many other transcriptional regulatory proteins, but there are no other similarities between the two proteins (35, 43, 47). The Cyc8 protein contains 10 copies of the 34-amino-acid tetratricopeptide repeat (TPR) near the N terminus (11), and these repeats are required for Cyc8 function (36). TPR domains are found in several other nuclear proteins, one of which, nuc2 of *Schizosaccharomyces pombe*, is tightly associated with the nuclear scaffold (11). The Tup1 protein contains six or seven repeats of about 40 amino acids each, called WD-40 (or simply WD) or β-transducin repeats, first found in the β subunit of heterotrimeric G proteins and now known to exist in a large family of proteins including Ste4, Cdc4, Cdc20, Mak11, and the *Drosophila* gene Enhancer of split involved in neural development (22, 28, 47).

Both the TPR and WD repeats are believed to mediate protein-protein interactions. Models for the structures of TPRs suggested that they should interact with each other to form coiled coils (16). A direct role for protein-protein interactions was demonstrated for one of the TPRs of Cdc27 of *Saccharomyces cerevisiae* (25). One of the WD repeats of Tup1 was shown to interact with the α2 repressor (22). It was pointed out that in several regulatory systems, functionally related proteins with TPR and WD repeats are found: Cyc8-Tup1 in glucose repression, Prp6-Prp4 in mRNA splicing, Cdc16 and Cdc23-Cdc20 in chromosome segregation, and Ski3-Mak11 in maintenance of the yeast RNA virus (11). We were the first to demonstrate a physical association between TPR and WD proteins, namely, Cyc8 and Tup1 (48).

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Two general mechanisms have been proposed to explain repression by Cyc8-Tup1: (i) control of nucleosome positioning so as to mask DNA targets for activators or transcription factors and (ii) direct inhibition of the transcription apparatus. The evidence for the first explanation was presented by Simpson and coworkers (27, 32), who found that the α2 operator was capable of controlling nucleosome positioning, which was directly implicated in repression of transcription. This nucleosome positioning was dependent on both Cyc8 (Ssn6) and Tup1, but *tup1* mutations had more severe effects on chromatin structure than did *cyc8* mutations (7). Recently, Tup1 was shown to interact directly with the amino-terminal ends of histones H3 and H4 (9). In favor of the second mode of repression, α2 operators were found to mediate repression of transcription when purified α2 protein was added to in vitro transcription reactions (14). Repression was found only when the extracts for transcription were prepared from yeast strains overexpressing both Cyc8 and Tup1. The authors argued that this result indicated that Cyc8-Tup1 acts directly to inhibit the general transcription machinery. As further support for this mechanism, mutations affecting repression have recently been identified in genes encoding subunits of the RNA polymerase II mediator complex (1, 23). Therefore, it seems likely that the Cyc8-Tup1 complex can repress transcription by two different mechanisms, nucleosome positioning and inhibition of the general transcription machinery.

We have shown that Cyc8 and Tup1 proteins are associated in a complex with an apparent size of 1,200 kDa, as determined by gel electrophoresis under native conditions (48). This large apparent size raised questions about the actual composition of the complex: How many subunits of Cyc8 and Tup1 are present in the complex? Are there other proteins in the complex besides these two? Several large complexes involved in transcriptional regulation, such as the Swi-Snf complex, are composed of many different subunits (3, 8). The purpose of the work described here was to answer these two questions.

MATERIALS AND METHODS

Yeast strains and plasmids. Strains BJ2168 (*MAT***a** *leu2 trp1 ura3-52 prc1-407 prb1-1122 pep4-3*), RTY493 (*MAT***a** *leu2 trp1 ura3-52 prc1-407 prb1-1122 pep4-3 cyc8-Δ1*::*LEU2*), and RTY535 (*MAT***a** *leu2 trp1 ura3-52 prc1-407 prb1-1122 pep4-3 tup1-Δ1*::*TRP1*) were described earlier (48). RTY545 (*MAT***a** *leu2 trp1 ura3-52 prc1-407 prb1-1122 pep4-3 tup1-Δ1*::*TRP1 cyc8-Δ1*::*LEU2*) was derived from RTY535 by transformation with pDSB (43). Yeast strains were transformed with plasmids by the lithium acetate method (19). pTXL63 contains both *CYC8* and *TUP1* cloned into YEp24 (48). pMB3 and pUV4-Δ129-282 are multicopy yeast vectors for the expression of Tup1Δ1-51 and Tup1Δ129-282, respectively (45a).

Myc epitope tagging of Cyc8. The 3′ end of the *CYC8* coding sequence was modified to encode the 10-amino-acid epitope recognized by the Myc1-9E10 monoclonal antibody (10). The C-terminal end of Cyc8 was chosen, because it was shown previously to be dispensable for function (36, 43). A 1.1-kb *Hin*cII-*Xba*I fragment containing the C-terminal coding sequence of *CYC8* was subcloned into pTZ19U (26), producing pUV16. This plasmid was mutagenized by the method of Kunkel et al. (24) with the Myc epitope-specific primer 5′TTAG TCGTCGTAGTTTAAATCTTCTTCAGAAATAAGCTTTTGTTCTCTTGG AAGTG3′ to produce pUV25 (Fig. 1A). A 3.1-kb *Hin*cII-*Xba*I fragment, a 2.4-kb *Hin*cII-*Xba*I fragment, both from pRT75 (43), and a 1.1-kb *Hin*cII-*Xba*I fragment from pUV25 were ligated to produce pUV28. A 2.4-kb *Kpn*I-*Bam*HI fragment from pUV28 replaced the corresponding fragment of pRT81 (*CYC8* in YEp24) (43) to yield pUV30, a 2μm *URA3* yeast expression vector for Cyc8-Myc. A 3.5-kb *Xho*I-*Sph*I fragment from pTXL6 (47) containing *TUP1* was ligated into the *Sal*I-*Sph*I site of pUV30 to get pUV31, a 2μm *URA3* yeast vector for coexpressing Cyc8-Myc and Tup1.

Construction of plasmids for in vitro translation. For expression of Tup1 and Cyc8 proteins in vitro, the coding sequences of these genes were subcloned into pCITE2a (Novagen). Plasmid pCITE2a carries a segment of the encephalomyocarditis virus RNA 5′ noncoding region that functions as a translational enhancer, increasing by typically 10-fold the in vitro translation efficiency of synthetic RNA in rabbit reticulocyte lysates. Plasmid pUV3 contains the entire *TUP1* gene in pBS(+) (Stratagene). A 1.2-kb *Nde*I-*Bam*HI fragment from pUV3 was subcloned into pJDH119 (18). This plasmid was then mutagenized by the Kunkel method with the primer 5′CAA ACC CAT ATG ACT GCC3′ (the *Nde*I site is underlined; the mismatch is in boldface) to create an *Nde*I site at the start codon. The 1.2-kb *Nde*I-*Bam*HI fragment produced by *Bam*HI-partial *Nde*I digestion was then shuttled back to pUV3 to create pUV11. pRT63 contains a 2-kb *Hin*dIII fragment containing the N-terminal coding region of *CYC8* in pBS(+). This plasmid was mutagenized with the primer 5′AAA AAA ATT AGG A**CC ATG GC**T CCG GGC GGT GAA3′ (the *Nco*I site is underlined; mismatches are in boldface) to create an *Nco*I site at the *CYC8* start codon. A 665-bp *Bgl*I fragment containing this *Nco*I site replaced the corresponding fragment of pRT75 (43) to yield pUV12. A 2.4-kb *Nde*I-*Xba*I fragment containing the *TUP1* coding sequence from pUV11 and a 3.2-kb *Nco*I-*Bam*HI fragment containing *CYC8* from pUV12 were ligated into the respective sites of pCITE2a to yield pJG1 and pJG2, respectively.

Yeast protein extractions. Cells were harvested in logarithmic phase of growth (A_{600} = 0.5 to 1.5). The cells were washed twice in extraction buffer [200 mM Tris-HCl (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 7 mM β-mercaptoethanol]. The cells were resuspended in 0.5 ml of extraction buffer with protease inhibitors (1 mM phenylmethylsulfonyl fluoride plus leupeptin, pepstatin, and aprotinin at 1 μg of each per ml). The cell suspension was transferred to a 1.5-ml microcentrifuge tube, an equal volume of glass beads was added, and the cells were broken by vortexing for three 1-min bursts followed by cooling on ice. The extract was centrifuged at maximum speed in a microcentrifuge for 20 min at 4°C, and the supernatant was collected. Protein was quantitated by the Bradford microassay (2).

Immunological procedures. Western blotting (immunoblotting) was performed as described previously (48). Antibodies against the Myc epitope EQKLISEEDL were expressed from the Myc1-9E10 cell line obtained as a gift from B. Errede, Chemistry Department, University of North Carolina, Chapel

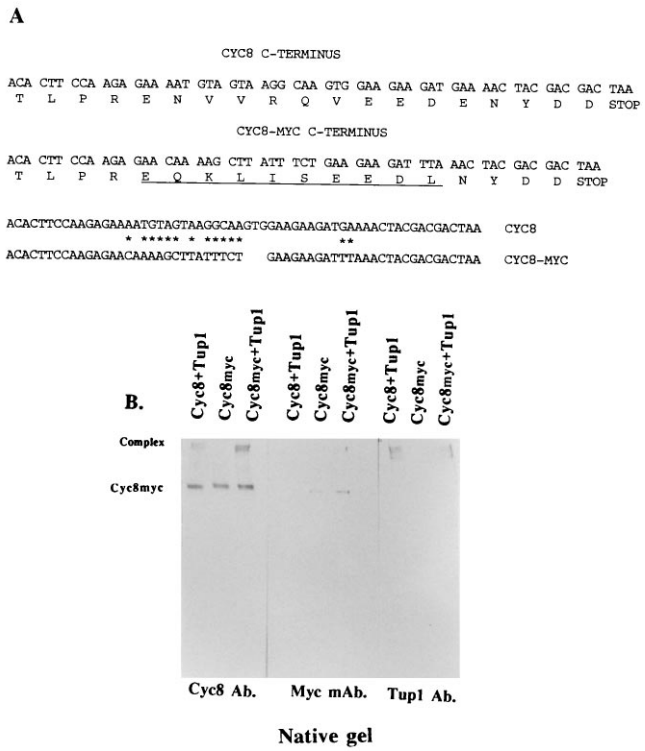

FIG. 1. Myc epitope tagging of Cyc8. (A) Design of the primer for epitope tagging of Cyc8. The top sequence shows the C-terminal coding region of wild-type *CYC8*. The middle sequence is this sequence altered by oligonucleotide-mediated mutagenesis to encode the Myc1-9E10 epitope (underlined). The bottom sequences show an alignment of the original and mutant sequences, with mismatches identified by asterisks. The mutant sequence shown here is the complement of the 56-mer oligonucleotide used for mutagenesis, with the addition of one nucleotide at the 5′ end. This primer was designed to minimize the number of base and amino acid changes; 4 of the 10 amino acids of the epitope were already present in the original sequence. (B) Expression of the epitope-tagged protein was tested by separation of crude yeast cell extracts on 6% acrylamide native gels and Western blotting with either Cyc8, Myc, or Tup1 antibody (Ab). The following yeast strains were used: BJ2168(pTXL63) (overexpressing both Cyc8 and Tup1), RTY493(pUV31) (overexpressing both Cyc8-Myc and Tup1 in a Δ*cyc8* background), and RTY545(pUV30) (overexpressing Cyc8-Myc in a Δ*cyc8* Δ*tup1* background).

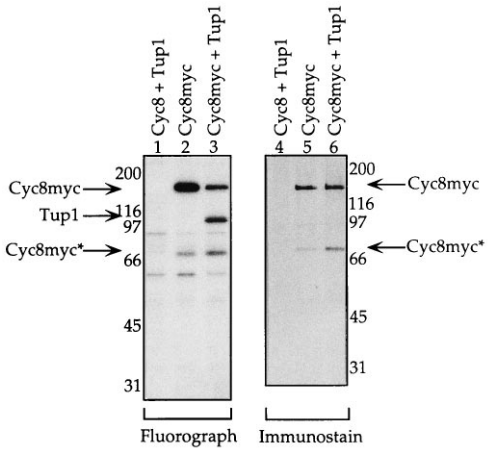

FIG. 2. Immunoprecipitation of the epitope-tagged complex. Yeast cells were grown in the presence of [^{35}S]methionine and [^{35}S]cysteine, and proteins were immunoprecipitated with Myc antibody as described in Materials and Methods. Fifteen-microliter aliquots of the total 50-μl samples were loaded on duplicate SDS–7.5% acrylamide gels. One gel was stained with Coomassie blue to visualize the size standards, treated with Fluoro-Hance (Research Products International Corp.), dried, and exposed to film for fluorography. The other gel was blotted to nitrocellulose and probed with Cyc8 antibodies. Protein size standards (broad range; Bio-Rad) were run on both gels. The yeast strains were the same as in Fig. 1. Cyc8myc* denotes a band present on the fluorogram and recognized by the Cyc8 antibodies that is presumably a proteolytic product of Cyc8. Sizes are indicated in kilodaltons.

Hill. This cell line was maintained by using standard protocols, and tissue culture supernatants were collected. For purification of the antibody in appreciable quantities, these cells were injected in BALB/c mice to induce tumors, and ascites fluid was collected (13). The antibody was affinity purified by using the Bio-Rad Affi-Gel protein A monoclonal antibody purification system as instructed by the manufacturer. Briefly, either 50 ml of tissue culture supernatant or 5 ml of ascites fluid diluted 1:1 with binding buffer was applied to a 0.5-ml Affi-Gel column preequilibrated with 5 bed volumes of binding buffer. After the column was washed with 15 bed volumes of binding buffer, antibody was eluted with 15 volumes of elution buffer. The eluate was collected into a tube containing 0.8 ml of 1 M Tris-HCl buffer (pH 9) and concentrated to 1 ml by ultrafiltration. The Myc monoclonal antibody concentration was determined by immunostaining after resolution on a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel and Western blotting. This antibody preparation was used in the experiment in Fig. 1B and in preliminary immunoprecipitation experiments, while a commercial anti-Myc antibody (Santa Cruz Biotechnology) was used for the experiment in Fig. 2.

Immunoprecipitation of the epitope-tagged complex. Immunoprecipitations were performed basically as described by Kolodziej and Young (21). Cells were metabolically labeled as follows (13). The cells were grown in low-sulfate medium, consisting of 0.67% Yeast Nitrogen Base without amino acids and nitrogen, 2% glucose, 38 mM ammonium chloride, and amino acids for auxotrophic requirements. Overnight cultures were diluted to an A_{600} of 0.2 and then grown for 6 h at 30°C. Radiolabeled [^{35}S]methionine and [^{35}S]cysteine (EXPRESS Protein Labeling Mix; NEN) were added to 20 μCi/ml. After an additional 2 h of growth, the cells were harvested by centrifugation and suspended in 2 volumes buffer A (21) containing 200 mM ammonium sulfate and 0.1% Triton X-100. All subsequent steps were carried out on ice or at 4°C. The cells were disrupted by vortexing five times for 1 min each with glass beads. The supernatants were recovered after centrifugation for 10 min. Aliquots of the samples containing 4×10^7 cpm were diluted to 50 μl with buffer A containing 200 mM ammonium sulfate and 0.1% Triton X-100, to which was added 150 μl of buffer A containing 0.1% Triton X-100 to bring the final ammonium sulfate concentration to 50 mM. These samples were precleared by addition of 20 μl of protein G PLUS-Agarose beads (Santa Cruz Biotechnology), incubation for 30 min, and centrifugation for 10 min. After the supernatants were transferred to a new tube, 2 μg of c-Myc (9E10) monoclonal antibody (Santa Cruz Biotechnology) was added, followed by incubation for 1 h. The samples were centrifuged for 10 min, and 20 μl of Protein G PLUS-Agarose beads was added to the supernatants. After 1 h of incubation, the samples were centrifuged for 1 min, and the pellets were washed twice with buffer B (21) containing 100 mM ammonium sulfate and 0.1% Triton X-100 and twice with buffer B containing 50 mM ammonium sulfate. The pellets were suspended in 50 μl of SDS sample buffer and boiled for 3 min. After centrifugation for 2 min, the supernatants were recovered and applied to SDS–7.5% acrylamide gels. The antigens were visualized by immunostaining or fluorography.

In vitro translations. Plasmids pJG1 and pJG2, encoding Tup1 and Cyc8, respectively, were transcribed and translated by the Single Tube Protein System 2 (Novagen). Five microliters of the reaction mixture was diluted to 50 μl with 10% glycerol containing bromophenol blue for loading onto native gels, and another 5 μl of reaction was diluted to 50 μl with SDS sample buffer and boiled for 3 min for loading onto SDS-gels. Ten microliters of the prepared sample, corresponding to 1 μl of translation reaction mixture (1.3×10^6 cpm), was resolved on either 4 to 20% acrylamide gradient native or SDS–7.5% acrylamide gels, electroblotted, and immunostained with either Cyc8 or Tup1 affinity-purified antibody. The dried blots were then exposed to Kodak X-ray film for 24 h at room temperature before development of the film.

Stokes radii. Both gel filtration chromatography (37) and pore gradient gel electrophoresis (PGGE) (33) were used to determine Stokes radii. For gel filtration chromatography, we used a Sepharose 4B column (46 by 0.53 cm) equilibrated with 200 mM Tris-HCl buffer (pH 8.0) containing 400 mM ammonium sulfate, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin, leupeptin, and aprotinin, each to a final concentration of 1 μg/ml). Two hundred microliters of 40% ammonium sulfate fraction containing 10 μg of protein was fractionated at a flow rate of 10 ml/h. Forty fractions were collected. The column was calibrated by fractionating size standards: phosphorylase *b* kinase (121 Å [1 Å = 0.1 nm]), thyroglobulin (85 Å), ferritin (78 Å), catalase (52 Å), and bovine serum albumin (BSA; 35 Å).

Stokes radii were also determined by PGGE. Ten-microgram aliquots of crude proteins were resolved on native linear polyacrylamide gradient gels with a constant acrylamide-to-*N*,*N*-methylenebisacrylamide ratio (29.2:0.8) in a range of approximately 4 to 20% total monomer at 4°C for 3 to 24 h at 15 V/cm, using 0.5× Tris-borate-EDTA as the running buffer. Protein standards were thyroglobulin dimer (667 kDa, 85 Å), ferritin (440 kDa, 61 Å), catalase (232 kDa, 52 Å), and lactate dehydrogenase (140 kDa, 42 Å). After transfer to the nitrocellulose membrane, markers were detected by staining with 0.55% Ponceau S in 1% acetic acid, and the Cyc8 and Tup1 proteins were detected by immunostaining. Time-dependent migration distances (D[mm]) of proteins were measured directly from the blots with a ruler, and the resulting data were evaluated by a two-step procedure. In the first step, the maximum migration distance (D_{max}) was calculated by plotting the double-logarithmized time-dependent migration distance [ln (ln D)] of the protein versus the reciprocal of the square root of its migration time ($t^{1/2}$ (t[h]). During the second step, the intercept values were delogarithmized once (ln D_{max}) and were used to construct the calibration lines. The ln D_{max} values were plotted versus the logarithm of their Stokes radii.

Sedimentation coefficients. Sedimentation coefficients were determined by density gradient centrifugation (37) for 12 h, using per tube, 4.5 ml of 5 to 20% linear gradients of sucrose in 50 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ and 5 mM EDTA. The fractions were analyzed by Western blotting. Marker proteins catalase (11.3S), aldolase (7.63S), and BSA (4.3S) were centrifuged in separate tubes in parallel and were detected by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis (PAGE).

RESULTS

Immunoprecipitation of the epitope-tagged complex. The composition of the Cyc8-Tup1 complex was first examined by immunoprecipitation. We found previously that immunoprecipitation of yeast cell extracts with Cyc8 antibodies coprecipitates Tup1, and vice versa (48). When we used these antibodies to analyze the complex by immunoprecipitation of extracts of ^{35}S-labeled cells, the background of nonspecific bands was too high. To circumvent this problem, we used oligonucleotide mutagenesis to place the epitope recognized by the Myc1-9E10 monoclonal antibody at the C terminus of Cyc8. Expression of the epitope-tagged Cyc8 (Cyc8-Myc) complemented a Δ*cyc8* mutant strain for flocculation, suggesting that Cyc8-Myc was functional. Expression of the Cyc8-Myc protein and its ability to associate with Tup1 were assayed by Western blots of native gels (Fig. 1B). In the strain expressing untagged Cyc8 and Tup1, the complex was recognized by both Cyc8 and Tup1 antibodies, but no bands were observed with the Myc antibody. In strains expressing Cyc8-Myc and Tup1, Cyc8-Myc comigrates with Tup1 with the same mobility as the normal complex. In this experiment, significant amounts of both tagged and untagged Cyc8 migrated as a monomer free of Tup1, probably because these proteins were overexpressed. These experiments demonstrate that the Cyc8-Myc protein is functionally expressed in yeast and associates with Tup1 to form a complex with the same electrophoretic mobility as the wild-type complex.

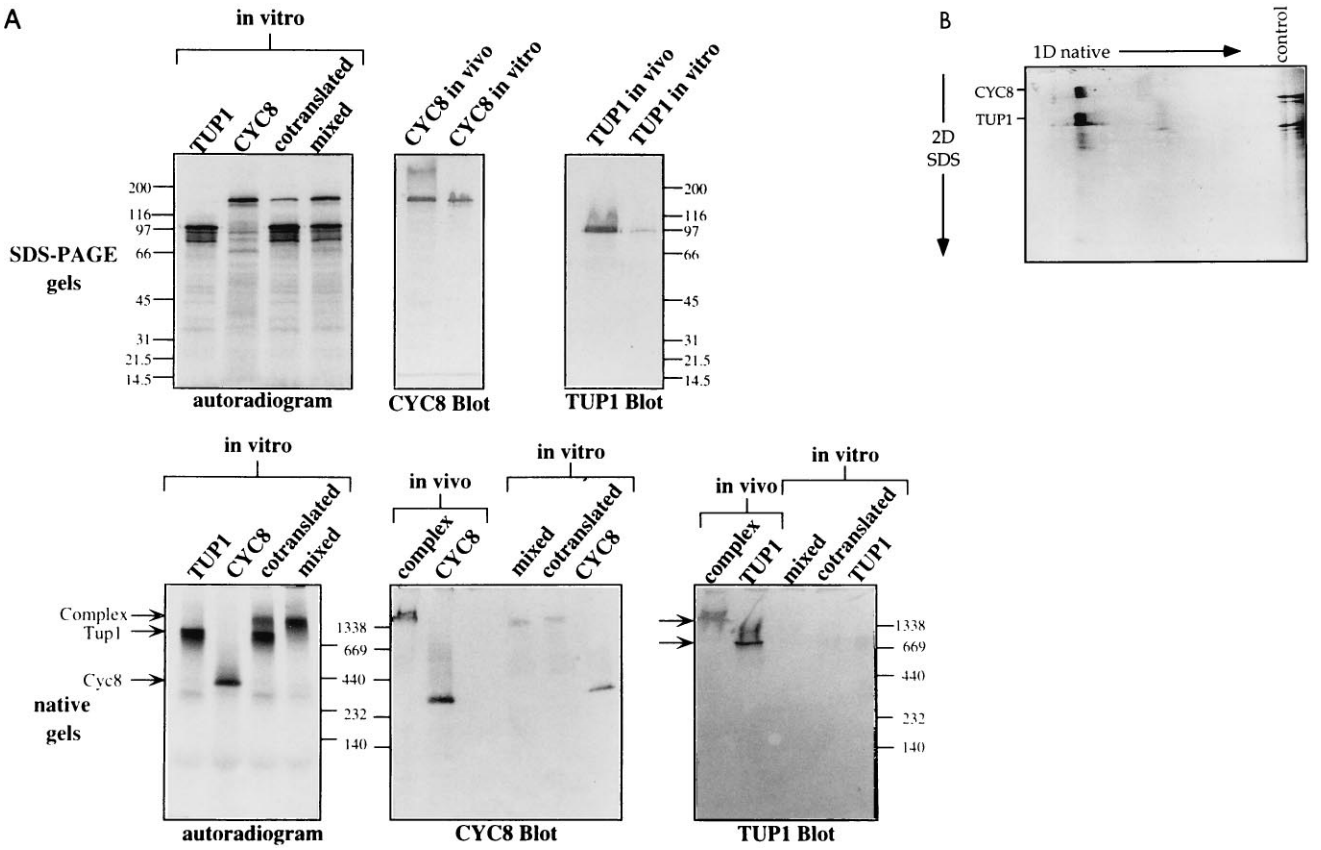

FIG. 3. In vitro expression of Cyc8 and Tup1. (A) RNA synthesized in vitro from pJG1 (TUP1) and pJG2 (CYC8) was translated in the presence of [^{35}S]methionine as described in Materials and Methods. The in vitro translation reaction products were resolved on either SDS–7.5% polyacrylamide gels or 4 to 20% polyacrylamide gradient native gels along with crude cell extracts as in vivo controls and markers. Duplicate gels were either dried and autoradiographed or blotted followed by staining with either Tup1 or Cyc8 antibody. The size standards for the SDS-gels were Bio-Rad broad-range standards; for the native gels, the Pharmacia standards described in the legend to Fig. 4A were used. The thyroglobulin dimer (1,338 kDa) was not resolved on the native gel used for the autoradiogram. (B) The complex formed by mixing the separate Cyc8 and Tup1 reactions was resolved by two-dimensional (2D) electrophoresis. The first dimension was a 4 to 20% acrylamide gradient slab gel. A lane from this gel was excised and run in a second dimension on a SDS–7.5% acrylamide gel. The spots were quantitated by scanning autoradiograms and by direct scintillation counting of gel slices.

The Cyc8-Tup1 complex was immunoprecipitated with the Myc antibody from extracts from yeast cells grown in the presence of [^{35}S]methionine and [^{35}S]cysteine (Fig. 2). Protein bands specific to the complex should be present in the strain expressing Cyc8-Myc and Tup1 (lane 3) and absent in the strain expressing untagged Cyc8 and Tup1 (lane 1). Only three specific bands, labeled Cyc8myc, Tup1, and Cyc8myc*, were observed. Other faint bands were present in all lanes and represent nonspecific background. The band labeled Cyc8myc* was identified as a proteolytic product of Cyc8, since a band of identical mobility was recognized by Cyc8 antibody on Western blots (lanes 5 and 6). This proteolytic product was also observed in crude extracts from yeast cells (data not shown). We concluded that only Cyc8 and Tup1 are components of the complex as determined by immunoprecipitation.

The native sizes of Cyc8, Tup1, and the complex expressed in vitro are similar to those expressed in vivo. As an independent approach to determine the composition of the complex, we attempted to reconstitute the complex from Cyc8 and Tup1 synthesized in vitro. The Cyc8 and Tup1 proteins were made by single-tube in vitro transcription-translation containing T7 RNA polymerase and rabbit reticulocyte lysate (Fig. 3A). The in vitro reactions produced mostly full-length proteins with some truncated products. Cyc8 and Tup1 made in vitro were detectable on Western blots, with similar mobilities on SDS-containing or nondenaturing gels as the proteins expressed in yeast. The in vitro Cyc8 products were clearly visible on the native Cyc8 blot, while in the native Tup1 blot the in vitro products were faint and barely visible in Fig. 3. When the Cyc8 and Tup1 translation reactions were mixed, or the two proteins were synthesized in the same reaction, a band that migrated with a slightly greater mobility compared to the full-length complex from yeast cells was produced. From a comparison with size standards, the in vitro complex in this experiment has an apparent size about 96% of that of the in vivo complex. A significant part, and perhaps all, of this difference can be attributed to the presence of incomplete in vitro products, especially Tup1. It is also possible that differences in posttranslational modifications such as phosphorylation contribute to the difference in apparent size. These results lend further support to the conclusion that the Cyc8 and Tup1 proteins are sufficient for complex formation.

To determine the stoichiometry of the complex, the reconstituted complex from Cyc8 and Tup1 made in vitro was resolved by two-dimensional electrophoresis (Fig. 3B). The first dimension was an acrylamide gradient gel as in Fig. 3A, and the second dimension was a standard SDS-polyacrylamide gel. The proteins were resolved as rectangles. The relative amounts of the two proteins were determined by scanning the autoradiograms or direct scintillation counting of solubilized gel

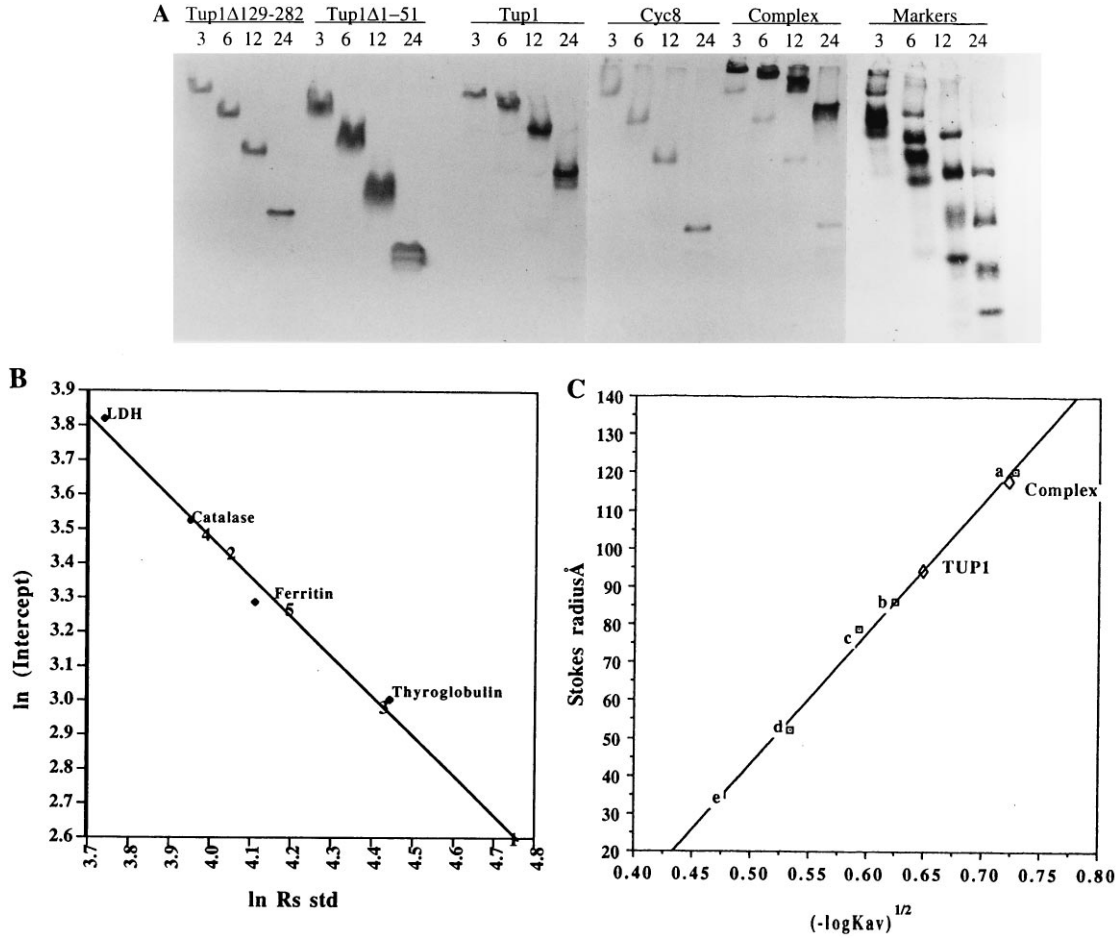

FIG. 4. Stokes radii. (A) Time-dependent migration patterns of test proteins and markers were determined by PGGE. Yeast cell extracts containing 10 μg of protein were resolved on native (4 to 20% total monomer) gradient gel at 4°C for 3 to 24 h at 15 V/cm, using 0.5× Tris-borate-EDTA as the running buffer. The yeast strains were BJ2198(pTXL63) expressing both Cyc8 and Tup1, RTY535(pRT81) expressing Cyc8 in the absence of Tup1, RTY493(pFW28) expressing Tup1 in the absence of Cyc8, RTY545(pMB3) expressing Tup1Δ1-51 in the absence of Cyc8, and RTY545(pUV4-Δ129-282) expressing Tup1Δ129-282 in the absence of Cyc8. The protein size markers (Pharmacia no. 17-0445-01) thyroglobulin (667 kDa, 85 Å), ferritin (440 kDa, 61 Å), catalase (232 kDa, 52 Å), and lactate dehydrogenase (140 kDa, 42 Å) were run on the gradient gels simultaneously. Proteins were detected by immunostaining, and markers were detected by staining with 0.55% Ponceau S in 1% acetic acid. The test protein and time of migration in hours are indicated above each lane. (B) Calibration lines used to calculate Stokes radii (Rs) of the test proteins. The test proteins (1, complex; 2, Cyc8; 3, Tup1; 4, Tup1Δ1-51; 5, Tup1Δ129-282) were constructed by plotting the ln D_{max} versus ln Rs. Std, standard. (C) Plot of $(-\log K_{av})^{1/2}$ versus Stokes radius. K_{av} was determined by gel filtration chromatography on a Sepharose 4B column. The column was calibrated by fractionating size standards (a, phosphorylase kinase [121 Å]; b, thyroglobulin [85 Å]; c, ferritin [78 Å]; d, catalase [52 Å]; e, BSA [35 Å]) separately. Elution profiles of the complex and Tup1 were determined by Western blotting, and those of marker proteins were monitored by reading A_{280}.

slices. The two methods gave very similar results for individual gels, but some variation was noted between gels. The ratios of Tup1 to Cyc8 were calculated, taking into account the number of methionines in each protein. In six replicate gels, the Tup1/Cyc8 ratios were determined to be 3.65 ± 0.28 by scanning and 3.55 ± 0.243 by counting. From examination of the gels, we found that a small amount of less than full-length Tup1 that associated in the complex was visible. When this rectangle was included in the total for Tup1, Tup1/Cyc8 ratios of 4.00 and 3.88 were obtained from two gels by scintillation counting. We consider that 4 to 1 represents the best estimate for the ratio of Tup1 to Cyc8.

The hydrodynamic properties of the complex and its constituents. To determine the actual number of subunits of each protein present in the complex, we used hydrodynamic methods to obtain estimates of the molecular weights of the complex and its constituents. Although a single method such as gel filtration cannot provide accurate estimates of molecular mass for nonglobular proteins, a combination of the sedimentation coefficient from sucrose gradients and the Stokes radius can yield accurate estimates of mass and other molecular parameters (37). We used both gel filtration and PGGE for determination of Stokes radii. Although both of these techniques are frequently used for determination of molecular weights, both rely upon molecular sieving, which separates on the basis of molecular size or Stokes radius rather than molecular weight. The migration patterns of test proteins and size markers subjected to PGGE are shown in Fig. 4A. The time-dependent migration distances were used to calculate the D_{max} as described in Materials and Methods. The ln D_{max} plotted against ln Stokes radius of the standards provided estimates of the Stokes radii of the sample proteins (Fig. 4B). The Stokes radii of the complex and Tup1 determined by both PGGE and gel filtration on Sepharose 4B were in good agreement. The Stokes radius of the complex calculated from gel filtration was 120 Å (Fig. 4C), while that calculated from the gradient gel was 116 Å (Fig. 4B); for Tup1 the Stokes radii were 93 and 90 Å, respectively.

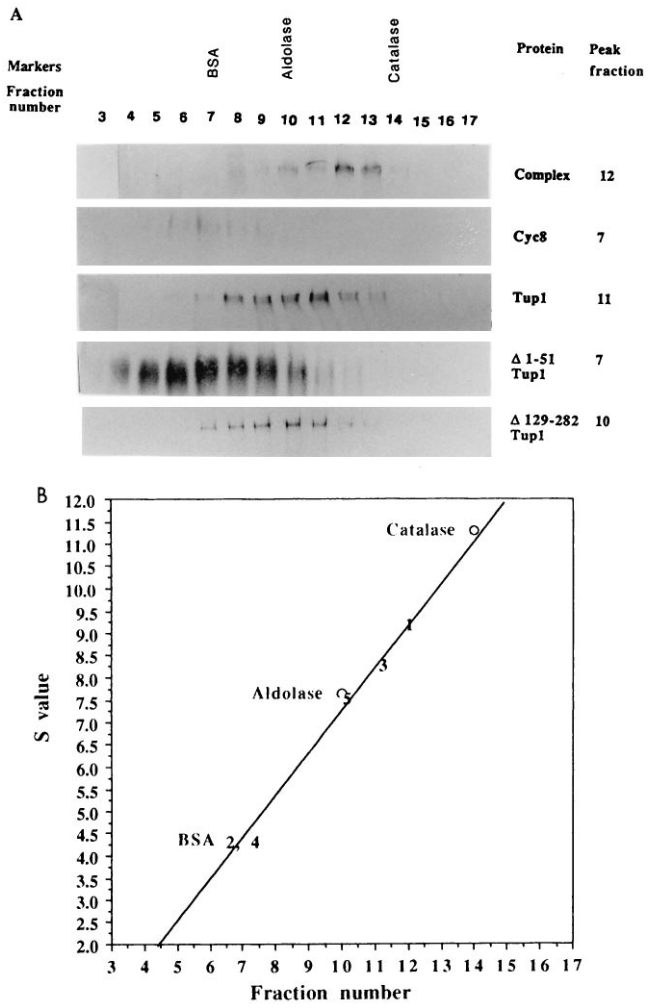

FIG. 5. Sedimentation coefficients (A) Profiles of test proteins. 1, complex; 2, Cyc8; 3, Tup1; 4, Tup1Δ1-51; 5, Tup1Δ129-282. Sedimentation coefficients were determined by density gradient centrifugation in 4.5 ml of 5 to 20% linear sucrose gradients per tube for 12 h. The samples were yeast cell extracts containing 240 to 660 μg of protein, prepared from the strains used for Fig. 4. The fractions were collected from the top by pipetting and were analyzed by Western blotting using affinity-purified Tup1 antibodies or Cyc8 antiserum (for Cyc8 and the complex). Peak positions of the markers are indicated. Markers BSA (67 kDa, 4.3S), aldolase (158 kDa, 7.35S), and catalase (232 kDa, 11.3S) were centrifuged in parallel in a separate tube and were detected by Coomassie blue staining. (B) Plot of sedimentation coefficients versus fraction numbers. Details are given in Materials and Methods.

The sedimentation coefficients of the complex and its constituents were determined by sucrose gradient centrifugation (Fig. 5A) and interpolation with the sedimentation coefficients of protein standards (Fig. 5B). Using the experimentally determined values for the Stokes radius and sedimentation coefficient, and the partial specific volume estimated from the amino acid composition (6), we estimated the native molecular masses of the complex, Cyc8, Tup1 oligomer, and Tup1 N-terminal mutants as described by Siegel and Monty (37) (Table 1). The molecular masses of the complex, free Cyc8, and free Tup1 are 437, 100, and 306 kDa, respectively. The calculated molecular mass of Cyc8 of 100 kDa is close to the size predicted from the amino acid sequence (107 kDa), suggesting that in the absence of Tup1, Cyc8 exists as a monomer. For Tup1, the calculated mass is 306 kDa, compared to 78 kDa, the size of the monomer predicted from the amino acid sequence. The ratio of these two numbers is 3.92, suggesting that Tup1 exists as a tetramer in the absence of Cyc8. The molecular mass of the complex, 437 kDa, is close to the sum (100 + 306 kDa) of the masses of free Cyc8 and Tup1. These data suggest that the complex is simply the combination of free Cyc8 and Tup1 and thus consists of one Cyc8 subunit and four Tup1 subunits.

Tup1 deletion mutants found previously to strongly affect electrophoretic mobility on native gels were also characterized (45a). Tup1Δ1-51 had greatly increased mobility and did not associate with Cyc8. Tup1Δ129-282 had some increase in mobility but still associated with Cyc8. These Tup1 deletion mutants were expressed in yeast cells in the absence of Cyc8 to directly determine the oligomeric state of Tup1. The Stokes radii and sedimentation coefficients of these proteins were determined (Fig. 4 and 5) and used to estimate their molecular parameters (Table 1). The molecular mass of Tup1Δ1-51 was estimated to be 96.2 kDa, suggesting that this species is a monomer. This finding is consistent with previous results showing that the N terminus of Tup1 is required for its oligomerization (45, 45a). Tup1Δ129-282 had an estimated mass of 208 kDa (Table 1), approximately 3.3 times the predicted monomeric size of 62 kDa. This deletion mutant protein may be a trimer, or allowing for experimental error, it could be a tetramer as is the wild-type Tup1.

We have calculated frictional ratios, f/f_0, of the complex, Cyc8, Tup1 proteins, and Tup1 mutants as described by Siegel and Monty (37). The frictional ratios reveal unusually high values for all of the proteins (Table 1), suggesting that they may be asymmetric.

DISCUSSION

We originally identified the Cyc8-Tup1 complex by nondenaturing gel electrophoresis as a species with an apparent size of 1,200 kDa (48). In this work, we determined the composition of the complex by using multiple experimental approaches. All of these methods yielded results consistent with a composition of one Cyc8 subunit and four Tup1 subunits per complex.

This result is surprising from several aspects. The large apparent size of the complex suggested that proteins other than Cyc8 and Tup1 might be present. Cyc8-Tup1 is believed to function by interaction with other proteins, including repressors, transcription factors, and histones, which might have been essential parts of the complex. Our results suggest that the interaction between the complex and these other proteins is transitory or not maintained during preparation of cell extracts. It is possible that some of these interactions are maintained, but these other proteins are present in minor substoichiometric amounts. Although we have usually overexpressed both Cyc8 and Tup1 to facilitate characterization of the complex, the same complex is the major species from nonoverexpressing cells (48). The complex behaves consistently when separated by gel electrophoresis, gel filtration, or sucrose gradient sedimentation. The biological significance of the complex is supported by genetic studies. Null mutations in *CYC8* or *TUP1* have nearly identical phenotypes, suggesting that the complex itself is the functional entity.

Another surprising feature of the complex is the 1:4 ratio of Cyc8 to Tup1 subunits. The subunits in heteromeric transcriptional regulatory proteins are usually in a 1:1 ratio. The surplus of Tup1 over Cyc8 subunits may reflect their different functional roles. Tup1 is believed to directly mediate repression by contacting elements of the transcriptional apparatus and also perhaps nucleosomes. Cyc8 is primarily responsible for interaction with the DNA-bound repressor proteins. This model is

TABLE 1. Physical parameters of the complex, Cyc8, Tup1, and Tup1 mutants

Mutant	Partial specific vol[a]	Sedimentation coefficient[b] (10^{13})	Stokes radius[c] (Å)	f/f_0[d]	Axial ratio, a/b[e]	Molecular mass (kDa)		
						Predicted[f]	Apparent[g]	Corrected[d]
Complex	0.723	9.2S	116	2.32	27	186	1480	437
Cyc8	0.720	4.25S	58	1.91	17	107	304	100
Tup1	0.725	8.25S	90	2.01	20	78	834	306
Tup1Δ1-51	0.725	4.25S	55	1.82	15	71	270	96.2
Tup1Δ129-282	0.726	7.5S	67	1.72	13	62	445	208

[a] Calculated from the predicted amino acid composition (6).
[b] Determined by isokinetic centrifugation in 5 to 20% sucrose gradients.
[c] Determined by PGGE.
[d] Frictional coefficients were calculated from the equation $f/f_0 = R_s/(3\upsilon M/4\pi N)^{1/3}$, where N is Avogadro's number, υ is partial specific volume, R_s is Stokes radius, and S is sedimentation coefficient (37), and corrected masses (M) were calculated from the equation $M = 6\pi\eta_l N R_s S/(1 - \upsilon\rho)$.
[e] Axial ratios for prolate ellipsoids were determined from a standard curve as a function of f/f_0 (4).
[f] Calculated from the amino acid sequence, considering the complex to be a heterodimer and the individual proteins to be monomers.
[g] Determined by PGGE.

supported by deletions of different TPR domains of Cyc8 that differentially affect the ability to repress genes regulated by the different repressor proteins (45). Also, repression mediated by LexA-Cyc8 fusion proteins requires Tup1, but not vice versa, indicating that Tup1 is the "business end" of the complex (20, 44). In the absence of Tup1, Cyc8 behaves as a monomer (Table 1), although its apparent size by native gel electrophoresis previously suggested it could be a multimer (48). The TPR domains of Cyc8 and other proteins were proposed to form coiled coils capable of dimerization (11). The TPR domains of Cdc16, Cdc23, and Cdc27 can mediate homomeric and heteromeric interaction between these proteins (25). The TPR domains of Cyc8 do not appear to foster dimerization under our experimental conditions.

Oligomerization of Tup1 is not absolutely necessary for repression. Deletions of the N terminus of Tup1 prevent its oligomerization but also eliminate association with Cyc8 (44, 45a). These N-terminal Tup1 deletions can still repress transcription in situations where association with Cyc8 is not required. For example, LexA-Tup1 fusions lacking the N terminus required for oligomerization were competent for repression, since binding to DNA does not require mediation of Cyc8 and repressor (44). Interestingly, repression by full-length LexA-Tup1 was higher in cells expressing chromosomal Tup1, while repression by N-terminally deleted LexA-Tup1 was not affected by chromosomal Tup1. These results suggest that the chromosomally encoded Tup1 associates with LexA-Tup1 resulting in increased repression, and this interaction does not take place with the LexA-Tup1 lacking the N terminus. Association with Cyc8 is also not required for repression by Matα2, since Tup1 interacts directly with this repressor (22). Mating defects in Δ*ssn6* Δ*tup1* double mutants were suppressed by overexpression of either full-length Tup1 or Tup1(336-713) (22). We have found that Tup1Δ1-51, a deletion mutant unable to associate with Cyc8, could repress *MFA2* (repressed by Matα2) but not *SUC2* (repressed by Mig1) (45a). These results imply that Cyc8 has an accessory role to Tup1 in repression of genes regulated by mating type. Recently the TPR domains of Cyc8 were shown to associate with the homeodomain of Matα2 (38).

Our results directly demonstrate that deletion of the N terminus of Tup1 converts it from a tetramer to a monomer. Previous studies using more indirect methods including column chromatography and the two-hybrid system have shown that the N terminus can mediate self-association of Tup1 (45). The N terminus of Tup1 contains regions highly likely to form coiled coils, which may be implicated in oligomerization (45a). The role of the Tup1 N terminus of Tup1 in association with Cyc8 is not understood, since all reported N-terminal deletions eliminate Tup1 multimerization and association with Cyc8. Oligomerization of Tup1 could be prerequisite for association with Cyc8, or there could be distinct interaction domains, both of which are removed in the known deletions.

The multiple Tup1 subunits could make the complex a more efficient repressor in several ways. The different Tup1 subunits of a single complex could contact multiple targets to mediate repression, including one or more elements of the transcription apparatus and histones in the nucleosomes. Multiple subunits might allow the complex to make effective contact with targets when assuming different orientations under the constraints imposed at different promoters. In the case of genes under mating-type regulation, one Tup1 subunit of the complex could contact Matα2, while another Tup1 subunit could contact the transcriptional apparatus.

The overestimation of the apparent molecular weights of the complex and its constituents can be explained by their high frictional coefficients. The frictional ratio, f/f_0, represents deviation of the hydrodynamic behavior of the protein particle from that of a sphere, which has an f/f_0 of 1.0 (4). The complete complex had the highest f/f_0 ratio, 2.32, but all of the forms of Cyc8 and Tup1 had ratios much greater than 1.0 (Table 1), signifying appreciable deviations from a spherical shape. A sample of nine globular proteins had an average f/f_0 of 1.22 ± 0.075, whereas a sample of four proteins considered to be fibrous had an average f/f_0 of 3.97 ± 1.79 (39). The f/f_0 for the complex falls in the lower range for fibrous proteins, similar to that of fibrinogen (2.34). The frictional coefficient can be related to molecular shape by considering the molecules as ellipsoids of revolution. A theoretical curve was used to relate the frictional coefficients of Cyc8-Tup1 and its components to the axial ratios (a/b) of a hypothetical prolate ellipsoid (Table 1). The complete complex had the highest axial ratio of 27, indicative of a highly asymmetric protein. The elongated shape of the complex may allow it to more easily make contact with proteins bound to different sites on the chromosome, for example, between repressors and the transcriptional apparatus. Several other transcriptional regulatory proteins, including GA-binding protein (40) and heat shock transcription factor (46), have been shown to be highly asymmetric, using methods similar to those employed in this paper. Standard methods had originally led to greatly inflated estimates of the molecular weights of these two proteins, as in the case of Cyc8-Tup1. These examples suggest that a characterization of the hydrodynamic properties of other transcription factors may reveal that pronounced asymmetry is a common feature with important functional implications.

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