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CAP Is a Bifunctional Component of the *Saccharomyces cerevisiae* Adenylyl Cyclase Complex

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CAP, a protein from *Saccharomyces cerevisiae* that copurifies with adenylyl cyclase, appears to be required for yeast cells to be fully responsive to RAS proteins. CAP also appears to be required for normal cell morphology and responsiveness to nutrient deprivation and excess. We describe here a molecular and phenotypic analysis of the CAP protein. The N-terminal domain is necessary and sufficient for cellular response to activated RAS protein, while the C-terminal domain is necessary and sufficient for normal cellular morphology and responses to nutrient extremes. Thus, CAP is a novel example of a bifunctional component involved in the regulation of diverse signal transduction pathways.

RAS proteins are highly conserved in evolution (27), and homologs of the mammalian *RAS* oncogenes are found in yeasts (6, 10, 22). RAS proteins stimulate adenylyl cyclase in the yeast *Saccharomyces cerevisiae* (4, 30), but the function of RAS in other yeasts and vertebrates is not known. Mammalian RAS proteins can stimulate yeast adenylyl cyclase, but they appear incapable of stimulating vertebrate adenylyl cyclase (2, 3). We have been studying the function of RAS in yeast cells in the hope that this will lead to insights into the function of RAS in other organisms.

S. cerevisiae contains the *RAS* genes *RAS1* and *RAS2*. Their major role appears to be the control of adenylyl cyclase. Deficiencies in RAS function lead to the failure to grow, and these defects can be suppressed by activation of the cyclic AMP-dependent protein kinase (30). Activated mutant alleles of *RAS2*, such as $RAS2^{Val\text{-}19}$, lead to phenotypes essentially identical to phenotypes which result from activation of the cyclic AMP pathway (14, 25). These phenotypes include an exquisite sensitivity to heat shock and nitrogen deprivation. However, certain experiments indicate that RAS proteins may have other functions in *S. cerevisiae* as well (31), and it has never been demonstrated that RAS proteins interact directly with the adenylyl cyclase catalytic protein. To explore more carefully the nature of RAS protein interaction with yeast adenylyl cyclase, we purified a RAS-responsive adenylyl cyclase complex from *S. cerevisiae*. In this complex was a protein we called CAP, for cyclase-associated protein (8, 9). The gene encoding CAP (*CAP*) was isolated and was found to encode a protein of 526 amino acids (9). A gene identical to *CAP* was isolated independently by others (7), who called it *SRV2*. Yeast cells having disruptions in the *CAP* gene display a complex set of phenotypes: they appear unresponsive to activated $RAS2^{Val\text{-}19}$, in that this allele no longer induces heat shock sensitivity, yet they are exquisitely sensitive to nutrient extremes; they are temperature sensitive; and they show gross morphological abnormalities (7, 9). The first phenotype clearly places CAP on the RAS/adenylyl cyclase pathway, but the other phenotypes suggest that CAP has other functions. Here we report that CAP is indeed bifunctional, and we have localized these functions to separate domains on the protein. These conclusions were foreshadowed by an allele of *CAP* that we called *supC*. *supC* strains are resistant to the effects of the activated $RAS2^{Val\text{-}19}$ gene but do not display the other defects of *cap* mutant strains (9).

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MATERIALS AND METHODS

Microbial culture. Standard methods were used to introduce plasmids into the various yeast strains (26). Strains were grown at 30°C in either rich medium (YPD; yeast extract–Bacto-Peptone–dextrose), synthetic complete minimal medium (SC), or SC drop-out medium lacking an essential amino acid or nucleotide base. The drop-out media used included SC lacking leucine (SC-leu), SC lacking histidine (SC-his), SC lacking uracil (SC-ura), and SC lacking both histidine and uracil (SC-his,ura). Drop-out media were used to maintain plasmids. Growth media were prepared according to Sherman et al. (26). Yeast extract, Bacto-Peptone, Casamino Acids, and yeast nitrogen base (YNB, lacking both ammonium sulfate and amino acids) were purchased from Difco. Amino acids used in cell culture were purchased from Sigma. Yeast strains SKN34 and SKN37 were grown either on SC medium or on SC medium lacking valine (a nonessential amino acid) unless otherwise indicated. *Escherichia coli* HB101 was used for plasmid transformations and plasmid DNA preparations (17).

Yeast strains. Yeast strains SP1 (*MAT***a** *leu2 his3 ura3 trp1 ade8 can1*) and TK161-R2V (*MAT***a** *leu2 his3 ura3 trp1 ade8 can1* $RAS2^{Val\text{-}19}$) have been described previously (30), as have strains SKN34 (*Mat***a** *leu2 ura3 trp1 ade8 can1 cap*::*HIS3*), SKN37 (*Mat***a** *leu2 ura3 trp1 ade8 can1* $RAS2^{Val\text{-}19}$ *cap*::*HIS3*), and TKR1-2LS (*Mat***a** *leu2 his3 trp1 ade8* $RAS2^{Val\text{-}19}$ *ras1*::*URA3* cap^{supC}) (9). The *cap*::*HIS3* allele lacks amino acids 78 to 451 of the coding region of *CAP* and, in effect, constitutes the null allele. Strain H1393 (*Mat***a** *ura3 gcn4*::*LEU2*) is defective at the *GCN4* locus and was the generous gift of A. G. Hinnebusch. Strain JG1 (*Mat***a** *leu2 ura3 trp1 ade8 can1* $RAS2^{Val\text{-}19}$ *cap*::*HIS3*::*ADH1-cap*$^{\Delta 4}$) was constructed as described below and expresses the carboxy-terminal 236 amino acids of CAP.

Plasmids. Plasmids used in this study included these vectors: YEp13M4, a YEp-based plasmid bearing the *LEU2* selectable marker, which is identical to the vector YEpM4 described previously (20); pAD4Δ, a YEp-based plasmid

bearing the *LEU2* selectable marker and the yeast *ADH1* promoter (1); pUV2, a YEp-based plasmid, similar to YEp13M4, bearing the *URA3* selectable marker; and pHV1, a YEp-based plasmid bearing the *HIS3* selectable marker. For *CAP* expression studies, the following plasmids were used: pADH-CAP, which has the coding region of *CAP* cloned into the *Sma*I site of pAD4Δ and whose expression is under the control of the *ADH1* promoter (9); pUADH-CAP, a plasmid that has the *Sph*I fragment of pADH-CAP cloned into the *Sph*I site of pUV2 and also allows for expression of *CAP* under control of the *ADH1* promoter; and pADH-CAPΔ1 through -16, which are plasmids that have various deletion mutants of *CAP* cloned into the *Sal*I and *Sac*I sites of pAD4Δ (see below). For *CAP* deletion mutant construction, plasmid pUCAP, which has the *Sal*I-*Sac*I fragment of *CAP* cloned into the *Sal*I and *Sac*I restriction endonuclease sites of pUC118, was used. Plasmids used for disrupting the chromosomal *CAP* locus, pHSPN5 and pHADΔ4, are described below.

For assaying the yeast general control nondepressing (GCN) pathway, we used the following plasmids: p238, a *URA3* centromere plasmid that constitutively expresses *GCN4* (19); and p180, a *URA3* centromere plasmid which bears a gene fusion between the upstream regulatory sequence of *GCN4* and the *lacZ* gene of *E. coli* (11). Both plasmids were a gift of A. G. Hinnebusch. Other fusion plasmids used in these experiments included pAB177 and pAB93, which are *URA3* centromere plasmids bearing gene fusions between the upstream regulatory regions of *HIS4* and *HIS3*, respectively, and the *lacZ* gene. These plasmids were the gift of K. Arndt.

Phenotypic assays. To assay the ability to grow on YPD, transformed yeast strains grown on SC-leu were replica plated onto YPD plates, incubated at 30°C, and examined 48 h later. To assay for temperature sensitivity, transformed yeast strains grown on SC-leu were replica plated onto prewarmed SC-leu plates, incubated at 37°C, and examined 72 h later. To assay for heat shock sensitivity (25), transformed yeast strains were grown on SC-leu for 48 h prior to heat shock. Cells were replica plated onto preheated SC-leu plates, which were then further incubated at 55°C for various time intervals (5 to 20 min). After heat shock, plates were allowed to cool and were incubated at 30°C for 72 h. To assay for nitrogen starvation sensitivity, transformed yeast strains were patched onto SC-leu plates, allowed to grow for 2 days, and then replica plated onto YNB plates containing 2% glucose. The YNB plates were then incubated for 9 days at 30°C. After this time, replicas were made onto SC-leu plates and then incubated for another 72 h at 30°C to monitor viability (30).

To assay for amino acid toxicity, yeast strains were seeded at a density of 2×10^5 cells per ml into SC containing various amino acids. Cultures were allowed to grow for 48 to 72 h at 25°C, after which aliquots were removed to determine cell density. Cell density was determined by direct counting using a hemacytometer or by the measurement of optical density at 595 nm (OD_{595}). A blank value for the medium was subtracted from the optical density reading obtained from each sample.

To assay for defects in the GCN pathway, we first tested for the toxicity of 3-aminotriazole (3AT; 3-amino-1,2,4-triazole; Sigma). Transformed yeast strains carrying a *HIS3* selectable marker were grown as patches on synthetic selective medium prior to replica plating onto SC-his plates containing 10 mM 3AT and 5 mM leucine. The presence of an inducible *HIS3* allele is required by yeast cells to overcome the toxicity of 3AT (reviewed by Hinnebusch [12]). Excess leucine is added to enhance the toxicity in *gcn4* mutant cells. After replica plating, the plates were allowed to grow for 48 h at 30°C and were then examined. To assess the integrity of the GCN pathway, we tested plasmids bearing gene fusions between the upstream regulatory sequences of *GCN4*, *HIS4*, or *HIS3* and a reporter gene, *lacZ* (see above). In wild-type yeast cells induced with 3AT, these gene fusions express β-galactosidase, whose activity can be assayed by using a chromogenic substrate, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Boehringer Mannheim). Induction of β-galactosidase activity from the *GCN4-lacZ* fusion occurs when the GCN pathway upstream of *GCN4* is functional. Induction of β-galactosidase activity from the *HIS4-lacZ* and *HIS3-lacZ* fusions occurs when the GCN pathway is functional with respect to the *HIS4* and *HIS3* loci. Yeast strains transformed with plasmids carrying gene fusions were grown on selective medium prior to replica plating onto SC-his plates containing 40 μg of X-Gal per ml with or without added 3AT (10 mM). Yeast strains were also replica plated onto YPD plates as a control for the growth of *cap* strains. Yeast patches which turned blue as a function of β-galactosidase activity were scored as positive in their response to challenge with 3AT. For enzymatic assays, liquid cultures of the above-mentioned strains were grown in SC-his,ura. During logarithmic phase growth, 3AT was added to a final concentration of 10 mM, and culture samples were removed at 0, 4, and 8 h after addition. Cells were collected for assay by centrifugation, washed, and resuspended in 0.01 volume of ice-cold extraction buffer containing 0.1 M Tris-HCl (pH 8.0), 20% (vol/vol) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 10 μg of soybean trypsin inhibitor per ml. Cell lysates were prepared according to Rose et al. (23). β-Galactosidase assays were performed as described by Miller (18). β-Galactosidase activity measured in the various lysates was normalized for protein concentration. During culture, cell growth was monitored by optical density. After addition of 3AT, *CAP* strains continued to grow, while *cap* mutant strains showed reduced growth. No cell lysis was observed after the addition of 3AT.

DNA manipulations. DNA restriction endonucleases, *Taq* polymerase, and T4 DNA ligase were used as recommended by the suppliers (New England BioLabs, Inc.; United States Biochemical Corp.; Cetus Corp.). Molecular cloning techniques were performed as described by Maniatis et al. (17). The polymerase chain reaction (PCR) (24) was carried out as follows: DNA amplification was done in a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 1 U of *Taq* polymerase, 100 pmol of oligonucleotide primers, and 10 ng of *Sca*I-linearized pUCAP (see below) in a final volume of 50 μl. The conditions used for PCR were 30 cycles of denaturation (95°C, 1.5 min), annealing (50°C, 3 min), and extension (72°C, 5 min). The PCR products were cleaved with the appropriate restriction enzymes, size fractionated by using gel electrophoresis, electroeluted, and subcloned into pAD4Δ (see below).

Construction of deletion mutants of *CAP*. We created three types of *CAP* deletions: internal deletion mutants, 5′ truncation mutants, and 3′ truncation mutants. Mutant *CAP* genes are designated Δ*x-y*, where the nucleic acid sequence between *x* and *y*, inclusive, is removed. Numbering of the nucleic acid sequence of *CAP* is as appears in Field et al. (9). For all deletion mutants, we first subcloned *CAP* into pUC118 to create plasmid pUCAP (see above).

For internal deletions within the gene, we used existing

restriction endonuclease sites. pUCAP was cleaved with the appropriate restriction endonucleases; the ends were religated and transformed into the bacterial strain HB101. The resulting plasmids were analyzed by restriction endonuclease cleavage to verify their structures. For deletion mutant Δ505-1498 (Δ6), restriction enzymes *Eco*47III and *Sna*BI were used. For deletion mutant Δ505-1105 (Δ7), restriction enzymes *Eco*47III and *Stu*I were used. For deletion mutant Δ1105-1498 (Δ8), restriction enzymes *Stu*I and *Sna*BI were used. For deletion mutant Δ1067-1413 (Δ14), restriction enzyme *Sty*I was used. All of these deletion mutants except Δ14 maintained the reading frame of the gene. The Δ14 mutation resulted in a frameshift at bp 1067 and the creation of a termination codon 15 bases downstream. In effect, therefore, Δ14 is a 3′ truncation mutant. These mutant genes were digested with restriction endonucleases *Sal*I and *Sac*I prior to subcloning in pAD4Δ (see below for additional details).

Truncations at either the 5′ or 3′ end of *CAP* were made by using PCR. PCR amplification of mutant *CAP* genes used oligonucleotide primers complementary to the coding region of CAP and to the region flanking the polycloning site in pUCAP. For 5′ deletions, a forward oligonucleotide primer complementary to the 5′ coding region of *CAP* and a reverse oligonucleotide primer complementary to the 3′ vector sequence were used. For 3′ deletions, a forward oligonucleotide primer complementary to the 5′ vector sequence and a reverse oligonucleotide primer complementary to the 3′ coding region of *CAP* were used. Oligonucleotide primers complementary to the coding region of *CAP* contained mismatches in order to create the proper initiation or termination codons, as well as restriction endonuclease sites, in the resulting *CAP* PCR product. A *Sal*I site is found 5′ to *CAP*, and a *Sac*I site is present 3′ to *CAP* in the polycloning site of pUCAP. Forward oligonucleotide primers complementary to the coding region of *CAP* contain a *Sal*I restriction endonuclease site. Reverse oligonucleotide primers complementary to the coding region of *CAP* contain a *Sac*I restriction endonuclease site. Therefore, all PCR products contain a 5′ *Sal*I site and a 3′ *Sac*I site. The oligonucleotide primers used for creating the various 5′ and 3′ deletions in *CAP* are listed below:

To express the various deletion mutants of *CAP*, we subcloned the mutant genes into the unique *Sal*I and *Sac*I restriction endonuclease sites in the high-copy-number yeast expression vector pAD4Δ (1). This vector carries the *S. cerevisiae LEU2* auxotrophic marker and a polycloning site downstream of the *S. cerevisiae ADH1* promoter. After subcloning, the correct structures of the resulting plasmids were determined by restriction endonuclease cleavage.

Chromosomal integration of a mutant allele of *CAP*. To explore more fully the effect of expressing the C-terminal domain of CAP in yeast cells, we created a strain that stably expresses a mutant $cap^{\Delta 4}$ gene from the *CAP* locus. The integration plasmid pHADΔ4 was created by subcloning an *Eco*RV-*Sna*BI fragment of pADH-CAPΔ4, which contains the *ADH1* promoter and the $cap^{\Delta 4}$ mutant gene, into the *Sna*BI site of *CAP* in the pHSPN5 *CAP* disruption plasmid (9). Plasmid pHSPN5 contains the *CAP* gene disrupted between *Nsi*I and *Hpa*I sites with the *HIS3* gene and was used to create strains SKN34 and SKN37 as previously described (9). This new plasmid, pHADΔ4, was digested with *Eco*RI, and the resulting fragments were used to transform yeast strain TK161-R2V. When yeast cells possessing the $RAS2^{Val\text{-}19}$ allele are transformed with this plasmid, they become resistant to prolonged heat shock and nitrogen starvation. One transformant strain was designated JG1. The disruption of *CAP* in this strain was confirmed by Southern blot analysis, and expression of the $cap^{\Delta 4}$ gene product was verified by Western immunoblot analysis. We call this allele *cap::HIS3::ADH1-cap*$^{\Delta 4}$.

Western analysis of mutant *CAP* proteins. The expression of mutant *CAP* proteins was analyzed by Western blotting. Briefly, 100 to 150 ml of log-phase cultures (OD_{595} = 0.5) of *cap* SKN37 cells, transformed with various deletion mutants of *CAP*, were harvested by centrifugation. Cell pellets were washed twice in 10 mM Tris-HCl–1 mM EDTA (pH 7.0) and frozen at −70°C for later use. Thawed cell pellets (150 μl) were resuspended in 350 μl of ice-cold extraction buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), aprotinin (10 μg/ml), and 100 μM phenylmethylsulfonyl fluoride. Washed glass beads (300 μl) were then added,

Δ4-93 (Δ1)	forward:	5′-GAT TAG AGG ATG TCG ACA TGT ATC AAG AAG GTT-3′
	reverse:	5′-CAC ACA GGA AAC AGC TAT GAC CAT-3′
Δ4-300 (Δ2)	forward:	5′-TAT CAG GAA AGG TCG ACA TGG TGG TCT TAG ATG-3′
	reverse:	5′-CAC ACA GGA AAC AGC TAT GAC CAT-3′
Δ4-561 (Δ3)	forward:	5′-CCT GGG TTG CAG TCG ACA CTC CCG TGT-3′
	reverse:	5′-CAC ACA GGA AAC AGC TAT GAC CAT-3′
Δ4-870 (Δ4)	forward:	5′-CAG CTT CCG TCG ACG AAA TCT CTA ATG ATA CAC-3′
	reverse:	5′-CAC ACA GGA AAC AGC TAT GAC CAT-3′
Δ4-1068 (Δ5)	forward:	5′-AAT CCG GTC GAC CCA CAA TGC CAA AAA AGC-3′
	reverse:	5′-CAC ACA GGA AAC AGC TAT GAC CAT-3′
Δ1495-1581 (Δ11)	reverse:	5′-CAG GGA TTG GGA GCT CTA CTT AAT CAT CGT-3′
	forward:	5′-ACG ACG TTG TAA ACC GAC GGC CAG T-3′
Δ1369-1581 (Δ12)	reverse:	5′-TAT CAA TGG AGC TCT AAG GTA GGG AAT-3′
	forward:	5′-ACG ACG TTG TAA AAC GAC GGC CAG T-3′
Δ853-1581 (Δ15)	reverse:	5′-TTT CAA AGA CGG GAG CTC ATG GGG CTG GTG-3′
	forward:	5′-ACG ACG TTG TAA AAC GAC GGC CAG T-3′
Δ577-1581 (Δ16)	reverse:	5′-TCC AAA ACT GAG CTC CGT CCT AGA AAT CTG TGA-3′
	forward:	5′-ACG ACG TTG TAA AAC GAC GGC CAG T-3′

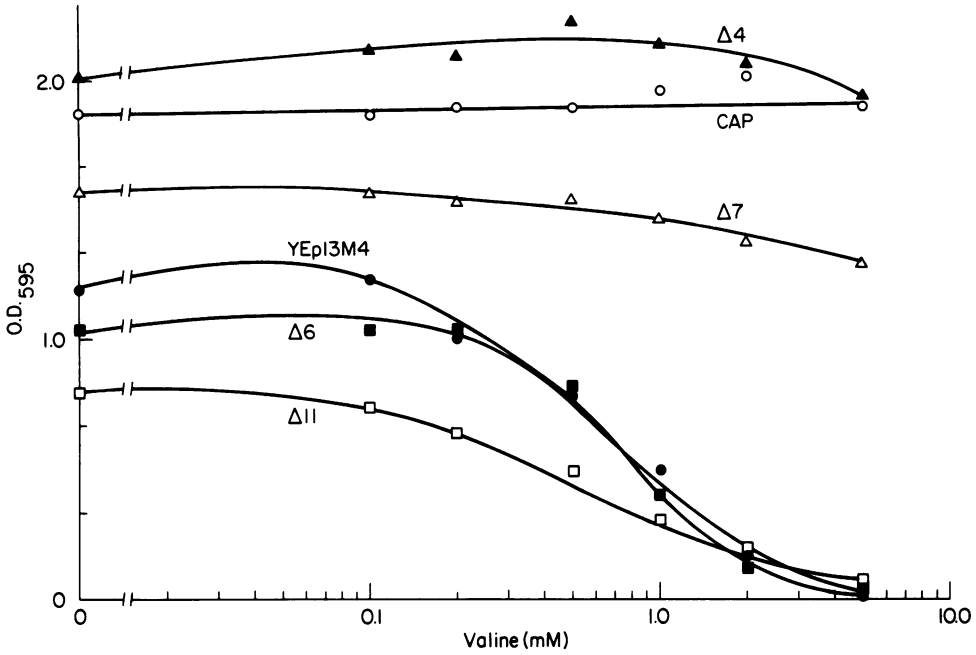


FIG. 1. Effects of increasing concentrations of valine on the growth of *cap* cells. Synthetic medium lacking valine was inoculated with SKN37 cells transformed with plasmids expressing either full-length *CAP* (CAP) or deletion mutants of *CAP* (Δ4, Δ6, Δ7, and Δ11) or with a yeast expression vector alone (YEp13M4). Valine was added in increasing concentrations (0 to 5 mM). Cultures were allowed to grow for 48 h at 25°C before measurements of optical density were taken. One optical density unit corresponds to approximately 6.0×10^6 cells per ml. The deletion mutant of *CAP*Δ4 expresses the C-terminal domain of CAP. Deletion mutants *CAP*Δ6 and -Δ11 express the N-terminal domain, while Δ7 contains both domains (see Fig. 4).

and the cells were disrupted by prolonged vortexing at 4°C (1-min intervals followed by chilling) using a Bead Beater (Biospec Products). The resulting extracts were clarified by centrifugation for 1 min at 12,000 × *g*, and the protein concentration of each sample was determined by using the BCA protein assay method (Pierce). Samples (50 to 125 μg per lane) were loaded on to 10% polyacrylamide gels prepared according to Laemmli (15). Samples were gel electrophoresed and blotted onto GeneScreen Plus membranes (New England Nuclear) as described by Peluso and Rosenburg (21). Membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS). After repeated washing of membranes in PBS containing 1.5% bovine serum albumin and 0.5% gelatin (PBG), they were incubated with polyclonal antisera (1:200 dilution) raised against full-length CAP expressed in *E. coli* (9). After the membranes were washed in PBG, they were probed with 5 μCi of ^{125}I-protein A (ICN Biomedicals), washed, dried, and autoradiographed on Kodak XAR-5 film.

RESULTS

Nutrient responses of *cap* cells. We have previously shown that disruption of the *CAP* allele, by deletion of the coding region and insertion of a selective marker gene, results in haploid yeast strains that grow in synthetic minimal medium but fail to grow in rich medium (YPD) (9). To examine this phenotype further, we separately tested yeast extract and Bacto-Peptone, two components of rich medium. For these experiments, we used the *cap* mutant strain SKN37 (see Materials and Methods for strain descriptions). When added to synthetic minimal medium at standard concentrations, both yeast extract (10 mg/ml) and Bacto-Peptone (20 mg/ml) were toxic to this strain (data not shown). This toxicity was found to be dose dependent. Both Bacto-Peptone and yeast extract are rich in amino acids. We therefore next tested the effect of Casamino Acids, an acid hydrolysate of casein, on the growth of SKN37 cells. This supplement was also toxic at concentrations that highly stimulate the growth of wild-type yeast cells. We then tested individual amino acids for toxicity by adding them to synthetic medium. Two aliphatic amino acids, valine and alanine, were found to be toxic to SKN37 cells, at concentrations which were not toxic to wild-type cells. To confirm that this phenotype resulted from the *cap* genotype, we transformed SKN37 cells with plasmids expressing either full-length or truncated *CAP*, or with vector alone, and seeded transformants in synthetic liquid medium containing increasing concentrations of valine. Growth was monitored by measuring optical density. Cells expressing full-length *CAP* grew as expected, while cells lacking *CAP* showed a concentration-dependent growth inhibition (Fig. 1). Half-maximal inhibition of the growth of *cap* cells was observed at 0.6 mM valine. The concentration of valine in YPD is approximately 10-fold higher than this (Difco amino acid analysis, unpublished). The response of cells expressing truncated *CAP* will be discussed later.

Toxicity of excess amino acids has been seen in yeast cells defective in the GCN pathway and can be attributed to indirect starvation, due to deficiencies in the synthesis of other amino acids (12). GCN4, a transcriptional regulator of various amino acid biosynthetic enzymes, is directly involved in the response to amino acid starvation in yeast cells (12). 3AT, which produces a metabolic block for the synthesis of histidine, is known to arrest the growth of *gcn4* cells, because such cells are unable to derepress transcription of the histidine biosynthetic enzymes (13). Similarly, we found

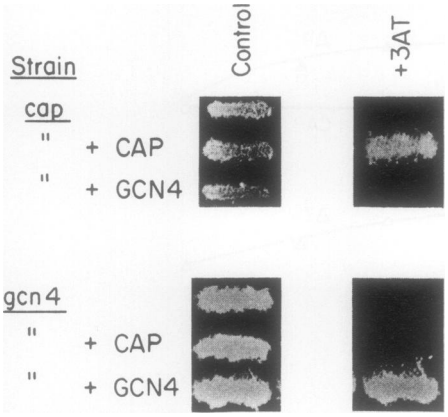


FIG. 2. Effects of CAP and GCN4 on the growth of *cap* and *gcn4* yeast strains on media containing 3AT. Strains SKN37 (*cap*) and H1393 (*gcn4*) were transformed with either a high-copy-number plasmid expressing full-length *CAP* (pUADH-CAP) or a centromere plasmid constitutively expressing *GCN4* or with yeast expression vector pUV2 (blank). All plasmids contained the *URA3* selectable marker (see Materials and Methods). Transformants were grown and plated as described in Materials and Methods. The column labeled Control represents plates containing synthetic medium lacking 3AT; the column labeled +3AT represents plates containing synthetic medium plus 10 mM 3AT.

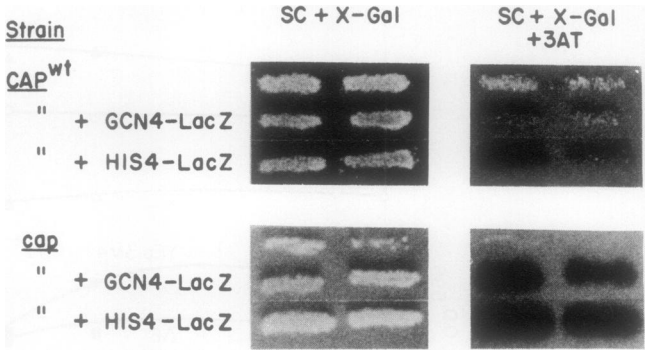


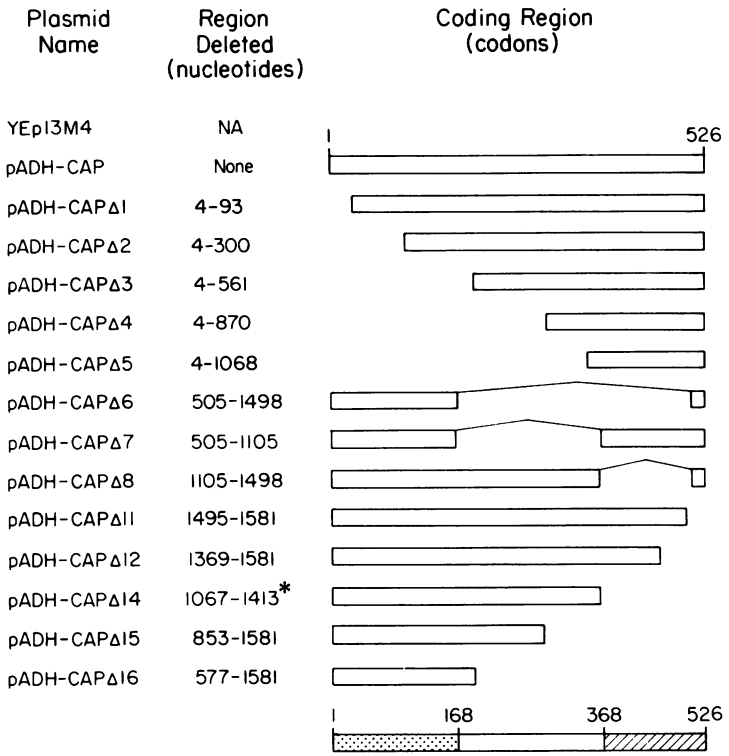
FIG. 3. Effects of 3AT on *GCN4-lacZ* and *HIS4-lacZ* expression in CAP^{wt} and *cap* strains. Strains SP1 (CAP^{wt}) and SKN37 (*cap*) were transformed with centromere plasmids bearing fusions between *lacZ* and the upstream regulatory sequences of GCN4 (*GCN4-lacZ*) or HIS4 (*HIS4-lacZ*) or with yeast expression vector pUV2 (blank). All plasmids carried the *URA3* selectable marker. β-Galactosidase activity was visualized on synthetic selective medium containing either X-Gal (40 μg/ml) alone (SC+X-Gal) or X-Gal and 10 mM 3AT (SC+X-Gal+3AT) to induce the GCN pathway. The dark patches in this black-and-white photograph reflect the induction of the blue pigment resulting from the production of β-galactosidase. See Materials and Methods for further description.

that *cap* cells also failed to grow on synthetic medium containing 10 mM 3AT, while wild-type cells were unaffected (not shown). To determine whether the GCN pathway was defective in *cap* mutant cells and thus unable to correct for a possible amino acid imbalance in *cap* mutant cells, we transformed *cap* mutant strain SKN37 and *gcn4* strain H1393 with plasmids expressing either CAP or GCN4. We then tested the ability of transformants to grow on synthetic medium containing 3AT (Fig. 2). A control plasmid, pUV2, was also used in these experiments (see Materials and Methods for plasmid descriptions). The toxicity of 3AT to *cap* mutant cells was corrected by the expression of CAP but not by the constitutive expression of GCN4. Correspondingly, the defect in *gcn4* cells could be corrected by the expression of GCN4 but not by the expression of CAP. Since GCN4 is a transactivator of the histidine biosynthetic enzymes, this result makes it highly unlikely that *cap* mutant cells are defective in growth in the presence of 3AT because of defects in the GCN pathway. This conclusion is further strengthened by additional experiments using *lacZ* fusion genes as reporters for induction of the GCN response (11, 12, 16). We determined that the GCN pathway was functioning properly in *cap* mutant cells. Reporters for *HIS4*, *HIS3*, and *GCN4* are induced with 3AT, as can be seen in plate assays (Fig. 3) and enzymatic assays (data not shown).

For plate assays (see Materials and Methods for description), we used the wild-type CAP (CAP^{wt}) strain SP1 and *cap* mutant strain SKN37 transformed with reporters for both *GCN4* and *HIS4* as well as control plasmids pUV2 and pHV1. Both strains carried the *URA3* and *HIS3* selectable markers. Wild-type yeast cells were induced for both reporter genes by growth on synthetic medium containing 3AT, as judged by the accumulation of blue color. Similarly, the reporter genes in *cap* mutant cells were strongly induced in the presence of 3AT and accumulated even more color than did wild-type strains. In addition, *cap* mutant strains showed some accumulation of color even in the absence of 3AT. These results suggest that the GCN pathway is intact and functional in *cap* mutant cells. To verify the results of the plate assays and to rule out the possibility that the strong color accumulation in *cap* mutant cells results from cell lysis and the leakage of β-galactosidase into the medium, we performed β-galactosidase enzymatic assays on cells cultured in liquid medium. For this experiment (see Materials and Methods for description), we also tested a *HIS3-lacZ* reporter. β-Galactosidase enzymatic assays performed on wild-type and *cap* mutant yeast cells demonstrated, in both cases, a strong stimulation of β-galactosidase activity within 4 to 8 h after the addition of 3AT to the culture medium (not shown). The levels of reporter gene activity in *cap* mutant strains were also higher than that of the wild-type SP1 strain both before (zero time) and after the addition of 3AT (data not shown).

Deletion analysis of CAP. The sequence of CAP is noteworthy: this 526-amino-acid protein contains a sequence of six consecutive prolines, and amino acid positions 273 through 286 consist solely of proline or alanine residues (9). This proline-rich stretch divides CAP into two domains. To test the function of these domains, various deletion mutants in the *CAP* gene were made (Fig. 4). Deletion mutants of *CAP* were tested for biological activity following transformation into various yeast strains. Three strains were used: SKN34 (*cap* mutant $RAS2^{wt}$), SKN37 (*cap* mutant $RAS2^{Val-19}$), and TKR1-2LS (cap^{supC} $RAS2^{Val-19}$). We monitored these phenotypes: temperature sensitivity; ability to grow on rich medium (YPD); ability to withstand deprivation of a nitrogen source; and heat shock sensitivity. Western blots confirmed that our stains expressed mutant CAP proteins of the correct size (Fig. 5). Such analysis also demonstrated that the amount of CAP in transformed strains was similar to the amount of CAP expressed by wild-type cells (data not shown).

cap mutant SKN34 cells show temperature-sensitive growth on synthetic medium, are unable to grow on rich medium, and are acutely sensitive to nitrogen starvation. SKN34 cells transformed with the full-length *CAP* gene regain the wild-type phenotypes (Fig. 4). Expression of a



Plasmid Name	Region Deleted (nucleotides)	Strain: SKN34(cap RAS2) YPD	SKN34(cap RAS2) TS	SKN34(cap RAS2) NS^r	SKN37(cap RAS2^{val19}) YPD	SKN37(cap RAS2^{val19}) TS	SKN37(cap RAS2^{val19}) NS^r	SKN37(cap RAS2^{val19}) HS^r	TKR1-2LS(cap^{supC} RAS2^{val19}) NS^r	TKR1-2LS(cap^{supC} RAS2^{val19}) HS^r	Summary: RAS Response	Summary: Nutrient Response
Phenotype:		YPD	TS	NS^r	YPD	TS	NS^r	HS^r	NS^r	HS^r		
YEp13M4	NA	−	−	−	−	−	−	+	+	+	−	−
pADH-CAP	None	+	+	+	+	+	−	−	−	−	+	+
pADH-CAPΔ1	4-93	+	+	+	+	+	+	+	+	+	−	+
pADH-CAPΔ2	4-300		ND		+	+	+	+	+	+	−	+
pADH-CAPΔ3	4-561		ND		+	+	+	+	+	+	−	+
pADH-CAPΔ4	4-870	+	+	+	+	+	+	+	+	+	−	+
pADH-CAPΔ5	4-1068		ND		+	+	+	+	+	+	−	+
pADH-CAPΔ6	505-1498		ND		−	−	−	−	−	−	+	−
pADH-CAPΔ7	505-1105	+	+	+	+	+	−	−	−	−	+	+
pADH-CAPΔ8	1105-1498		ND		−	−	−	−	−	−	+	−
pADH-CAPΔ11	1495-1581		ND		−	−	−	−	−	−	+	−
pADH-CAPΔ12	1369-1581	−	−	−	−	−	−	−	−	−	+	−
pADH-CAPΔ14	1067-1413*		ND		−	−	−	−	−	−	+	−
pADH-CAPΔ15	853-1581	−	−	−	−	−	−	−	−	−	+	−
pADH-CAPΔ16	577-1581		ND		−	−	−	−	−	−	+	−

ND - not determined
NA - not applicable

FIG. 4. Deletion analysis of *CAP*. Deletion mutants of *CAP* were tested for functional complementation in three yeast strains: SKN34 (*cap RAS2*^{wt}), SKN37 (*cap RAS2*$^{Val-19}$), and TKR1-2LS (*cap*^{supC} *RAS2*$^{Val-19}$). The various regions of the nucleic acid sequence deleted and the corresponding coding regions expressed are as indicated. CAPΔ14 has a frameshift mutation at nucleotide 1413 (see Materials and Methods for details). A yeast expression vector, YEp13M4, was used as a control plasmid and had no effect on the phenotypes examined. Phenotypes are represented as follows: +, either growth or survival under the conditions tested; −, either lack of growth or failure to survive under the conditions tested. The conditions tested were as follows: YPD, ability of cells to grow on rich media; TS, ability of cells to grow on synthetic medium at 37°C; NS^r, ability of cells to survive a prolonged nitrogen deprivation; HS^r, ability of cells to survive prolonged heat shock (see Materials and Methods for details). The last two columns summarize our findings; + under the RAS Response column indicates that the mutant gene confers penetrance of the activated *RAS* phenotypes; + under the Nutrient Response column indicates that the mutant gene confers the nutrient responses seen in wild-type cells (see text for details).

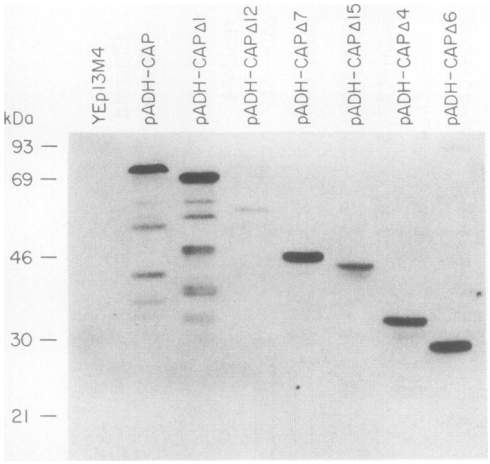


FIG. 5. Western analysis of mutant CAP proteins. The expression of mutant CAP proteins was demonstrated by Western analysis. Whole-cell extracts (125 μg of protein per lane) from SKN37 cells transformed with full-length *CAP* (pADH-CAP), with deletion mutants of *CAP* (pADH-CAPΔ1, -Δ4, -Δ6, -Δ7, -Δ12, and -Δ15; see Fig. 4), or with a yeast expression vector (YEp13M4) as a control were electrophoresed on a 10% polyacrylamide gel and blotted as described in Materials and Methods. Blots were subsequently incubated with polyclonal antisera (1:200) raised against *E. coli*-expressed full-length CAP and were probed with ^{125}I-protein A. The migration of prestained molecular weight markers is shown on the left.

C-terminal region of CAP as small as 237 amino acids appears to be sufficient for restoring the wild-type phenotypes (Fig. 4). In contrast, expression of the N-terminal region of CAP, even when including the middle proline-rich region, had no effect on the *cap* mutant phenotypes of this strain. Thus, the C-terminal domain is required for suppression of these *cap* mutant phenotypes and appears sufficient by itself. In addition, cells expressing the C-terminal domain of CAP were also resistant to valine and 3AT toxicity, while cells expressing the N-terminal domain alone remained sensitive (Fig. 1 and data not shown). Thus, the toxicity of valine and 3AT, temperature sensitivity, sensitivity to starvation, and sensitivity to rich media are all associated with the function of the carboxy-terminal region of CAP.

To explore the effects of deletions in *CAP* on the response of cells to activated *RAS*, we introduced the *CAP* mutants into strain SKN37, which expresses the activated $RAS2^{Val\text{-}19}$ gene. CAP^{wt} strains are sensitive to both heat shock and nitrogen starvation when they express the $RAS2^{Val\text{-}19}$ gene (25, 30). SKN37, however, is not heat shock sensitive yet remains sensitive to nitrogen deprivation and displays the other defects typically seen in *cap* mutant strains. The sensitivity to nitrogen deprivation seen in *cap* mutant $RAS2^{Val\text{-}19}$ cells probably results from the disruption of *CAP* and not from penetrance of the activated *RAS2* allele. Consistent with this idea and with the results described above, the introduction into SKN37 of mutants expressing only the C-terminal domain of CAP restored the ability to grow in rich medium, survive nitrogen deprivation, and grow at 37°C. However, these mutant *CAP* genes did not restore heat shock sensitivity to SKN37 and thus do not confer penetrance of the activated $RAS2^{Val\text{-}19}$ allele. In contrast, expression of as little as the first 192 amino acids of CAP was sufficient to render SKN37 cells sensitive to heat shock. Even small deletions of the N-terminal portion of CAP destroy its ability to render SKN37 cells sensitive to activated RAS (Fig. 4). Suppression of glycogen accumulation, another phenotype of activated *RAS* (14, 28), also requires expression of the CAP N-terminal domain (data not shown). Therefore, the N-terminal domain, comprising amino acids 1 through 192, appears both necessary and sufficient for the expression of the phenotypes associated with activated *RAS*.

Similar conclusions about the N-terminal domain of CAP can also be inferred from our experiments with the cap^{supC} $RAS2^{Val\text{-}19}$ strain TKR1-2LS. Experiments with this strain (Fig. 4) further demonstrate that the N-terminal domain of CAP, when expressed separately, has a dominant effect over the mutant allele.

Cells expressing mutant CAP proteins containing complete N-terminal and C-terminal domains, but entirely lacking the middle portion of the gene, behave like CAP^{wt} cells in our assays. Hence the proline-rich region does not appear to be essential for the functions that we have assayed.

We further note that expression of the C-terminal domain of CAP in CAP^{wt} $RAS2^{Val\text{-}19}$ cells was unable to reverse either the heat shock or nitrogen starvation phenotype (data not shown). In addition, the N-terminal domain of CAP did not render a CAP^{wt} RAS^{wt} strain heat shock sensitive (data not shown). Thus, the truncated CAP proteins do not appear to have dominant interfering effects in CAP^{wt} cells.

Morphological abnormalities in cells expressing only the C-terminal domain of CAP. We next examined the effects of expressing the domains of CAP on the morphology of yeast cells. We observe a wide range of cell sizes in growing cultures of *cap* mutant cells (9). Many *cap* mutant cells are extremely rounded and large. *cap* mutant strain SKN37 was transformed with high-copy-number plasmids expressing various domains of CAP. N-terminal portions of CAP that were capable of restoring the sensitivity to cells to activated *RAS* were not capable of restoring normal cell morphology (data not shown). On the other hand, cultures of cells expressing the C-terminal portion of CAP contained greater numbers of morphologically normal cells. Even in such cultures, however, morphologically abnormal cells are seen. From these observations, we conclude that the morphologic abnormality of *cap* mutant cells results from loss of the C-terminal portion of CAP and can be at least partially restored by expressing that region.

The incomplete suppression of morphologic abnormalities seen in *cap* mutant cells expressing C-terminal portions of CAP from high-copy-number plasmids may have been due to plasmid instability. To circumvent the problems associated with the instability of high-copy-number plasmids, we created strain JG1. This strain expresses the C-terminal 237 amino acids of CAP ($cap^{\Delta 4}$) from an *ADH1* promoter integrated at the *CAP* locus. Expression of the wild-type *CAP* gene is completely ablated (see Materials and Methods). JG1 grows well in rich medium, but it is not heat shock sensitive or sensitive to starvation even though it contains the $RAS2^{Val\text{-}19}$ allele. We have determined by Western blot analysis that the level of expression of truncated CAP protein in JG1 is comparable to the level of expression of full-length CAP protein seen in wild-type cells (data not shown). Expression of full-length CAP in JG1 restored the phenotypes associated with activated RAS (data not shown). When JG1 cells are in log phase in synthetic medium they are similar in size to CAP^{wt} $RAS2^{Val\text{-}19}$ cells, although they are more round in shape. In contrast, cultures grown in rich medium or in synthetic medium containing elevated concentrations of valine display the distinctly enlarged cells seen in

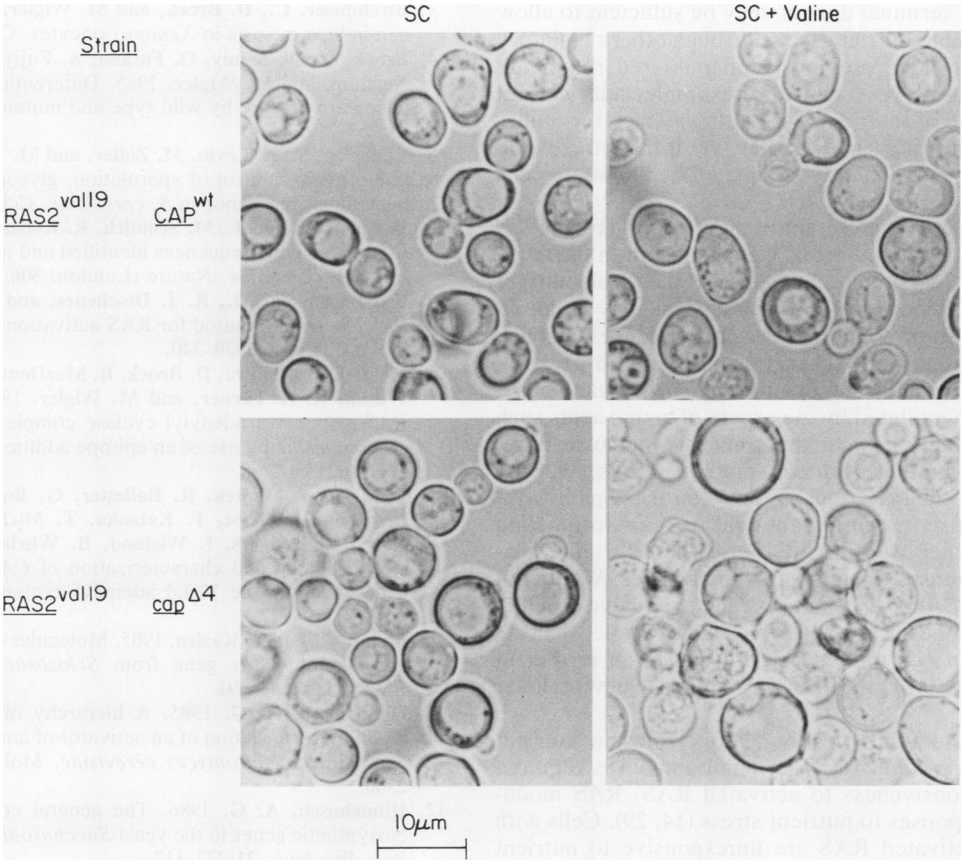


FIG. 6. Effect of valine on the morphology of CAP^{wt} and $cap^{\Delta 4}$ strains. Strains TK161-R2V ($RAS2^{Val-19}$ CAP^{wt}) and JG1 ($RAS2^{Val-19}$ $ADH1\text{-}cap^{\Delta 4}$) were seeded and grown in liquid culture with either synthetic medium lacking valine (SC) or synthetic medium containing 5 mM valine (SC + Valine). Cultures in log-phase growth were harvested by centrifugation (5 min at 1,500 × *g*) and examined microscopically. See text for details.

cultures of *cap* mutant strains (Fig. 6). Many of these cells also appear to have enlarged vacuoles, a feature typical of *cap* mutant cells. Thus, the constitutive expression of the C-terminal domain of CAP does not fully compensate for the morphological effects caused by disruption of the wild-type allele.

DISCUSSION

RAS proteins are ubiquitous in evolution. Their function is unknown except in *S. cerevisiae*. In that yeast, RAS regulates adenylyl cyclase but probably has other functions as well (31). While investigating the mechanism by which RAS proteins stimulate adenylyl cyclase in *S. cerevisiae*, we found a protein, which we have called CAP, that copurifies with RAS-responsive adenylyl cyclase.

CAP appears to be a truly bifunctional protein, and each function is associated with a separate protein domain. *cap* mutant cells display two sets of seemingly unrelated phenotypes. The first includes morphologic abnormality, temperature sensitivity, sensitivity to nitrogen starvation (9), inability to grow in rich medium, amino acid toxicity, and sensitivity to metabolic block by 3AT (this report); the second is a loss of cellular responsiveness to activated *RAS* (9). The first set of phenotypes appears to correlate with loss of expression of the C-terminal domain of CAP and is restored to *cap* mutant cells by expression of that domain. As we have demonstrated previously, the phenotypes of temperature sensitivity, morphologic abnormality, and failure to grow in rich medium associated with loss of CAP function are not reversed by overexpression of the yeast A kinase or cyclic AMP phosphodiesterase gene (9). Thus, the functions of the C-terminal domain of CAP do not appear to be related to the one known target for RAS action, namely, adenylyl cyclase. In contrast, loss of responsiveness to activated RAS correlates with a loss of expression of the N-terminal domain of CAP and can be restored to *cap* mutant cells by expression of that domain. The phenotypes associated with activated RAS are undoubtedly due, at least in part, to its effects on adenylyl cyclase (25, 30). Cells that contain the *supC* or *srv2* allele of *CAP* are resistant to the effects of $RAS2^{Val-19}$ but show none of the other phenotypes of *cap* cells (7, 9). We speculate that these alleles contain mutations that alter the N-terminal domain of CAP but do not alter the function of the C-terminal domain of CAP.

The two domains of CAP can function independently. Nevertheless, the C-terminal domain does not appear to function completely correctly in the absence of the N-terminal domain. Indeed, strain JG1, which expresses only the C-terminal domain of CAP, has a normal growth response to nutrient extremes, but cultures of JG1 accumulate morphologically abnormal cells upon prolonged growth in rich medium. Our assays for CAP function may not be sufficiently sensitive to detect important interactions between the N-terminal and C-terminal domains. A low level of

function of the C-terminal domain may be sufficient to allow the cell to respond to nutrient stress through other regulatory mechanisms. We have previously encountered redundant regulatory controls in yeast cells. For example, cells without regulatory control of their A kinase pathway lack the ability to respond to some nutrient extremes, yet if the unregulated A kinase activity is sufficiently attenuated by mutation, cells regain this ability (5).

cap mutant cells are intolerant of either nitrogen deprivation or an excess of amino acids. Cells deficient in the GCN pathway are also sensitive to extremes in the availability of amino acids (12). The growth of *gcn4* strains may be inhibited by the excess of some amino acids because feedback inhibition decreases the synthesis of other amino acids. A similar mechanism may be operant in *cap* mutant cells, which are growth inhibited by the excess of amino acids such as valine. However, despite the similarity in nutrient responses of *gcn* mutant and *cap* mutant strains, we can observe no functional relationship between these pathways. The GCN response in *cap* mutant cells appears to function properly, and hence we must look elsewhere for an explanation of its responses to amino acid imbalances. We do not know, in fact, whether *cap* mutant cells even have a defect in protein biosynthesis. It is possible, for example, that the primary defect in *cap* mutant cells results in an inefficient assembly of newly synthesized protein into new cellular components.

CAP appears to fulfill two regulatory roles in nutrient sensing in *S. cerevisiae*. Its N-terminal domain is required for cellular responsiveness to activated RAS. RAS modulates cellular responses to nutrient stress (14, 29). Cells with constitutively activated RAS are unresponsive to nutrient deprivation. They remain heat shock sensitive even when growth is arrested and lose viability when starved. These responses are probably mediated by the effects of RAS on adenylyl cyclase (25, 30). We do not understand why the presence of the N-terminal of CAP is needed for the normal cellular responses to activated RAS, but we presume that this CAP domain is required for the proper regulation of adenylyl cyclase (9). Our present work demonstrates that the C-terminal domain of CAP is required for another pathway regulating responses to nutrient stresses. We presume that the two regulatory pathways, which are both embedded in the CAP protein, are in some way functionally linked. Many possible models can be envisioned which incorporate RAS, adenylyl cyclase, CAP, and possibly cytoskeletal components into the growth-regulatory circuitry of the cell. Additional biochemical and genetic experiments are required to develop a model.

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