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A yeast TCP-1-like protein is required for actin function *in vivo*

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ABSTRACT **We previously identified the *ANC2* gene in a screen for mutations that enhance the defects caused by yeast actin mutations. Here we report that *ANC2* is an essential gene that encodes a member of the TCP-1 family. TCP-1-related proteins are subunits of cytosolic heteromeric protein complexes referred to as chaperonins. These complexes can bind to newly synthesized actin and tubulin *in vitro* and can convert these proteins into an assembly-competent state. We show that *anc2-1* mutants contain abnormal and disorganized actin structures, are defective in cellular morphogenesis, and are hypersensitive to the microtubule inhibitor benomyl. Furthermore, overexpression of wild-type Anc2p ameliorates defects in actin organization and cell growth caused by actin overproduction. Mutations in *BIN2* and *BIN3*, two other genes that encode TCP-1-like proteins, also enhance the phenotypes of actin mutants. Taken together, these findings demonstrate that TCP-1-like proteins are required for actin and tubulin function *in vivo*.**

TCP-1 (t-complex polypeptide 1) is a subunit of eukaryotic cytosolic chaperonins. It has sequence homology to the subunits of bacterial (GroEL/hsp60), plastid, and mitochondrial (chaperonin 60) chaperonins (1, 2), protein complexes that facilitate protein folding in cells (3–5). The structure of the cytosolic chaperonin, a two-layered torus composed of multiple subunits (6–8), is similar to that of the bacterial, mitochondrial, and plastid chaperonins, but the subunit composition is more complex. Recent studies have identified distinct families of TCP-1-related proteins, referred to as CCT (chaperonin containing TCP-1) proteins (9). Within a family, the CCT proteins share ≈60% sequence identity across species lines, while between families the identity is only ≈30%, even within a single species such as mouse or yeast.

TCP-1-containing chaperonins from rabbit, mouse, and bovine tissues can facilitate the folding of newly synthesized actin and tubulin (8, 10, 11) as well as actin-related and tubulin-related proteins (12). In addition, in CHO cell lysates, tubulin and a 43-kDa protein that comigrates with actin on polyacrylamide gels are bound to chaperonins (13). Genetic evidence for the functional importance of the chaperonin–tubulin interaction *in vivo* has been obtained in budding yeast (14), but similar evidence pertaining to actin has not yet been reported. Also, while seven CCT families have been identified in vertebrates, the extent to which all of these families are conserved across species lines has not been fully explored. Functional tests of the different family members in a biologically relevant context are required to address issues such as whether the different family members have similar functions.

Genes that encode a CCTα and a CCTβ have been identified in budding yeast (14, 15), and their gene products have been shown to be components of the same complex (15). Mutations in either gene cause defects in microtubule functions, particularly in proper segregation of nuclei. We report here the identification of a CCT protein in yeast, the product of the *ANC2* (actin-noncomplementing) gene.* *ANC2* was previously identified as an extragenic mutation that exacerbates the phenotype of a temperature-sensitive actin (*act1*) mutant (16). Interactions between mutant alleles of the *ANC2* gene and genes that encode actin and actin-binding proteins strongly suggest that Anc2p is involved in actin function (17). We show here that Anc2p is essential for cell viability and is required for actin function in living cells. Concurrent with our studies, mutations in two additional yeast genes, *BIN2* and *BIN3*, were also found to disrupt the actin and microtubule cytoskeletons (18). Together, these data demonstrate that multiple CCT proteins contribute to tubulin and actin function *in vivo*.

MATERIALS AND METHODS

Cloning and Sequencing. A DNA fragment that complements the temperature-sensitive phenotype of *anc2-1* cells was isolated from a centromere-based plasmid (YCp50) yeast genomic library (19). For sequencing, a 2.7-kb fragment containing the *ANC2* gene was first subcloned into pRS316 (20) to create pDV211. A nested deletion series was created from pDV211 by using *Exo* III and was used for sequencing the sense strand of *ANC2* (nt 1–2727; Biomolecular Resource Center sequencing facility, University of California, San Francisco). The antisense strand of *ANC2* was sequenced from nt 2307–439 by using oligonucleotide primers.

***ANC2* Deletion and Genetic Linkage Analysis.** The *ANC2* null allele was generated by replacing nt 530–2305 (*Eco*NI/*Hpa* I fragment), which include the entire chromosomal *ANC2* coding sequence plus 194 bp upstream, in two different wild-type diploid strains (DDY426 and DDY665) with the integrating plasmid pDV219, which contains the *LEU2* gene with *ANC2* flanking sequences at the 5′ (*Bam*HI/*Eco*NI; 530 bp) and the 3′ (*Hpa* I/*Bam*HI; 435 bp) ends. A total of 60 tetrads derived from six independent transformants for both parental strains were dissected and segregation of 2 viable:2 inviable spore colonies was observed for each tetrad. In all cases, viable colonies were Leu$^-$. Linkage analysis confirmed that the *ANC2* gene was cloned. *anc2-1*/*ANC2* heterozygotes were transformed with pDV219 to disrupt the *anc2-1* or *ANC2* locus, and the resulting tetrads were dissected (60 tetrads). Again, 2 viable:2 inviable spore segregation was observed and viable spores were never found to be Leu$^+$. All viable spores were Ts$^+$, suggesting that integration occurred only at the *anc2-1* locus and there was selective pressure against recovery of *anc2-1*/*anc2*Δ diploids. Integration at the *ANC2* locus was also analyzed by Southern hybridization of genomic DNA from diploid strains. In addition to a complete open reading frame encoding Anc2p (nt 724–2307), a partial open reading frame encoding 150 amino acids was identified on the antisense strand (nt 1–450). To eliminate the possibility that the lethality of the *ANC2* deletion results from disruption of the neighboring gene, we demonstrated that a plasmid containing only the *ANC2* coding sequence plus 255 bp upstream (pDV218; *Xba* I/*Xba* I 2.3-kb fragment from pDV211 subcloned into pRS316) could

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*The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z33504).

suppress the lethality of *anc2Δ::LEU2*. *anc2Δ::LEU2/ANC2* cells were transformed with pDV218 and tetrads were dissected. In 30 tetrads exhibiting four viable spores, two Leu$^+$ versus two Leu$^-$ spore segregation was observed. When tested for plasmid-dependent growth, 66 of 66 Leu$^+$ (*anc2Δ*) spores failed to grow in the absence of pDV218. These results demonstrate that the *anc2Δ::LEU2* disruption is lethal because it abolishes *ANC2* gene function.

Viability, Morphology, and Fluorescence Studies. In viability studies, diploid wild-type (DDY665) and *anc2-1* mutant (DDY666) cells were plated onto rich (yeast extract/peptone/dextrose) medium and allowed to form colonies for 3 days at 25°C. Aliquots of cells from the same cultures were fixed with formaldehyde (13.4%), and the number of cells per ml was determined with a Coulter Counter. Percentage viability was determined by dividing the number of colonies formed by the total number of cells that were plated. In both morphology and immunofluorescence studies, cells grown to early logarithmic phase (≈2 × 10^6 cells per ml) were fixed in formaldehyde and sonicated to disrupt clumps. An aliquot of fixed cells was mounted on a glass slide to analyze cell morphology by phase microscopy, and the remaining cells were processed for actin immunofluorescence and nuclear staining with 4′,6-diamidino-2-phenylindole as described (21).

***ACT1* Overexpression.** Haploid wild-type (DDY186) or *anc2-1* mutant (DDY299) cells were doubly transformed with a *URA3* plasmid and a *LEU2* plasmid (see below), and the plasmids were selected for by plating on minimal synthetic dextrose (SD) medium. Stable transformants were tested for growth by either streaking to single colonies or spotting with a multipoint inoculator on SD medium and allowed to grow at different temperatures. Plasmids used include control plasmids without inserts (pRS316 and pRS425) (20), *ACT1–CEN* plasmid (pKFW29) (17), and *ANC2* 2-μm plasmid (pDV220 and pDV222; the *ANC2*-containing 2.7- and 2.3-kb fragments, respectively, isolated from pDV211 were cloned into pRS425).

RESULTS

***ANC2* Sequence and Chromosomal Deletion.** The *ANC2* gene was cloned by isolating a genomic DNA fragment that

A

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Yeast Anc2p    MSAKVPS.............NATFKNKEK..PQEVRKANIIAARS   45
Mouse Cctdp    MPENVASRSGAPTAGPGSRGKSAYQDRDK..PAQIRFSNISAAKA
Yeast Tcp1p              MSQLFNNSRSDTLFLGGEKISGDDIRNQNVLATMA

  *****    * *   *                  **        *     *

VADAIRTSLGPKGMDKMIKTSRGEIIISNDGHTILKQMAILHPVARMLVEVSAAQDSEAG  105
VADAIRTSLGPKGMDKMIQDGKGDVTITNDGATILKQMQVLHPAARMLVELSKAQDIEAG
VANVVKSSLGPVGLDKMLVDDIGDFTVTNDGATILSLLDVQHPAGKILVELAQQQDREIG

                        *  *          *           *   *

DGTTSVVILTGALLGAAERLLNKGIHPTIIADSFQSAAKRSVDILLE.MCHKVSLSDREQ  165
DGTTSVVIIAGSLLDSCTKLLQKGIHPTIISESFQKALEKGLEILTD.MSRPVQLSDRET
DGTTSVVIIASELLKRANELVKNKIHPTTIITGFRVALREAIRFINEVLSTSVDTLGKET

              **                                *     *     *

LVRAASTSLSSKIVSQYSSFLAPLAVDSVLKISD.ENSKNVD..LNDIRLVKKVGGTIDD  225
LLNSATTSLNSKVVSQYSSLLSPMSVNAVMKVIDPATATSVD..LRDIKIVKKLGGTIDD
LINIAKTSMSSKIIGADSDFFSNMVVDALLAVKTQNSKGEIKYPVKAVNVLKAHGKSATE

       *  *        * *     *   **      *       *       *     *

TEMIDGVVLTQTAIKSAGGPTRK..EKAKIGLIQFQISPPKPDTENNIIANDYRQMDKIL  285
CELVEGLVLTQKVANS..GITRV..EKAKIGLIQFCLSAPKTDMDNQIVVSDYAQMDRVL
SLLVPGYALNCTVA.SQAMPKRIAGGNVKIACLDLNLQKARMAMGVQINIDDPEQLEQIR

  *   *  *                          *       * * *       **  *  *

KEERAYLLNICKKIKKAKCNVLLIQKSILRDAVNDLALHFLSKLNIMVVKDIEREEIEFL  345
REERAYILNLVKQIKKTGCNVLLIQKSILRDALSDLALHFLNKMKIMVVKDVEREDIEFI
KREAGIVLERVKKIIDAGAQVVLTTKGI.....DDLCLKEFVEAKIMGVRRCKKEDLRRI

                        *  *          *           *

SKGLGCKPIADIELF.TEDRLGSA......DLVEEIDSDGS.KIVRVTGIRNNNARPTVS  405
CKTIGTKPVAHIDQF.TADMLGSA......ELAEEVSLNGSGKLFKITGC..TSPGKIVT
ARATGATLVSSMSNLEGEETFESSYLGLCDEVVQAKFSDDECILIKGTSKHSSS.....S

  *                       *** **   **      * *     *     *   *

VVIRGANNMIIDETERSLHDALCVIRCLVKERGLIAGGGAPEIEISRRLSKEARSMEGVQ  465
IVVRGSNKLVIEEAERSIHDALCVIRCLVKKRALIAGGGAPEIELALRLTEYSRTLSGME
IILRGANDYSLDEMERSLHDSLSVVKRTLESGNVVPGGGCVEAALNIYLDNFATTVGSRE

          *        *  * *       *         *                * *

AFIWQEFASALEVIPTTLAENAGLNSIKVVTELRSKHENGELND............GISV  525
SYCVRAFADAMEVIPSTLAENAGLNPISTVTELRNRHAQGEKTT............GINV
QLAIAEFAAALLIIPKTLAVNAAKDSSELVAKLRSYHAASQMAKPEDVKRRSYRNYGLDL

       *   *   *   * *

RRSGTTNTYEEHILQPVLVSTSAITLASECVKSILRIDDIAFSR..........      (528)
RKGGISNILEEMVVQPLLVSVSALTLATETVRSILKIDDVVNTR..........      (539)
IRGKIVDEIHAGVLEPTISKVKSLKLALEACVAILRIDTMITVDPEPPKEDPHDH     (559)
```

B

	Mouse Cctδp	Yeast Bin3p	Mouse Cctαp	Yeast Tcp1p	Yeast Bin2p
Yeast Anc2p	59.4	32.1	32.0	31.0	27.9
Mouse Cctδp		32.9	32.8	33.2	32.6
Yeast Bin3p			35.3	35.1	28.4
Mouse Cctαp				61.9	29.8
Yeast Tcp1p					31.9

FIG. 1. Anc2p sequence and sequence comparisons. (*A*) Alignment of the Anc2p, mouse Cctδp, and yeast Tcp1p protein sequences. Identical residues are indicated in black. Asterisks indicate residues that are identical between Anc2p and mouse Cctδp but that differ from residues highly conserved among yeast, human, mouse, and *Drosophila* Tcp1p (Cctαp) sequences. Numbers on each row indicate residue positions (including gaps) in the Anc2p sequence, and numbers at the end of each sequence indicate the total number of residues in each protein. Alignments were made using the PILEUP computer program (Genetics Computer Group software). (*B*) Percentage identity determined for pairwise sequence comparisons using the GAP computer program (Genetics Computer Group software). For yeast Bin2p and Bin3p sequences, see ref. 18.

complements the temperature-sensitive phenotype of the *anc2-1* mutant. Sequence analysis revealed that the *ANC2* gene encodes a protein with a predicted molecular weight of 58,080 (528 amino acids; Fig. 1*A*). By searching protein sequence data bases, Anc2p was found to be homologous throughout its entire sequence (≥28% identical) to members of the TCP-1 family (Fig. 1). A new nomenclature, CCT for chaperonin containing TCP-1, has recently been proposed for this family of cytoplasmic chaperonin proteins (9) (with this nomenclature, Tcp1p is called Cctαp). This family includes Bin2p and Bin3p, two yeast proteins identified in a screen for mutants defective in chromosome segregation (18). Bin3p was independently identified as a yeast Cctβp in connection with the yeast genome sequencing project (ref. 15; T. Huffaker, personal communication). While two pairs of TCP-1-related proteins, yeast (14) and mouse (22) Tcp1p (Cctαp), and yeast Anc2p and mouse Cctδp (9) are ≈60% identical to each other across species lines, the yeast proteins Anc2p, Bin2p, Bin3p, and Tcp1p are only ≈30% identical to each other (Fig. 1*B*). These observations suggest that each yeast protein is a member of a different subfamily of TCP-1-like proteins. Fig. 1*A* also indicates residues (asterisks) that are identical between Anc2p and mouse Cctδp but that differ from residues highly conserved in the Cctαp family, including yeast Tcp1p.

An *ANC2* null allele has a recessive lethal phenotype (see *Materials and Methods*), demonstrating that Anc2p is essential for cell viability. *BIN2* and *BIN3* have also been shown to be essential genes (18). Overexpression of *BIN2*, *BIN3*, or *ANC2* cannot suppress the temperature-conditional lethality of mutations in either of the other two genes (data not shown; see ref. 18).

Actin-Associated Phenotypes of *anc2-1* Cells. Because a mutation in *ANC2* enhances the phenotype of actin mutations, we determined whether the *anc2-1* mutation causes defects in the actin-dependent process of bud formation. The prevalence of unbudded *anc2-1* cells and the viability of these cells were assessed at the nonpermissive temperature of 35°C (Table 1). After 4 hr at 35°C, the viability of *anc2-1* cells is high, and unbudded cells (61% compared to 40% for wild-type cells) accumulate. The proportion of unbudded cells increases to 84% after 6 hr. About 1% of *anc2-1* cells grown at the permissive or nonpermissive temperature are abnormally large and multinucleate (data not shown), and 5–10% are normal in size but appear pear-shaped (Fig. 2*Ad*) rather than ellipsoid like wild-type cells (Fig. 2*A a* and *b*). Finally, between 0.5% and 2.2% of the mutant cells grown at the permissive (Fig. 2*A c*, *e*, and *f*) or the nonpermissive (data not shown) temperature have more than one bud. These phenotypes are similar to those reported for actin mutants (23).

Table 1. Phenotypes of *anc2-1* mutants

	Time, hr	% cells with phenotype			
		Wild type		*anc2-1*	
		25°C	35°C	25°C	35°C
Viability	0	100	100	100	100
	4	100	100	100	88
	6	100	100	74	30
Unbudded	0	44.9	44.9	42.3	42.3
	4	38.8	40.4	33.5	61.1
	6	ND	34.2	38.4	83.5
Delocalized actin in unbudded cells	0	ND	12.5	ND	6.0
	4	ND	19.8	ND	82.6

Aliquots of cells were collected at different times after shifting from 25°C to 35°C and either plated onto rich medium for viability determination or fixed and processed for morphology and immunofluorescence studies. At least 200 cells were analyzed for each entry. ND, not determined.

Actin organization in *anc2-1* cells was analyzed by immunofluorescence microscopy. In wild-type cells and in *anc2-1* cells grown at the permissive temperature, cortical actin patches concentrate at one end of unbudded cells to mediate bud formation (Fig. 2*Bg*). In *anc2-1* cells grown at the nonpermissive temperature, cortical actin structures are scattered over the surface of unbudded cells (Fig. 2*Bi*). The proportion of unbudded *anc2-1* cells exhibiting delocalized actin patches increases as unbudded cells accumulate at the nonpermissive temperature (Table 1), suggesting that the failure of *anc2-1* cells to form buds results from a failure to organize cortical actin structures.

***In Vivo* Interactions of Anc2p and Actin.** At the permissive and nonpermissive temperatures, ≈2% of the *anc2-1* cells contain abnormal actin bars randomly oriented in the cytoplasm (Fig. 2*Bl*; Table 2). In addition, we report here that 15–18% of wild-type cells containing the actin gene on a centromere-based (low copy number) plasmid exhibit similar actin bars. The increased actin dosage also makes wild-type and *anc2-1* yeast cells grow poorly (Fig. 2*C*). Because diminished Anc2p function can cause actin bar formation, we tested whether elevation of Anc2p levels can suppress the actin bar phenotype in cells with elevated actin levels. Increasing the dosage of *ANC2* by expression on a high-copy-number plasmid (2-μm based) has no effect on wild-type cells but improves the growth of *anc2-1* cells. Moreover, high copy number of *ANC2* suppressed both the growth defects and actin bar phenotype caused by increased *ACT1* dosage in wild-type and *anc2-1* cells (Fig. 2*C*; Table 2). Transformations with control plasmids lacking the *ACT1* or *ANC2* inserts demonstrated that the effects on cell growth and actin organization reported above depended on the *ACT1* and/or *ANC2* genes (Fig. 2*C*; Table 2).

Other Cytoskeletal Defects. Because different TCP-1-related proteins have been biochemically implicated in actin and tubulin function, we also examined microtubule organization and nuclear segregation in *anc2-1* cells. At both the permissive and nonpermissive temperatures, microtubule structures appear normal in *anc2-1* cells, but ≈2.1% of cells (compared to <0.1% for wild type) have two or more nuclei (Fig. 2*Bk*), and the mitotic spindle is often aligned randomly rather than spanning the bud neck in budded cells undergoing nuclear division (data not shown). In addition, growth of *anc2-1* cells is inhibited at 20°C by the anti-microtubule drug benomyl (5 μg/ml), about one-half the concentration needed to arrest wild-type cells (data not shown). The *anc2-1* mutants showed normal sensitivity to cycloheximide, indicating that the benomyl sensitivity is not due to nonspecific defects in membrane permeability.

Finally, we also determined that mutations in *BIN2* and *BIN3*, genes implicated in microtubule function and actin function (18), enhance the phenotype of actin mutants. Both *bin2-1* and *bin3-1* mutants, like *anc2-1* mutants, failed to complement the *act1-4* mutation but complemented *act1-1* (data not shown).

DISCUSSION

Our data and the data of Chen *et al.* (18) demonstrate that three yeast TCP-1-related proteins are critically important for actin function *in vivo*. The fact that mutations in *ANC2* and *ACT1* (the yeast actin gene) cause similar defects (delocalized actin patches and defective budding) (23, 24) indicates that actin and perhaps actin assembly are defective in *anc2-1* cells. These findings demonstrate that the biochemically identified chaperonin–actin interaction is functionally important *in vivo*. Moreover, the formation of actin bars in *anc2-1* mutants and in cells with elevated actin levels as well as the prevention of bar formation by Anc2p overproduction suggests that one function of Anc2p is to prevent actin from forming nonfunctional protein aggregates, a function that was

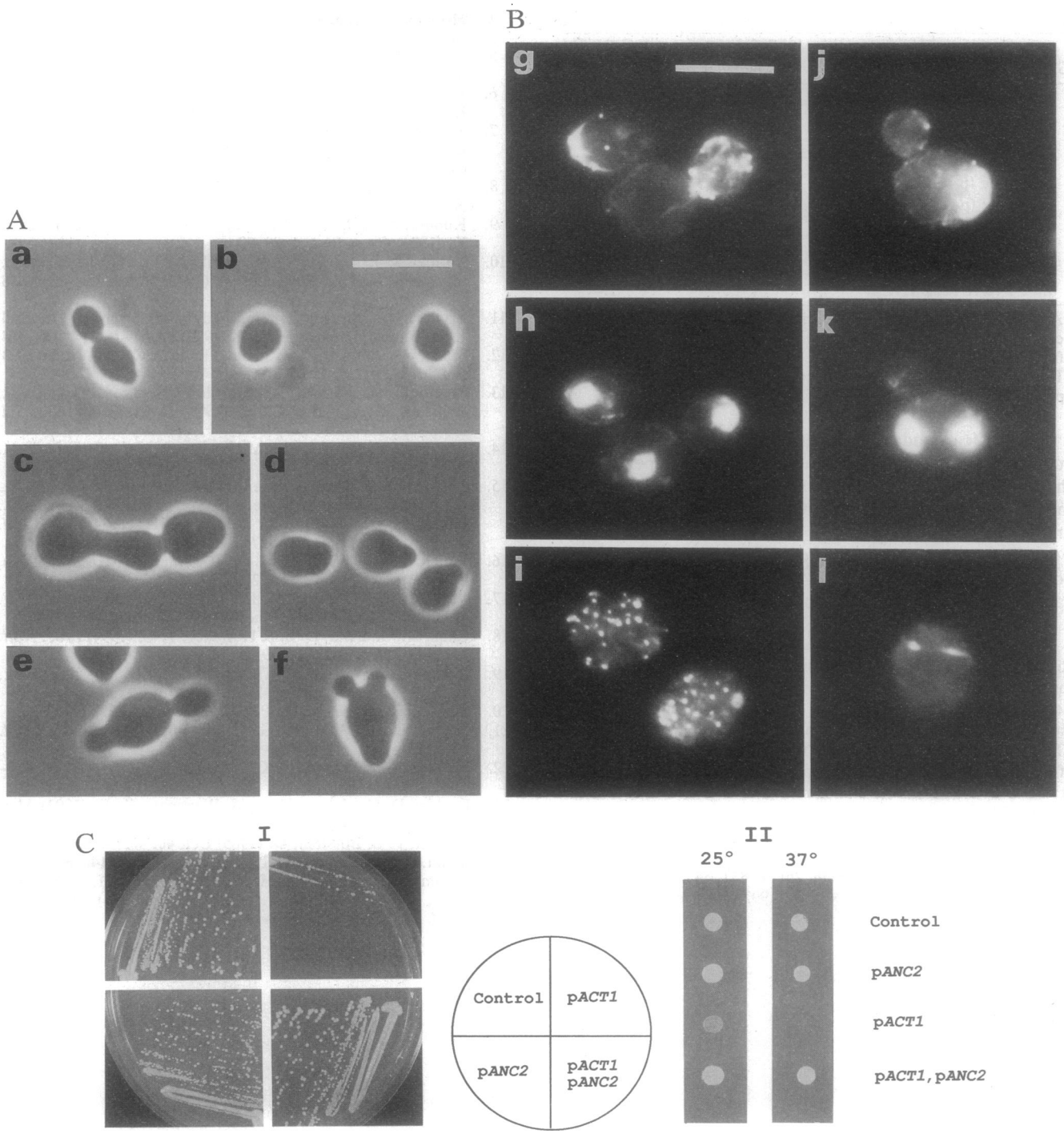

FIG. 2. Phenotypes resulting from changes in the sequence or dosage of *ANC2*. Phase-contrast (*A*) and fluorescence (*B*) micrographs of wild-type (*a* and *b*) and *anc2-1* (*c–l*) cells grown at 25°C (*c* and *e–h*) or at 36°C (*a*, *b*, *d*, and *i–l*) for 4 hr. (*g*, *i*, *j*, and *l*) Immunofluorescence using anti-actin antibodies. (*h* and *k*) 4′,6-Diamidino-2-phenylindole staining of the nuclei for the same cells shown in *g* and *j*, respectively. (Bars = 10 μm.) (*C*) (*I*) Growth at 25°C of *anc2-1* cells transformed with *ACT1* and/or *ANC2* plasmids or both control plasmids lacking *ACT1* and *ANC2* inserts, as indicated. (*II*) Growth at 25°C or 37°C of wild-type cells transformed with *ACT1* and/or *ANC2* plasmids or both control plasmids lacking *ACT1* and *ANC2* inserts, as indicated. Photographs were taken after 4 days (*I*) or 2 days (*II*).

also suggested from biochemical studies (25). Indeed, rhodamine-phalloidin, a probe specific for filamentous actin, does not stain the actin bars (D.B.-N.V., unpublished result), indicating that these bars do not consist of normal actin filaments.

The existence of four TCP-1-related proteins [Tcp1p (CCTα), Anc2p (CCTδ), Bin2p (CCTγ) (T. Huffaker, personal communication), and Bin3p (CCTβ)] in yeast raises two important questions: (*i*) Does each protein perform a similar function? (*ii*) Are the different proteins contained in a single complex? While TCP-1-related proteins identified within a species are ≈30% identical to each other, subfamilies of TCP-1-like proteins are 60% conserved across species lines. This high sequence conservation across species suggests conservation of function. Phenotypic studies indicate that each CCT contributes to cytoskeletal function in yeast. The fact that the *ANC2*, *BIN2*, *BIN3*, and *TCP1* genes, each of which encodes a member of a different CCT subfamily, are all essential suggests that each CCT has a distinct function, imparting a specific property to a chaperonin.

Table 2. Actin bar phenotype

Genotype (plasmid transformed)	Total cells analyzed	% cells with actin bars
Wild type (none)	>1000	0
anc2-1 (none)	>1000	2.2*
Wild type (p*ACT1*)†	201	18.4
Wild type (p*ACT1*)	111	15.3
Wild type (p*ACT1*, p*ANC2*)	263	2.7
Wild type (p*ACT1*, p*ANC2*)	165	2.4
anc2-1 (p*ACT1*)	205	24.4
anc2-1 (p*ACT1*)	208	10.6
anc2-1 (p*ACT1*, p*ANC2*)	206	1.5
anc2-1 (p*ACT1*, p*ANC2*)	207	1.4

*Average percentage for five experiments for which ≈200 cells were counted each time. Percentage of *anc2-1* cells with bars varied from 0.5% to 4.4%.

†Two independent cell transformants were analyzed for each type of plasmid transformed.

Biochemical studies in vertebrates have shown that the subunit composition of chaperonins can vary depending on the tissue source (6, 9, 11, 26). Currently, it is known that in mouse α, β, γ, and δ CCTs can all be in the same complex (9), and in yeast α and β CCTs can be in the same complex (15). Further studies are required to fully elucidate the subunit composition of cytosolic chaperonins and the contribution of each subunit to chaperonin function.

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