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Cloning and Characterization of *CAP*, the S. cerevisiae Gene Encoding the 70 kd Adenylyl Cyclase–Associated Protein

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Summary

Adenylyl cyclase from S. cerevisiae contains at least two subunits, a 200 kd catalytic subunit and a subunit with an apparent molecular size of 70 kd, which we now call CAP (cyclase-associated protein). We cloned a cDNA encoding CAP by screening a yeast cDNA expression library in E. coli with antisera raised against the purified protein. The cDNA contained an open reading frame capable of encoding a 526 amino acid protein that is not homologous to any sequences in the current data bases. Adenylyl cyclase activity in membranes from cells that lacked *CAP* was not stimulated by RAS2 proteins in vitro. These results suggest that *CAP* is required for at least some aspects of the RAS-responsive signaling system. Mutants lacking *CAP* had four additional phenotypes that appear to be unrelated to effects of the RAS/adenylyl cyclase pathway: the inability to grow on rich medium (YPD), temperature sensitivity on minimal medium, sensitivity to nitrogen starvation, and a swollen cell morphology.

Introduction

The *RAS* gene family is widely conserved in eukaryotic cells (Shilo and Weinberg, 1981; DeFeo-Jones et al., 1983; Reymond et al., 1984; Powers et al., 1984). Our laboratory has been studying *RAS1* and *RAS2*, the two *RAS* genes of Saccharomyces cerevisiae (Wigler et al., 1988). RAS proteins are about 90% identical to their mammalian counterparts in the N-terminal third of the protein. Besides being highly homologous, yeast and mammalian RAS proteins are active in their heterologous host cells (Kataoka et al., 1985a; DeFeo-Jones et al., 1985). RAS proteins share a number of biochemical properties: all bind GTP and GDP, possess intrinsic GTPase activity, and appear to be acted on by GTPase-activating proteins (Scolnick et al., 1979; Sweet et al., 1984; Gibbs et al., 1984; McGrath et al., 1984; Tamanoi et al., 1984; Temeles et al., 1985; Tamanoi et al., 1985; Trahey and McCormick, 1987; Ballester et al., 1989). RAS proteins activate effectors when bound to GTP and are turned off by GTP hydrolysis (De Vendittis et al., 1986; Field et al., 1987). Many oncogenic forms of mammalian RAS have a reduced GTPase activity and are resistant to the GTPase-activating proteins, owing to point mutations (Sweet et al., 1984; Gibbs et al., 1984; Trahey and McCormick, 1987). Analogous point mutations in the yeast *RAS2* gene also reduce the GTPase activity of the encoded protein and cause abnormal phenotypic responses to nutrient limitation (Toda et al., 1985; Kataoka et al., 1984; Tamanoi et al., 1985).

We previously demonstrated that the S. cerevisiae adenylyl cyclase is under the control of yeast RAS proteins (Toda et al., 1985). Activation of adenylyl cyclase by RAS protein can be demonstrated in vitro using either purified yeast or human RAS proteins (Toda et al., 1985; Broek et al., 1985). Although the effector system of mammalian RAS is not yet known, it is probably not adenylyl cyclase (Birchmeier et al., 1985). This evolutionary riddle has prompted us to scrutinize the RAS–adenylyl cyclase interactions in greater detail, and for this we have taken genetic and biochemical approaches. Central to both approaches has been the cloning and characterization of *CYR1*, the gene encoding yeast adenylyl cyclase (Kataoka et al., 1985b; Field et al., 1990; Colicelli et al., submitted).

In one of our genetic approaches we have sought mutations that could reverse the phenotypes induced by the $RAS2^{val19}$ allele, namely, heat shock and nitrogen starvation sensitivity. Several suppressor mutants of these phenotypes were found. Some of these, as expected, had mutations in *RAS2* (Powers et al., 1989) and *CYR1* (S. P., unpublished data). Some had mutations in the gene *RAM* (Powers et al., 1986), also called *DPR* (Fujiyama et al., 1987), which encodes a protein required for the maturation of RAS and the sex pheromone, a-factor. A number of additional complementation groups were found that could have mutations in genes encoding products needed for $RAS2^{val19}$ to activate its effectors. One of these was called *supC* (unpublished data).

In our biochemical approach we have sought to purify a RAS-responsive adenylyl cyclase from S. cerevisiae. To do this we engineered yeast to express an adenylyl cyclase fused to a small N-terminal peptide, expressing an epitope recognized by a monoclonal antibody. After immunoaffinity purification and glycerol gradient sedimentation, we purified a RAS-responsive adenylyl cyclase complex that contained at least two subunits: a 200 kd protein, which was the product of *CYR1*, and a protein with a relative molecular size on SDS–polyacrylamide gels of 70 kd, which we now call CAP for cyclase-associated protein (Field et al., 1988). We report here the cloning and analysis of *CAP*. *supC* appears to be an allele of *CAP*. We show that production of a RAS-responsive adenylyl cyclase requires the presence of CAP. Cells with full disruptions of *CAP* have a number of interesting properties.

Results

Raising Antisera to CAP

We previously described a method for purifying a RAS-responsive adenylyl cyclase from S. cerevisiae (Field et

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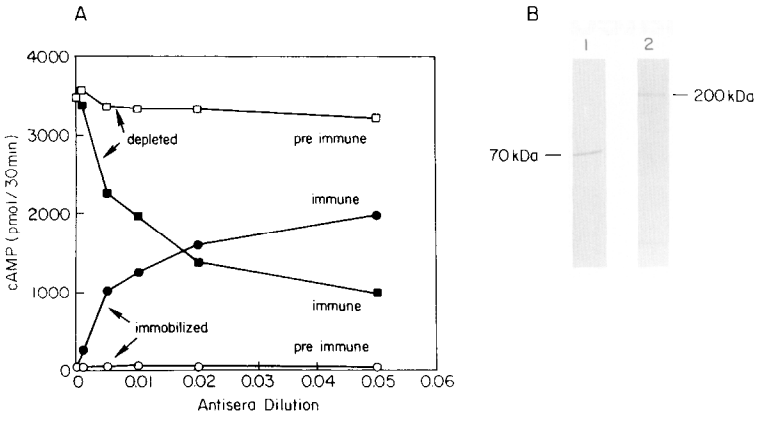

Figure 1. Characterization of Anti-70 kd Antisera

(A) Immunoprecipitation of adenylyl cyclase activity with anti-70 kd antisera. Solubilized yeast membranes from strain JF36A were subjected to immunoprecipitations as described in Experimental Procedures using the indicated dilutions of anti-70 kd antisera. "depleted" indicates adenylyl cyclase activity in the supernatant fraction after incubation with preimmune serum (open squares) or immune serum (solid squares); "immobilized," adenylyl cyclase activity in protein A–agarose bead pellets after incubation with preimmune serum (open circles) or immune serum (closed circles).

(B) Western blot analysis of purified adenylyl cyclase complex using anti-70 kd antisera (lane 1) or anti-adenylyl cyclase fusion protein monoclonal antibody 12CA5 (lane 2). Detection was by colloidal gold-conjugated second antibodies as described in Experimental Procedures.

al., 1988). To obtain enough protein for further studies, we scaled up the procedure. Extract from approximately 300 liters of cells was used to purify the adenylyl cyclase complex. The purified complex was subjected to SDS–polyacrylamide gel electrophoresis and then stained with Coomassie blue to visualize proteins. Gel slices containing the 70 kd band were homogenized and then injected into rabbits. After several injections we tested the rabbit sera for anti-CAP antibodies (Figure 1). While sera collected prior to immunization contained no detectable reactivity toward adenylyl cyclase, sera collected after immunization could immunoprecipitate about 50% of the adenylyl cyclase activity from a crude extract (Figure 1A). On Western blots of purified adenylyl cyclase, the antisera detected a 70 kd band, while a 200 kd band was detected with monoclonal antibody 12CA5, an antibody directed against the adenylyl cyclase fusion protein (Field et al., 1988) (Figure 1B). These experiments further support the hypothesis that S. cerevisiae adenylyl cyclase is a multisubunit complex containing at least two distinct subunits.

Cloning a cDNA Encoding CAP

To clone the gene encoding the 70 kd subunit, we used the antisera described above to screen a λ cDNA expression library (the generous gift of J. Kuret). Ten immunoreactive plaques were isolated after screening about 500,000 plaques. From restriction maps and direct DNA sequencing it became clear that we had cloned two genes. One of them, isolated six times, encodes the 70 kd subunit. The other, isolated four times, encodes a protein of about 90 kd. The sequence of the gene encoding the 90 kd protein is not homologous to the gene encoding the 70 kd protein (data not shown) or to any other known protein. The 90 kd protein is found at varying levels in purified adenylyl cyclase, as judged from Western blots (data not shown). At present we are trying to determine if the 90 kd protein is another, previously unidentified subunit of adenylyl cyclase or merely a purification artifact.

The sequence of one of the cDNAs encoding the 70 kd subunit is shown in Figure 2. There is a single open reading frame, initiated by an ATG, of 526 codons. Just 4 codons 5′ to the ATG is an in-frame stop codon, and 3′ to the open reading frame is a poly(A) sequence 13 nucleotides long, followed by an EcoRI site. This restriction site is presumably derived from the linkers used in cloning. The five other cDNAs were partially sequenced. At least four of these cDNAs arose from independent cloning events. We searched three data bases (GenBank, release 60; EMBL, release 19; and Swiss-Protein, release 11) but found no homologous genes. The predicted amino acid sequence is noteworthy for a proline-rich stretch located at the center (amino acids 275–285).

We further characterized the apparently full-length cDNA clone by expressing its encoded protein in Escherichia coli using a T7 promoter vector. Western blot analysis indicated that this gene directed synthesis of a protein of the appropriate size that reacted with anti-70 kd subunit antisera (Figure 3A). In addition, extracts from E. coli that expressed this protein blocked immunoprecipitation of adenylyl cyclase from yeast by the anti-70 kd antisera (Figure 3B). Finally, we purified the recombinant protein from E. coli extracts and used it to raise antisera in four other rabbits. These sera immunoprecipitated adenylyl cyclase from yeast extracts and visualized a 70 kd band in Western blots of purified adenylyl cyclase complex (data not shown). These data suggest that we indeed cloned a gene encoding the 70 kd subunit of adenylyl cyclase, which we named *CAP*.

Phenotypes Caused by Disrupting *CAP*

To study the function of *CAP*, we disrupted the gene in yeast as follows. First, we constructed plasmids that had either the *URA3* or *HIS3* genes inserted between two closely spaced HpaI sites. Later, constructs deleted the region between the NsiI site and the HpaI sites (Figure 4). These plasmids were digested with EcoRI and then used individually to transform a *ura3*$^-$ *his3*$^-$ strain, SP1. Disruption of the *CAP* genomic locus was confirmed by

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70K
-38                                                GAATTCGGGGCCAAGTTGCAACCGTGTGAAATCGAATC

1     ATGCCTGACTCTAAGTACACAATGCAAGGTTATAACCTTGTTAAGCTATTAAAAAGGCTAGAAGAAGCCACTGCA
1     METProAspSerLysTyrThrMetGlnGlyTyrAsnLeuValLysLeuLeuLysArgLeuGluGluAlaThrAla

76    AGATTAGAGGATGTCACCATCTATCAAGAAGGTTATATTCAGAATAAATTGGAGGCATCTAAAAATAACAAGCCT
26    ArgLeuGluAspValThrIleTyrGlnGluGlyTyrIleGlnAsnLysLeuGluAlaSerLysAsnAsnLysPro

151   TCCGACTCCGGGGCCGATGCGAATACTACGAATGAACCTTCTGCAGAAAATGCTCCTGAAGTAGAACAAGATCCG
51    SerAspSerGlyAlaAspAlaAsnThrThrAsnGluProSerAlaGluAsnAlaProGluValGluGlnAspPro

226   AAATGCATAACTGCGTTCCAATCTTACATCGGTGAGAATATTGATCCGCTGGTAGAATTATCAGGAAAGATCGAC
76    LysCysIleThrAlaPheGlnSerTyrIleGlyGluAsnIleAspProLeuValGluLeuSerGlyLysIleAsp

301   ACGGTGGTCTTAGATGCTTTGCAGTTGCTAAAGGGAGGCTTTCAATCGCAATTGACTTTTTTAAGAGCTGCTGTG
101   ThrValValLeuAspAlaLeuGlnLeuLeuLysGlyGlyPheGlnSerGlnLeuThrPheLeuArgAlaAlaVal

376   AGATCAAGAAAACCGGATTATTCTTCTCAAACTTTCGCTGATTCTTTAAGACCTATCAACGAAAATATTATAAAG
126   ArgSerArgLysProAspTyrSerSerGlnThrPheAlaAspSerLeuArgProIleAsnGluAsnIleIleLys

451   CTGGGTCAATTGAAGGAATCAAACCGTCAAAGCAAATACTTCGCATATTTGAGCGCTTTATCTGAGGGTGCTCCT
151   LeuGlyGlnLeuLysGluSerAsnArgGlnSerLysTyrPheAlaTyrLeuSerAlaLeuSerGluGlyAlaPro

526   TTGTTCTCCTGGGTTGCAGTGGACACTCCCGTGTCTATGGTCACAGATTTCAAGGACGCAGCACAGTTTTGGACT
176   LeuPheSerTrpValAlaValAspThrProValSerMetValThrAspPheLysAspAlaAlaGlnPheTrpThr

601   AATAGAATTTTGAAAGAATACAGAGAGTCTGATCCTAATGCTGTTGAATGGGTTAAGAAATTTTTGGCCTCTTTC
201   AsnArgIleLeuLysGluTyrArgGluSerAspProAsnAlaValGluTrpValLysLysPheLeuAlaSerPhe

676   GATAATTTGAAAGCCTACATTAAAGAGTATCATACTACTGGGGTTTCCTGGAAAAAAGACGGTATGGATTTTGCT
226   AspAsnLeuLysAlaTyrIleLysGluTyrHisThrThrGlyValSerTrpLysLysAspGlyMetAspPheAla

751   GACGCGATGGCACAATCAACGAAGAATACAGGTGCTACTTCATCTCCTTCGCCAGCAAGTGCTACAGCGGCTCCA
251   AspAlaMetAlaGlnSerThrLysAsnThrGlyAlaThrSerSerProSerProAlaSerAlaThrAlaAlaPro

826   GCACCACCACCTCCTCCACCAGCCCCACCAGCTTCCGTCTTTGAAATCTCTAATGATACACCAGCAACGAGTAGT
276   AlaProProProProProProAlaProProAlaSerValPheGluIleSerAsnAspThrProAlaThrSerSer

901   GATGCTAACAAAGGCGGTATTGGCGCGGTCTTCGCCGAACTAAATCAGGGTGAAAATATCACTAAGGGTTTGAAA
301   AspAlaAsnLysGlyGlyIleGlyAlaValPheAlaGluLeuAsnGlnGlyGluAsnIleThrLysGlyLeuLys

976   AAAGTAGACAAATCCCAACAAACTCACAAAAATCCTGAATTACGTCAATCCTCTACAGTTTCTTCCACAGGAAGT
326   LysValAspLysSerGlnGlnThrHisLysAsnProGluLeuArgGlnSerSerThrValSerSerThrGlySer

1051  AAATCCGGTCCACCACCAAGGCCAAAAAAGCCATCAACATTGAAAACTAAGAGGCCTCCTAGAAAGGAATTGGTA
351   LysSerGlyProProProArgProLysLysProSerThrLeuLysThrLysArgProProArgLysGluLeuVal

1126  GGAAACAAATGGTTTATTGAGAATTACGAAAATGAAACTGAATCTCTGGTTATTGATGCAAATAAAGATGAGTCT
376   GlyAsnLysTrpPheIleGluAsnTyrGluAsnGluThrGluSerLeuValIleAspAlaAsnLysAspGluSer

1201  ATCTTCATAGGTAAATGTTCTCAAGTTCTTGTTCAAATAAAAGGAAAAGTTAACGCTATCTCGTTGAGTGAAACT
401   IlePheIleGlyLysCysSerGlnValLeuValGlnIleLysGlyLysValAsnAlaIleSerLeuSerGluThr

1276  GAGTCATGCAGTGTTGTTCTTGATTCTAGCATTTCCGGTATGGATGTCATCAAATCCAACAAGTTTGGCATTCAA
426   GluSerCysSerValValLeuAspSerSerIleSerGlyMetAspValIleLysSerAsnLysPheGlyIleGln

1351  GTTAACCATTCCCTACCTCAAATCTCCATTGATAAATCTGACGGCGGTAACATCTATTTATCCAAGGAATCCTTG
451   ValAsnHisSerLeuProGlnIleSerIleAspLysSerAspGlyGlyAsnIleTyrLeuSerLysGluSerLeu

1426  AATACTGAAATCTACACCTCGTGCTCAACTGCTATTAACGTCAACTTACCAATCGGCGAGGACGATGATTACGTA
476   AsnThrGluIleTyrThrSerCysSerThrAlaIleAsnValAsnLeuProIleGlyGluAspAspAspTyrVal

1501  GAATTCCCAATCCCTGAACAGATGAAGCATAGCTTCGCTGATGGTAAGTTCAAATCTGCTGTTTTCGAACATGCT
501   GluPheProIleProGluGlnMetLysHisSerPheAlaAspGlyLysPheLysSerAlaValPheGluHisAla

1576  GGTTAATATTGCGAGGAGCATTAATTGTATTTAGTTAAAAGAAATAAATCACATTTGAATCAATCAGAAAGAAAA
526   GlyTER

1651  GTGAATGAATAAACAATTATATACTTTTGTAAAACCTTTTTTTCATGTGTAAAAAAAAAAAAACCCGAATTC
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Figure 2. Sequence of *CAP* cDNA

The sequence begins and ends with EcoRI sites presumably derived from the linkers used in cloning. TER indicates an in-frame termination codon. This sequence is taken from pCAP-2. Five other clones were partially sequenced. They were all identical to pCAP-2 except for the extreme 5′ and 3′ termini.

Southern blot analysis (data not shown). *cap*$^-$ cells displayed four phenotypes: an inability to grow on rich medium (Figure 5), temperature sensitivity on synthetic medium (Figure 5), sensitivity to nitrogen starvation (described below), and changes in cell morphology (Figure 6). Cells lacking *CAP* were rounder than wild-type cells, and many were unusually large. These phenotypes were recessive, since they were suppressed upon mating to wild-type cells and also suppressed by expression of the wild-type gene on a plasmid (data not shown).

We confirmed that the phenotypes described above were due to the loss of *CAP* by subjecting a heterozygous diploid strain to tetrad analysis. In a *his3*$^-$/*his3*$^-$ diploid strain, *CAP* was disrupted with *HIS3*. The phenotypes of

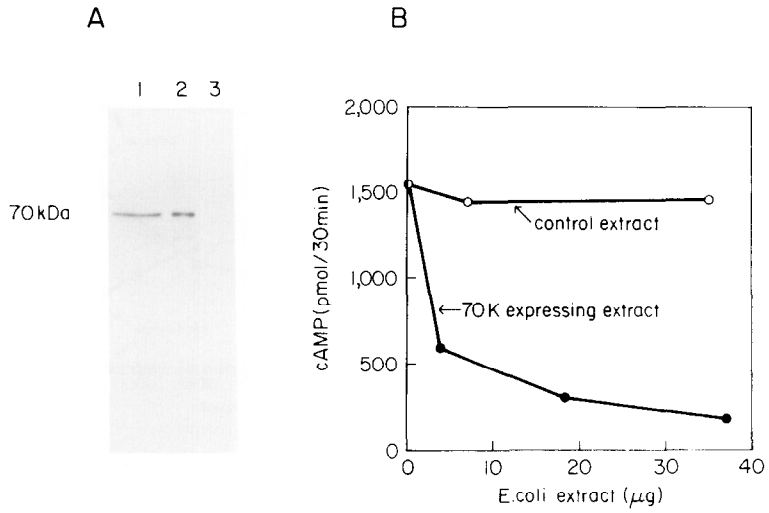

Figure 3. Analysis of Recombinant Protein Made in E. coli

(A) Western blot with anti–70 kd antisera. Lane 1, 20 ng of adenylyl cyclase complex purified from yeast; lane 2, 1.0 μg of crude extract from E. coli expressing *CAP* cDNA from the bacteriophage T7 promoter; lane 3, 1.0 μg of crude extract from E. coli without expression plasmid. (B) Competition for immunoprecipitation by the recombinant protein. Extract from yeast strain JF36A (100 μl) was incubated with the indicated amounts of crude extract from either E. coli expressing CAP (closed circles) or E. coli without the expression plasmid (open circles), 2 μl of anti–70 kd antisera, and 30 μl of 50% protein A–Sepharose beads as described in Experimental Procedures. Adenylyl cyclase assays were performed on the Sepharose bead pellets.

His$^+$, inability to grow on rich medium and large cell morphology, cosegregated 2:2 for each of nine tetrads examined (data not shown).

Finally, we verified that the wild-type phenotypes could be restored by a genomic *CAP* gene. To do this we screened a 2μm based genomic library for the ability to complement the phenotypic abnormalities of *CAP*-deficient cells. One clone was clearly able to reverse the four phenotypes of *cap*$^-$ cells described above. Restriction mapping indicated that it corresponded to the genomic version of the *CAP* cDNA we had cloned. Its restriction map is shown in Figure 4 aligned with that of the cDNA.

Genetic Interactions with *CYR1* and *RAS*

The phenotypes of sensitivity to rich medium and morphological changes described above do not correspond to any phenotypes we have previously observed in cells altered in the genes encoding components of the RAS/adenylyl cyclase signaling pathway. Nevertheless, to test if these phenotypes were related to the cAMP pathway, we carried out several high copy number plasmid suppression experiments. Neither the failure to grow on rich medium nor the morphological changes were suppressed by a plasmid expressing a catalytic subunit of the cAMP-dependent protein kinase, encoded by *TPK1*. We have pre-

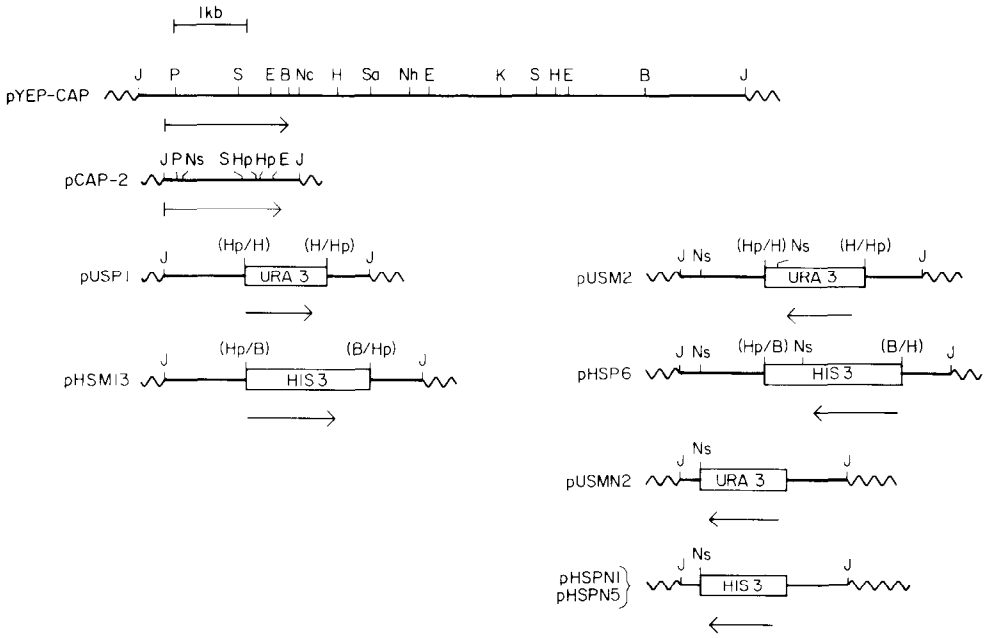

Figure 4. Restriction Maps of the *CAP* Gene and Disruption Plasmids

pYEP-*CAP*, genomic clone isolated by suppression of *cap*$^-$ phenotypes; pCAP-2 is a cDNA clone. Arrow indicates direction of transcription and locations of reading frames. pUSP1, pUSM2, pHSM13, pHSP6, pUSMN2, pHSPN1, and pHSPN5 are plasmids used for disruption of *CAP* in yeast as described in Experimental Procedures. Restriction enzymes abbreviated as follows: B, BamHI; E, EcoRI, H, HindIII; Hp, HpaI; J, junction with vector; K, KpnI, Nc, NcoI; Nh, NheI; Ns; NsiI; P, PstI; S, StuI; Sa, SacI.

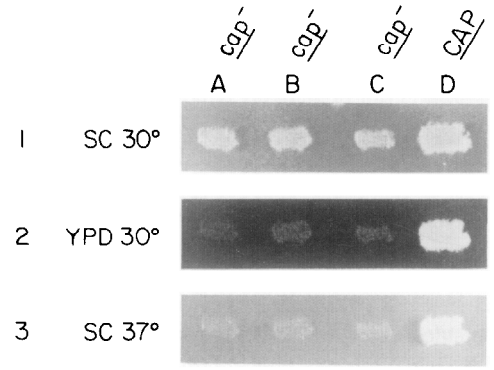

Figure 5. Sensitivity to Rich Medium and Temperature Sensitivity of *cap*⁻ Cells

CAP and *cap*⁻ strains were grown on SC plates (these are made from synthetic medium and contain a complete complement of amino acids) at 30°C and then replica plated to either SC or YPD plates (these are made from rich medium) and grown for 2 days. The cells in row 1 were grown on SC plates at 30°C, those in row 2 were grown on YPD plates at 30°C, and those in row 3 were grown on SC plates at 37°C. The strains were: A, JFSKN22 (*cap*⁻); B, JFSKN32 (*cap*⁻); C, JFSKN34 (*cap*⁻); D, SP1 (*CAP*). Complete genotypes are given in Table 2.

viously shown that this plasmid suppresses the loss of both *RAS* genes and also the loss of *CYR1* (Toda et al., 1987a). Moreover, the phenotypes were not suppressed by plasmids expressing *BCY1*, the gene encoding the regulatory subunit of the yeast cAMP-dependent protein kinase (Toda et al., 1987b), *PDE1*, a yeast phosphodiesterase (Nikawa et al., 1987), or *JUN1* (Nikawa et al., 1987). *JUN1* is the same gene as *MSI1* (Ruggieri et al., 1989). We have previously shown that these plasmids suppress the activation of the adenylyl cyclase pathway. Together, these results suggest that CAP has some functions that are not related to the RAS/adenylyl cyclase pathway.

Our laboratory has made extensive use of the phenotypes induced by the *RAS2*^{val19} gene to search for other genes in the RAS pathway. These phenotypes include heat shock sensitivity and nitrogen starvation sensitivity. Both phenotypes seem to be consequences of elevated levels of cAMP, because the same set of phenotypes result from mutations that activate the cAMP-dependent signaling pathway and both phenotypes can be suppressed in *RAS2*^{val19} cells by genes that lower levels of cAMP. We previously identified a chromosomal mutation, called *supC*, that suppresses these phenotypes (T. K., unpublished data). We cloned *SUPC* by screening for plasmids that cause a *RAS2*^{val19} *supC* strain to become heat shock sensitive again (S. P., unpublished data). By tetrad analysis, *supC* appeared to be genetically linked to the *RAS2* locus on chromosome 14 (S. P., unpublished data). By Southern blotting undigested and NotI-digested yeast DNA separated in pulse field gels (data not shown), we also found that *CAP* was physically linked to the *RAS2* locus. Moreover, the *SUPC* gene we identified had a restriction map identical to that of the *CAP* gene. Both the genomic clone of *CAP* described above and the *CAP* cDNA clone expressed in a yeast vector reversed the *supC* phenotype (data not shown).

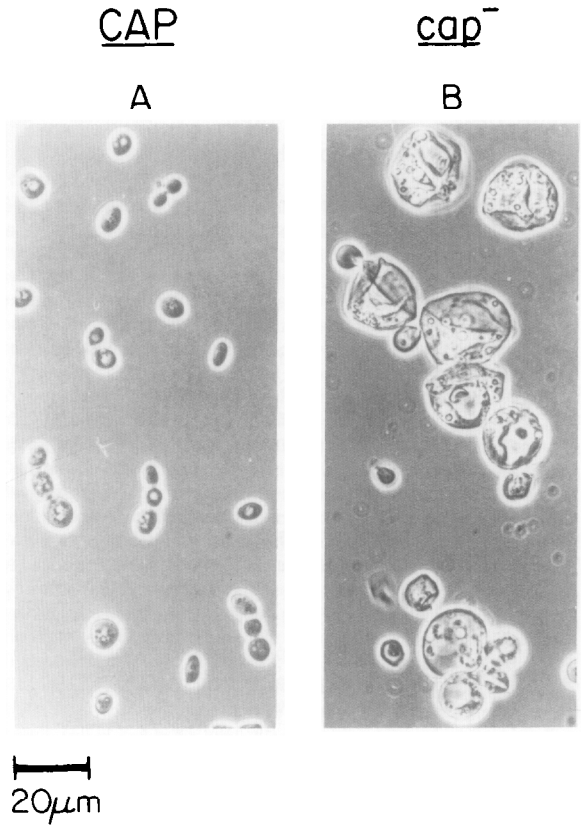

Figure 6. Light Micrograph of Yeast Strains Grown in SC Medium at 30°C

(A) SP1, a wild-type strain. (B) *cap*⁻ disruption in JC1.

The above results suggest that *supC* is an allele of *CAP*, and that CAP is involved in the RAS pathway. To test this more directly we examined the ability of strains with disruptions of *CAP* to suppress the heat shock sensitivity phenotype usually induced by the *RAS2*^{val19} gene. We first disrupted the *CAP* gene in TK161-R2V, a strain that contains the *RAS2*^{val19} allele. Next, the strains were transformed with either a YEp13 vector plasmid or a plasmid expressing the *CAP* cDNA and tested for heat shock sensitivity and growth on YPD (Figure 7; see Experimental Procedures). While cells with an intact chromosomal copy of *CAP* were sensitive to heat shock, cells with the disrupted *CAP* gene were resistant to heat shock; heat shock sensitivity was restored by introduction of the *CAP* expression plasmid. Likewise, cells with the disrupted *CAP* gene could not grow on YPD; growth on YPD was restored by introduction of the *CAP* expression plasmid (Figure 7). Finally, to test directly if *supC* and *cap*⁻ were allelic, we carried out genetic crosses between them and determined that they were in the same complementation group (data not shown).

One other major phenotype induced in normal cells by the presence of *RAS2*^{val19} is reduced viability upon nitrogen starvation. Indeed, the *supC* mutation was originally obtained based upon the reversion of nitrogen starvation sensitivity in a *RAS2*^{val19} strain. We therefore tested the

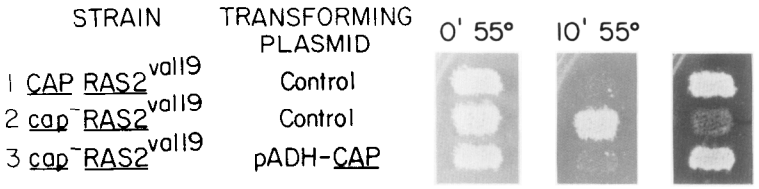

Figure 7. Heat Shock Sensitivity and Rich Medium Sensitivity Assays of *cap*⁻ *RAS2*^{val19} Cells

The *CAP* gene was disrupted in the *RAS2*^{val19} strain TK161-R2V (Table 2) to create strain JFSKN37. TK161-R2V or JFSKN37 was then transformed with the indicated plasmids, which are selected by using the *LEU2* marker, and then scored for growth on rich medium (YPD) or sensitivity to heat shock on SC plates (10 min at 55°C) as indicated. The strains and plasmids were as follows: row 1, TK161-R2V containing pYEp13M4 (a *LEU2* vector plasmid); row 2, JFSKN37 containing pYEp13M4; row 3, JFSKN37 containing pADH-*CAP* (a plasmid expressing *CAP* from the ADH-1 promoter).

ability of *CAP* disruptions to affect the nitrogen starvation sensitivity phenotype of *RAS2*^{val19} strains. To our initial surprise, cells lacking *CAP* become sensitive to nitrogen starvation whether or not they contain the *RAS2*^{val19} gene (Figure 8).

Biochemical Consequences of Disrupting *CAP*

We have shown that disruptions of *CAP* interfere with the phenotype of *RAS2*^{val19} and that CAP associates with adenylyl cyclase. We therefore examined the consequence of disrupting *CAP* on the responsiveness of adenylyl cyclase to RAS. For this purpose we transformed *cap*⁻ cells with the plasmid pYCYR. pYCYR expresses high levels of adenylyl cyclase fused at its N-terminus to a peptide epitope recognized by monoclonal antibody 12CA5 (Field et al., 1988, 1990). Cells were grown, and membranes were prepared as described previously and tested for adenylyl cyclase activity (Table 1). There was a substantial amount of activity measured in the presence of Mn^{2+}, which reflects the amount of catalytic subunit present (Casperson et al., 1983). Significantly less activity was seen in the presence of Mg^{2+}. In extracts from cells with an intact copy of *CAP*, adenylyl cyclase activity was greatly stimulated by RAS protein in the presence of Mg^{2+}. However, in two independent experiments analyzing a total of eight independently isolated strains, significantly less stimulation of Mg^{2+}-dependent activity occurred when RAS2 protein was added to extracts from *cap*⁻ cells. Thus, CAP appears to be required for the production of a RAS-responsive adenylyl cyclase. We also examined these extracts for the presence of the N-terminal epitope by Western blotting. Although full-length adenylyl cyclase fusion protein is readily observed in a *CAP* background, the full-length adenylyl cyclase fusion protein was not observed in *cap*⁻ cells (data not shown). Thus, CAP may be required to stabilize full-length adenylyl cyclase.

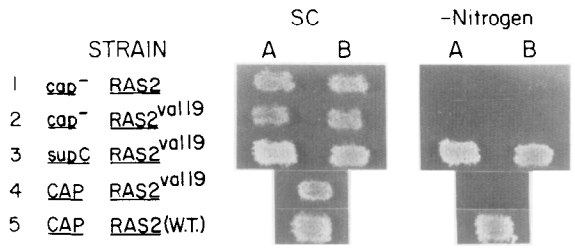

Figure 8. Nitrogen Starvation of *cap*⁻ Cells

The indicated strains were grown on SC plates (SC), replica plated to a plate that lacked nitrogen, incubated for 9 days at 30°C, then replica plated back onto SC plates and photographed after 2 days of growth. The genotypes of the strains are as follows. In row 1, both strains were *cap*⁻ *RAS2*; patch A was JFSKN22 and B was JFSKN25. In row 2, both strains were *cap*⁻ *RAS2*^{val19}; patch A was JFKSN43 and B was JFSKN46. In row 3, both strains were *supC RAS2*^{val19}; patch A was RS27-2B and B was TKR1-2LS. In row 4, the patch was *CAP RAS2*^{val19}; the strain was TK161-R2V. In row 5, the patch was *CAP RAS2*, the wild-type strain SP1.

Discussion

We have cloned a gene from S. cerevisiae, which we name *CAP*, that encodes the 70 kd subunit of the RAS-responsive adenylyl cyclase complex. The evidence that *CAP* is the correct gene is as follows. First, *CAP* cDNA was capable of directing the synthesis of a protein that reacts with an anti–70 kd antiserum and comigrated with the authentic 70 kd subunit on SDS gels. Second, an extract from E. coli expressing *CAP* blocked immunoprecipitation of adenylyl cyclase complex from yeast by the anti–70 kd antisera. Finally, recombinant protein purified from E. coli

Table 1. RAS Activation of Adenylyl Cyclase in *cap*⁻ Cells

		cAMP (pmol/min per mg)		
Strain	Genotype	Mn^{2+}	Mg^{2+}	Mg^{2+} + RAS2^{val19} + GTP
Experiment 1.				
TKB111	*CAP*	278	33.5	121.0
JFSKO13(a)	*cap*⁻	535	43.1	44.4
JFSKO13(b)	*cap*⁻	770	40.1	32.3
JFSKO13(c)	*cap*⁻	391	8.9	13.6
JFSKO13(d)	*cap*⁻	1626	104.0	133.0
JFSKO13(e)	*cap*⁻	246	18.5	21.9
Experiment 2.				
TKB111	*CAP*	166	13.4	165.0
JFSKN22(f)	*cap*⁻	298	43.0	70.0
JFSKN25(g)	*cap*⁻	290	64.5	95.4
JFSKN25(h)	*cap*⁻	439	69.3	106.0

The indicated strains were transformed with the adenylyl cyclase plasmid pYCYR (Field et al., 1988, 1990) and then grown in SC-Leu medium to a density of 1 × 10^7 cells per ml. Membrane extracts were prepared as previously described and adenylyl cyclase activity was measured in the presence of Mn^{2+}, Mg^{2+}, or Mg^{2+} plus, 12 μg of RAS2^{val19} and 50 μM GTP. See Table 2 for strain descriptions. a–e: independent transformants of JFSKO13 with pYCYR. f–h: independent transformants of JFSKN25 with pYCYR.

could be used to raise new antisera that also reacted with adenylyl cyclase complex from yeast. From its sequence, *CAP* encodes a protein of 526 amino acids bearing no obvious similarity to any other proteins in the current data bases. Recently, however, we have learned that *CAP* was cloned independently in another lab by using a genetic screen similar to the one we used to identify and clone *supC*. Their gene, called SRV2, predicts a protein identical to *CAP* (Fedor-Chaiken et al., 1990).

Two lines of evidence indicate that CAP is required for the activation of adenylyl cyclase by RAS. First, *cap*$^-$ cells are not heat shock sensitive when they contain the activated *RAS2*^{val19} gene. Furthermore, an allele of *CAP*, named *supC*, also does not exhibit nitrogen starvation sensitivity when it contains *RAS2*^{val19}. Both heat shock and nitrogen starvation sensitivity are hallmark responses to *RAS2*^{val19} in an otherwise wild-type background (Toda et al., 1985; Sass et al., 1986; Powers et al., 1986). Second, adenylyl cyclase activity in yeast membranes from *cap*$^-$ cells is not responsive to RAS. We cannot at present distinguish among a large number of possible modes of action of CAP.

One observation suggests that CAP is not solely a positive regulatory protein for adenylyl cyclase. Unlike cells that lack *RAS1* and *RAS2* or cells that lack *CYR1*, cells that lack *CAP* are clearly viable. This suggests that cAMP levels are higher in *cap*$^-$ strains than in *ras1*$^-$ *ras2*$^-$ strains or in *cyr*$^-$ strains. This suggests to us that CAP may inhibit the basal activity of adenylyl cyclase in the absence of RAS1 and RAS2. Since CAP appears to be required for cells to express full-length adenylyl cyclase, an increase in basal levels of adenylyl cyclase might result from the presence of a more active proteolytic degradation fragment of the enzyme.

Disruption of *CAP* has other interesting consequences. Four recessive phenotypes were observed in *cap*$^-$ cells. These were temperature sensitivity, inability to grow on rich medium, morphological abnormalities, and sensitivity to nitrogen starvation. These phenotypes do not appear to be related to the yeast RAS/adenylyl cyclase pathway because they are not suppressed by plasmids that otherwise suppress the activation or attenuation of the RAS/adenylyl cyclase pathway. Paradoxically, *cap*$^-$ cells are sensitive to nitrogen starvation and yet resistant to heat shock. We have not previously seen a separation of these phenotypes in cells with mutations in the RAS/adenylyl cyclase pathway. These effects can be readily explained if CAP is multifunctional. Thus, CAP may have an enzymatic function in its own right or may be required for other signaling systems. We have not yet determined if these other putative functions of CAP are dependent upon RAS.

The major impetus for these studies has been to identify the effector of yeast RAS proteins. Because yeast RAS proteins are structurally and functionally related to mammalian RAS proteins, identification of yeast RAS effectors may yield clues about mammalian RAS effector functions. The experiments here suggest that another protein is required for a RAS-responsive adenylyl cyclase. It is possible that CAP may be the yeast RAS effector and prove to be a useful reagent for identifying RAS effectors in other organisms.

Experimental Procedures

Raising *CAP* Antisera

Adenylyl cyclase was purified from the strain JF36A (Table 2) by scaling up procedures we previously described (Field et al., 1988). Approximately 300 liters of cells were grown in the Sloan Kettering fermenter facility to a density of 2 × 10^7 cells per ml in YPD medium (1% yeast extract, 2% peptone, 2% glucose [Difco Laboratories, Detroit, MI]), harvested, quick frozen in liquid nitrogen, and stored at −80°C until needed. Soluble membrane extracts were prepared from about 20% of the frozen cells at a time. About 10% of each extract was subjected to monoclonal antibody affinity chromatography at a time. Approximately 40 affinity-purified preparations were pooled, concentrated by ultrafiltration from a volume of 300 ml to a volume of 5.7 ml [Amicon, Danvers, MA], and then subjected to glycerol gradient sedimentation using 0.2 ml of concentrated enzyme per 5 ml of gradient. The tubes containing the gradients were punctured, and fractions were collected. Peaks of adenylyl cyclase activity were identified, pooled, concentrated by ultrafiltration, subjected to SDS gel electrophoresis, and then stained with Coomassie blue. Based on comparisons with standards, we calculate that a total of 20–50 μg of 70 kd subunit was purified from the 300 liters of cells. The 70 kd band was excised, homogenized, resuspended in Freund's adjuvant, and then used as an antigen. Subcutaneous injections of 1–2 μg were given to two rabbits (New Zealand white females, specific pathogen free, purchased from Hare Marland, Hewitt, NJ) at 2–4 week intervals; beginning after about 8 weeks the rabbits produced antisera. Rabbits were bled periodically, and sera was stored at −80°C until needed. For consistency, the results reported here were all obtained with antisera from the same bleed.

Immunological Assays

Immunoprecipitations were carried out typically with 100 μl of the solubilized membrane extract described above, the indicated amounts of antisera, and 30 μl of 50% protein A–Sepharose beads (Sigma, St. Louis, MO). Mixtures were rotated for 1 hr at 4°C. Then, 50 μl of the supernatant was withdrawn, the pellet was washed three times with buffer C (Field et al., 1988) containing 1% lubrol and 0.5 M NaCl, and both the supernatant sample and pellet were tested in adenylyl cyclase assays. Appropriate correction factors were used in analyzing the data to account for the dilutions and relative amounts of sample assayed.

Western blots were carried out using a blotting cell (Bio-Rad, Richmond, CA), following procedures recommended by the manufacturer for transfer to nitrocellulose membranes. Detection was by colloidal gold-conjugated second antibodies in Figure 1 (Bio-Rad) and by ^{125}I-labeled protein A in Figure 3 (ICN, Irvine, CA). A 1:300 dilution of the anti–70 kd antisera was used in each experiment. In Figure 1, a 3 μg/ml concentration of monoclonal antibody 12CA5 was used.

For screening, the cDNA library phage plaques were incubated for 3 hr with nitrocellulose filters soaked in 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma) and then subjected to immunological detection. Duplicate lifts were made from each plate. A 300-fold dilution of an E. coli lysate-treated CAP antisera was used. Detection was by goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad), using 3,3′-diaminobenzidine as a color reagent (Sigma). Because IPTG was added to phage after they had already formed plaques, positive signals had a halo shape that aided in detection.

Other Procedures

The cDNA library was a generous gift of Jeff Kuret of Cold Spring Harbor Laboratory. It was prepared from the S. cerevisiae strain SP1. The library was cloned into the EcoRI site of λ-Zap (Strategene, La Jolla, CA) using EcoRI linkers. Phage were grown in the strain BB4 under conditions recommended by the manufacturer. Excision and other manipulations were also as recommended by the manufacturer. DNA sequencing was carried out by a modification of the Sanger-Coulson method. Both strands were sequenced. The sequence of clone #2 (pCAP-2) is shown, beginning and ending with the EcoRI site presumably derived from the linkers. Clone #1 (pCAP-1) was partially se-

Table 2. Strains Used in This Study

Strain	Genotype	Reference
SP1	*MATa leu2 his3 ura3 trp1 ade8 can1*	(Toda et al., 1985)
TK161-R2V	*MATa leu2 his3 ura3 trp1 ade8 can1 RAS2*^{val19}	(Toda et al., 1985)
JF36A	*MATα leu2 his3 ura3 trp1 ade8 can1 ras1::HIS3 ras2::URA3* (pEF-*CYR1*)	(Field et al., 1988)
TKB111	*MATα leu2 his3 ura3 trp1 ade8 can1 ras1::HIS3 ras2::URA3* (pADH-*CYR1*)	(Field et al., 1987)
JC1	*MATα leu2 his3 ura3 trp1 ade8 lys2 can1*	
JC1T[a]	*MATα leu2 his3 ura3 ade8 lys2 can1*	
SPJCT[b]	*MATa/MATα leu2/leu2 his3/his3 ura3/ura3 trp1/+ ade8/ade8 lys2/+ can1/can1*	
JFSKN37[c]	*MATa leu2 ura3 trp1 ade8 can1 RAS2*^{val19} *cap::HIS3*	
JFSKN43[d]	*MATa leu2 his3 trp1 ade8 can1 RAS2*^{val19} *cap::URA3*	
JFSKN46[d]	Same as JFSKN43, independent isolate	
JFSKO13[e]	*MATa leu2 ura3 trp1 ade8 can1 cap::HIS3*	
JFSKN22[f]	*MATa leu2 his3 trp1 ade8 can1 cap::URA3*	
JFSKN25[f]	Same as JFSKN22, independent isolate	
JFSKN32[g]	*MATa leu2 ura3 trp1 ade8 can1 cap::HIS3*	
JFSKN34[g]	Same as JFSKN32, independent isolate	
RS27-2B	*MATα his3 ura3 trp1 ade8 RAS2*^{val19} *supC*	
TKR1-2LS	*MATa leu2 his3 trp1 ade8 RAS2*^{val19} *ras1::URA3 supC*	

[a] Trp$^+$ revertant of JC1.
[b] Cross between JC1T and SP1.
[c] *CAP* was disrupted in TK161-R2V with EcoRI fragment of pHSPN5.
[d] *CAP* was disrupted in TK161-R2V with EcoRI fragment of pUSMN2.
[e] *CAP* was disrupted in SP1 with EcoRI fragment of pHSM13.
[f] *CAP* was disrupted in SP1 with EcoRI fragment of pUSMN2.
[g] *CAP* was disrupted in SP1 with EcoRI fragment of pHSPN5.
Unless otherwise indicated, all strains are from this paper.

quenced. It differed only in the sequences near the linkers. Standard protocols were used for other DNA manipulations (Maniatis et al., 1982).

The T7 expression system was prepared as follows. The phagemid pT7.7f1A was derived from pPK1 (Field et al., 1988). The SalI HindIII-digested vector fragment of pPK1 was ligated to a SalI–HindIII fragment encoding the yeast gene SCH9 to form the plasmid pT7.SCH9 (Toda et al., 1988). The SCH9 gene was removed from pT7.SCH9 by SacI–HindIII digestion followed by treatment with T4 DNA polymerase in the presence of 80 μM dNTPs. Ligation of the blunt-ended vector fragment yielded pT7. A particular clone of this plasmid, pT7.7, was digested with EcoRV and ligated to the Klenow-filled BamHI–EcoRI fragment of pUCf1 (Pharmacia, Piscataway, NJ) that includes the bacteriophage f1 intergenic region. The two possible orientations of the f1 origin are designated A and B. The phagemid pT7.7f1A produces single-stranded phage that contain DNA complimentary to the T7 promoter.

Convenient restriction sites were added to pCAP-2 cDNA by DNA amplification using the polymerase chain reaction (Saiki et al., 1988). One oligonucleotide, 5′-GTGAAATCGACATATGCCTGACT, encoded an NdeI site that encompassed the initiator methionine, while the other, 5′-ATTCAAAGTCGACTTATTTCTTTTA-3′, encoded a SalI site 3′ of the termination codon. The polymerase chain reaction was performed according to the manufacturer's recommendations, using 10 ng of BamHI-digested pCAP-2 as the template. The thermal cycler was programmed for 1.5 min at 94°C, 3 min at 55°C, and 7 min at 72°C. The 1.6 kb amplified DNA fragment was isolated from an agarose gel following electrophoresis, treated with T4 DNA polymerase in the presence of 80 μM dNTPs, digested with NdeI and SalI, and ligated to SalI–NdeI-digested pT7.7f1A, yielding pT7.CAP. This plasmid was transformed into the E. coli strain BL-21(DE3) pLysS, and protein was expressed as described (Studier and Moffatt, 1986).

Plasmids used for gene disruption were constructed by subcloning a 1.2 kb HindIII fragment containing the *URA3* gene or a 1.7 kb BamHI fragment containing the *HIS3* gene between the HpaI sites of pCAP-1. Prior to ligation, the ends of the marker fragments were filled-in using Klenow fragment. The plasmid pUSP1 contains the *URA3* gene oriented so that *URA3* transcription is from the same strand as *CAP* transcription, and the plasmid pUSM2 contains the *URA3* gene oriented so that *URA3* transcription is from the opposite strand as *CAP* transcription. The plasmid pHSM13 contains the *HIS3* gene oriented so that *HIS3* transcription is from the same strand as *CAP* transcription, and the plasmid pHSP6 contains the *HIS3* gene oriented so that *HIS3* transcription is from the opposite strand as *CAP*. Other disruption plasmids were made by digesting the plasmids pHSP6 and pUSM2 with NsiI, isolating the vector fragment, and ligating them back together. The resulting plasmids were pUSMN2 for *URA3* and pHSPN1 or pHSPN5 for *HIS3*. These plasmids are shown in Figure 4. To disrupt the gene in yeast, these plasmids were digested with EcoRI, and the resulting fragments were used to transform strains JC1, SP1, TK161-R2V, or SPJCT. Disruption of *CAP* was confirmed by Southern blot analysis.

Standard yeast growth conditions and genetic manipulations were used (Sherman et al., 1986). Membrane extracts, RAS purifications, and adenylyl cyclase assays were as described previously (Salomon et al., 1974; Broek et al., 1985).

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