Adenylate Cyclase in *Saccharomyces cerevisiae* Is a Peripheral Membrane Protein

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The adenylate cyclase system of the yeast *Saccharomyces cerevisiae* contains the *CYR1* polypeptide, responsible for catalyzing formation of cyclic AMP (cAMP) from ATP, and two *RAS* polypeptides, which mediate stimulation of cAMP synthesis of guanine nucleotides. By analogy to the mammalian enzyme, models of yeast adenylate cyclase have depicted the enzyme as a membrane protein. We have concluded that adenylate cyclase is only peripherally bound to the yeast membrane, based on the following criteria: (i) substantial activity was found in cytoplasmic fractions; (ii) activity was released from membranes by the addition of 0.5 M NaCl; (iii) in the presence of 0.5 M NaCl, activity in detergent extracts had hydrodynamic properties identical to those of cytosolic or NaCl-extracted enzyme; (iv) antibodies to yeast adenylate cyclase identified a full-length adenylate cyclase in both membrane and cytosol fractions; and (v) activity from both cytosolic fractions and NaCl extracts could be functionally reconstituted into membranes lacking adenylate cyclase activity. The binding of adenylate cyclase to the membrane may have regulatory significance; the fraction of activity associated with the membrane increased as cultures approached stationary phase. In addition, binding of adenylate cyclase to membranes appeared to be inhibited by cAMP. These results indicate the existence of a protein anchoring adenylate cyclase to the membrane. The identity of this protein remains unknown.

Adenylate cyclase is a pivotal enzyme in the cells of both mammals and yeast cells. In mammals, the enzyme serves signalling functions that are critical in many homeostatic mechanisms (9). In yeast cells, nutritional signals are transmitted through adenylate cyclase, which in turn regulates cellular growth by altering cyclic AMP (cAMP) levels (18). Activity in both systems is modulated by GTP-binding proteins; G_s and G_i regulate adenylate cyclase in animals (9), and *RAS1* and *RAS2* regulate adenylate cyclase activity in *Saccharomyces cerevisiae* (26). In addition to sharing similar biochemical functions, the *RAS* proteins share amino acid sequence similarities with the superfamily of GTP-binding proteins that includes the G proteins and p21^{ras} products of the *ras* proto-oncogenes (1).

Models of the yeast enzyme have been based loosely on past experience with the more thoroughly studied animal adenylate cyclase. Thus, it has been suggested that the *RAS* proteins may interact with some form of receptor protein in a manner analogous to that of the G proteins. Candidates for genes encoding such proteins have been tentatively proposed (2, 3, 21, 24). In a similar way, it has been presumed that the yeast enzyme might be anchored directly to the phospholipid bilayer, resembling the mammalian enzyme, which is known to be an integral membrane protein. The best illustration of this assumption has been the use of detergents to solubilize the yeast adenylate cyclase from the membrane (7, 10).

Previous studies have indicated that adenylate cyclase in detergent extracts behaves like a large particle on gel filtration and sucrose density centrifugation. In the presence of 0.5 M NaCl, the size of this complex is reduced to approximately 600 kilodaltons (kDa) (7, 10). This is considerably larger than the size of a single catalytic subunit, indicating an oligomeric structure.

We have conducted experiments to probe the structure of the yeast adenylate cyclase enzyme complex. We present

evidence indicating that yeast adenylate cyclase is not an integral membrane protein but is attached to the membrane via some unidentified macromolecule. Activity can be extracted from the membranes by addition of NaCl. These extracts were used to functionally reconstitute activity into membranes devoid of endogenous activity, prepared from *cyr1* mutant yeast strains. Membranes prepared from *cyr1* mutants grown at high cAMP concentrations were less effective in reconstituting activity than membranes from mutants grown at lower cAMP levels. These experiments imply the presence of a component that anchors adenylate cyclase to the membrane. This component may serve a regulatory as well as a structural role.

MATERIALS AND METHODS

Abbreviations. GppNHp, 5'-guanylylimidodiphosphate; GDPβS, guanosine 5'-O-(2-thiodiphosphate); IBMX, 3-isobutyl-1-methylxanthine. Adenylate cyclase activity units are expressed as picomoles of cAMP produced per minute.

Materials. Adenylate cyclase grade [α -³²P]ATP (40 cpm/pmol) was from Amersham Corp., and [³H]cAMP (10,000 cpm per assay) was from New England Nuclear Corp. [³⁵S]methionine was from Irvine Chemical and Nuclear.

Media. Yeast cells (Table 1) were grown on either rich medium (YPD) containing 1% yeast extract, 2% Bacto-peptone (Difco Laboratories), and 2% glucose or on minimal medium (SD) containing 6.7 g of yeast nitrogen base per liter, 2% glucose, and the appropriate factors to support the growth of auxotrophic strains.

Expression of *CYR1* proteins with in-frame internal deletions. Plasmids encoding *CYR1* proteins with in-frame internal deletions were prepared from the plasmid pAC2 (4, 10). References to residue number are based on the predicted amino acid sequence of Kataoka et al. (13). pAC103 was prepared by removal of a 3.7-kilobase (kb) *SacI* fragment as described previously (10a). This produced an in-frame deletion of the codons for amino acids 27 to 1233. pAC104 was prepared by removal of 4.06 kb between the *SacI* site at

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
381G (wild type)	<i>MATa ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3 cryR</i>	9a
HR125 (wild type)	<i>MATa leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4</i>	4
NW23-9C (<i>cyr1-1</i> in HR125)	<i>MATa cyr1-1 cam leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4</i>	4
TC41-1 (<i>cyr1</i> deletion in NW23-9C)	<i>MATa cyr1::URA3 cam leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4</i>	10a
GC118-8A (<i>bcy1</i> in TC41-1)	<i>MATa cyr1::URA3 bcy1 leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4</i>	4
AM53-1A (<i>bcy1</i> mutant)	<i>MATa bcy1 his7 gal7-2</i>	K. Matsumoto

position 739 and the *SacII* site at position 4801. The ends were blunted with mung bean nuclease and religated to produce an in-frame deletion of the codons for amino acids 27 to 1381. pAC202 was produced by deletion of 1.5 kb between the *EcoRI* sites at positions 1066 and 2572, producing an in-frame deletion of the codons for amino acids 135 to 636. pAC203 was produced by deletion of a 0.88-kb *KpnI* to *HpaI* fragment, blunting the ends with T4 polymerase, and religation. This produced an in-frame deletion of the codons for amino acids 42 to 333. These plasmids and the wild-type pAC2 were used to transform the *cyr1-1 cam* yeast strain NW23-9C (4) by the lithium acetate method of Ito et al. (12). Transformants were grown on minimal medium lacking uracil and supplemented with 1 mM cAMP.

Preparation of yeast particulate fractions. Yeast cells were grown to an OD₆₆₀ of approximately 2 to 4. The cultures were centrifuged, and cells were disrupted by vortexing with glass beads in YMB buffer containing 50 mM MES (2[N-morpholino]ethanesulfonic acid [pH 6.0]), 0.1 mM EDTA, 0.1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 50 µg of leupeptin per ml as described previously (5). The homogenate was centrifuged in a Beckman J6 for 5 min at 1,000 rpm to remove nuclei and unbroken cells. The supernatant was then spun for 30 min at 14,000 rpm to separate the particulate and soluble fractions. Both particulate and soluble fractions were stored in YMB at -70°C until needed.

³⁵S-labeling of yeast proteins. Typically, a 100-ml culture was grown in minimal medium lacking methionine to which 1 mCi of [³⁵S]methionine was added. The cultures were grown at 30°C overnight to a density with an OD₆₆₀ of approximately 1, and the cells were disrupted as described above.

Immunoprecipitation. ³⁵S-labeled fractions were extracted with NDET.3 (20 mM Tris [pH 7.5], 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA 0.3% sodium dodecyl sulfate [SDS]). Typically, samples containing between 100 and 500 µg of protein and approximately 90 × 10⁶ cpm were extracted in 200 µl of NDET.3 for 30 min at room temperature, the mixture was centrifuged for 15 min in an Eppendorf microcentrifuge at 14,000 rpm, and the supernatant was incubated overnight at 4°C with 2 µg of antibody 31 (10a). Antibodies were precipitated with 15 µl of a 10% suspension of fixed *Staphylococcus aureus*. The *S. aureus* cells were collected by centrifugation, suspended in NDET.3, and washed twice by centrifugation through a 1-ml cushion of 30% sucrose in 0.5× NDET.3. The pellet was then washed in water, and immunoprecipitated proteins were released for SDS gel electrophoresis by boiling in sample buffer containing 2-mercaptoethanol (15a). Proteins were resolved on 5% polyacrylamide gels, and the gels were stained, treated with En³Hance, and dried for autoradiography.

Reconstitution assays. Yeast membranes from a *CYR1*⁺ strain (381G) were extracted by incubation in YMB containing 0.5 M NaCl for 30 min on ice. The suspension was centrifuged for 15 min at 14,000 rpm in an Eppendorf

microcentrifuge (substituting 1 h of ultracentrifugation at 100,000 × g did not affect the outcome), and the supernatant was withdrawn. The extracts were then reconstituted by mixing them with membranes prepared from a *cyr1* mutant strain (TC41-1) and reducing the NaCl concentration either by 10-fold dilution in reconstitution buffer (50 mM MES, 5 mM MgCl₂, pH 6.0) at 4°C for 1 h or by dialysis against this buffer at 4°C for 4 h (where indicated). The membranes were then isolated by centrifugation for 30 min at 15,000 rpm and assayed for adenylate cyclase activity.

Adenylate cyclase assay. Adenylate cyclase was assayed by a modification of the method of Salomon et al. (22) as described by Casperson et al. (4). Guanine nucleotides, when present, were used at 10 µM; manganese was present at 10 mM. Protein was assayed by the method of Lowry et al. (16).

RESULTS

Dissociation of adenylate cyclase activity from a large complex in detergent extracts. When we used gel filtration to measure the size of adenylate cyclase detergent solubilized from the membrane with taurocholate, we found that the enzyme could exist as two different-sized particles. At low ionic strength, the enzyme behaved like a very large particle of several million daltons; however, when 0.5 M NaCl was added, this size was dramatically reduced.

As shown in Fig. 1A, at low ionic strength, activity eluted from a Sepharose 4B column as a large particle, very near the void volume. Although beyond the range of the standards, this indicates a particle size of at least several million daltons. This does not represent incomplete solubilization; the enzyme remained soluble after centrifugation at 100,000 × g for 1 h.

When 0.5 M NaCl was added to the detergent extracts, activity eluted as a much smaller particle, corresponding to a protein of approximately 600 kDa (Fig. 1B) (10). A variable amount of activity generally migrated in the void volume and apparently represents unsolubilized enzyme, since it could be removed by spinning the sample at 100,000 × g prior to applying it to the column. Activity eluting in the included volume eluted at the same position when rechromatographed (not shown). In the presence of NaCl, detergent could be omitted from the column equilibration buffer without altering the mobility of the adenylate cyclase activity.

These results indicate that in detergent extracts at low ionic strength, the catalytic *CYR1* protein is associated with a large complex and that this association can be disrupted by addition of NaCl.

Release of soluble adenylate cyclase activity from membranes. The dissociation of adenylate cyclase activity from a larger particle induced by NaCl in detergent extracts was paralleled in membrane preparations. When crude membrane preparations were incubated with increasing concentrations of NaCl, adenylate cyclase activity was released

FIG. 1. Gel filtration of detergent extracts. Membranes (strain 381G, 15 mg) were centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge, and the pellet was suspended in 1.4 ml of YMB with 5% taurocholate. The mixture was incubated with periodic agitation on ice for 1 h. The mixture was then centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge, and the supernatant was withdrawn. (A) The supernatant (1 ml) was loaded onto a Sepharose 4B column (1 by 46 cm) equilibrated in 50 mM MES–250 mM sucrose–5 mM $MgCl_2$ –1 mM EDTA–0.02% sodium azide–5% sodium taurocholate. (B) The treatment was the same except that NaCl was added to the extract and equilibration buffer to a concentration of 500 mM. Fractions (1.1 ml) were collected and assayed for adenylate cyclase activity with Mn^{2+} . V₀, Void volume; IgM and IgG, immunoglobulin M and G, respectively; β-Gal, β-galactosidase.

from the membrane into the supernatant fraction (Fig. 2A). Typically, we observed release of approximately 60 to 70% of the total activity into the supernatant. This was a rapid process; complete release took less than 5 min at 0°C. Release was maximal at about 0.5 M NaCl. In most cases, a significant fraction of the total activity remained behind in the pellet fraction and could not be released by the addition of more NaCl. As shown in Fig. 2B, a portion of the particulate activity was also resistant to detergent extraction. In the experiment shown, extraction with 2% taurocholate was effective in solubilizing only 50% of the total activity from the pellet. We have screened several ionic and nonionic detergents, including cholate, deoxycholate, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, Lubrol PX, and Triton X-100, and have found taurocholate to be the most effective detergent for solubilizing adenylate cyclase, in terms of both total amount and percentage of activity released. Following extraction with NaCl, detergent extraction with 5% taurocholate was not effective in solubilizing additional activity from the pellet. With either NaCl or detergent extraction, the activity remaining in the pellet must be in some way aggregated or associated with an insoluble component of the cell.

Location of adenylate cyclase activity. Because activity was so readily released from the membranes with NaCl, we examined supernatant fractions from yeast membrane preparations for adenylate cyclase activity. In all cases, we found a significant amount of activity in the soluble fraction produced during the disruption of the yeast cells. The percent-

FIG. 2. NaCl and taurocholate extraction of adenylate cyclase. Strain 381G membranes (2 mg/ml) were treated with the indicated concentration of either NaCl (A) or sodium taurocholate (B) in YMB (final volume, 200 μ l). Samples were incubated on ice for 1 h (taurocholate) or 30 min (NaCl) and then centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge to separate pellet (dark bars) and supernatant (cross-hatched bars) fractions. Pellet fractions were suspended in 200 μ l of YMB, and all samples were adjusted to equal concentrations of either NaCl (A) or taurocholate (B) and assayed for adenylate cyclase activity with Mn^{2+} . Activity is expressed as the total activity in each fraction.

age of soluble activity varied from 20 to 80% of the total adenylate cyclase activity, depending on the strain and conditions. Typically, about 50% of the total activity was found in the soluble fraction. We do not know whether this represents cytosolic activity or activity released from the membrane by the cell disruption process. We will refer to this activity as cytosolic activity to distinguish it from soluble activity extracted with NaCl or detergent. Of the activity remaining in the membranes, about 60 to 70% (or approximately 30% of the starting total) could be released by the addition of NaCl (Fig. 2A). Thus, about 80% of the total adenylate cyclase activity was either soluble upon disruption of the cell or extractable with NaCl.

In order to compare the adenylate cyclase from detergent extracts, salt extracts, and cytosolic fractions, we measured the size of the enzyme in all three fractions by using gel filtration (Fig. 3). All samples were run on a Sepharose 4B column equilibrated in YMB containing 0.5 M NaCl. No detergent was added, because the mobility of the adenylate cyclase in detergent extracts was unchanged by the presence or absence of detergent in the column buffer. By hydrodynamic criteria, the three enzyme preparations were indistin-

FIG. 3. Gel filtration of detergent, NaCl, and cytosolic extracts. Sepharose 4B chromatography was as described in the legend to Fig. 1B except that taurocholate was omitted from the equilibration buffer. The column was loaded with (A) detergent extract as described for Fig. 1B; (B) the cytosolic fraction prepared during cell disruption as described in the text; or (C) NaCl extract prepared as described for the detergent extract, except that YMB with 500 mM NaCl was used in place of the detergent extraction buffer.

guishable. All of the preparations eluted from the column with a similar pattern, the major peak eluting with a Stokes radius of approximately 11 nm and a variable peak of activity eluting in the void volume. The peak of activity in the void volume was minimized by careful removal of the supernatant during the extraction process, and these fractions tended to be cloudy; thus, the void volume activity is probably still associated with a particulate fraction.

Immunoprecipitation of *CYR1* protein from membrane and cytosol fractions. The immunoprecipitation experiment shown in Fig. 4 demonstrates that after cell disruption, the *CYR1* polypeptide was found in both the soluble and particulate fractions. Membrane and cytosol fractions from a *CYR1*⁺ strain [HR125(pAC2)] and a control *cyr1* strain (TC41-1) labeled with [³⁵S]methionine were solubilized and immunoprecipitated with an antibody to the *CYR1* polypeptide (10a). An immunoreactive band at approximately 205 kDa, corresponding to the full-length *CYR1* protein, was observed in both the soluble and particulate fractions of the *CYR1*⁺ strain (lanes B and D) but not at all in the *cyr1* strain (lanes A and C). The *CYR1* protein was present in roughly equal amounts in both the membranes (lane B) and cytosol (lane D), corresponding well with the relative levels of adenylate cyclase activity observed in these two fractions.

Taken together, the results support a model in which adenylate cyclase is a soluble protein that can associate with a membrane-bound protein to form an oligomeric complex. With detergents, adenylate cyclase can be extracted from

FIG. 4. Immunoprecipitation of yeast adenylate cyclase. Cytosolic and membrane fractions from the indicated yeast strains were labeled with [³⁵S]methionine and prepared as described in the text. Membrane and cytosolic fractions were suspended in equivalent volumes of buffer so that abundances of immunoprecipitated products could be compared directly. Labeled proteins were immunoprecipitated as described in the text and run out on a 5% polyacrylamide gel. Samples to be immunoprecipitated contained: lane A, TC41-1 membranes, 78×10^6 cpm; lane B, HR125(pAC2) membranes, 88×10^6 cpm; lane C, TC41-1 cytosol, 181×10^6 cpm; lane D, HR125(pAC2) cytosol, 98×10^6 cpm. Autoradiograms were exposed for 48 h.

the membrane as a part of this complex. The association of adenylate cyclase with this complex can be disrupted by NaCl, reducing the size of the enzyme in detergent extracts, or releasing activity from the membranes.

Reconstitution of soluble adenylate cyclase activity into membranes. In order to determine whether dissociation of adenylate cyclase from the membrane is reversible, we reconstituted salt-extracted adenylate cyclase from a wild-type strain (381G) into membranes prepared from TC41-1, *cyr1* mutant yeast strain (Fig. 5). After mixing the wild-type extract with the *cyr1* acceptor membranes, the extraction process was reversed by diluting the NaCl concentration 10-fold, allowing adenylate cyclase to reassociate with the membranes. The addition of extract restored guanine nucleotide-sensitive adenylate cyclase activity to the membranes, indicating functional coupling between the *CYR1* protein and the *RAS* proteins on the membrane. Activity in the presence of the nonhydrolyzable GTP analog GppNHp was more than fourfold greater than that seen with the GDP analog GDPβS.

Without extract, no adenylate cyclase activity was ever observed in these membranes (not shown). The extract by itself contained Mn^{2+} -dependent activity, but only very low levels of activity with Mg^{2+} ATP, and this activity was not stimulated by the addition of GppNHp. Boiling the acceptor membranes prior to reconstitution markedly diminished the amount of activity in the pellet; however, even in the complete absence of acceptor membranes, some Mn^{2+} -dependent activity always appeared in the pellet fraction. Thus, there is a component of activity in the pellet fraction that represents simple precipitation of enzyme activity rather than functional reconstitution of adenylate cyclase into membranes.

The use of Mn^{2+} ATP as a substrate for adenylate cyclase produces high catalytic activity in the absence of any inter-

FIG. 5. Reconstitution of NaCl-extracted adenylate cyclase into membranes. Membranes from wild-type yeast cells (381G, 26.5 mg) were suspended in 1.4 ml of YMB containing 500 mM NaCl and extracted as described in the legend to Fig. 2. Samples containing 200 μ l of the extract were then mixed with 15 mg of membranes prepared from a *cyr1* mutant (TC41-1), diluted into 5 ml of reconstitution buffer, and incubated for 30 min at 4°C. One sample contained TC41-1 membranes stored on ice, while a control sample contained membranes that had been boiled for 5 min prior to reconstitution as indicated. The mixtures were centrifuged for 30 min at 15,000 rpm, and the pellets were suspended in 750 μ l of reconstitution buffer and assayed for adenylate cyclase activity in the presence of GDP β S (cross-hatched bars), GppNHp (light bars), or Mn²⁺ (dark bars). Activity is expressed as totals for each reconstitution.

action with other components. However, although this activity is useful for following the catalytic subunit, it is not of physiological importance. The restoration of guanine nucleotide sensitivity of the enzyme preparation was more significant than the appearance of Mn²⁺-dependent activity in the pellet. Although some Mn²⁺-dependent activity appeared in the pellet in the absence of membranes, stimulation by guanine nucleotides was never observed without the addition of native acceptor membranes. Thus, a hallmark of reconstitution into membranes was not so much the appearance of Mn²⁺-dependent activity in the pellet fraction but the restoration of guanine nucleotide sensitivity.

Reconstitution of soluble adenylate cyclase activity onto membranes appears to be a saturable binding process. In the reconstitution experiment shown in Fig. 6, the concentration of extract was varied while the quantity of acceptor membranes was held constant. Specific binding was obtained by subtracting the activity found in the pellet in the absence of acceptor membranes from the activity in the pellet with acceptor membranes present (Fig. 6, inset). The amount of activity associated with the membranes increased as the concentration of extract increased and reached a plateau at approximately 8 pmol of cAMP per min per assay (1.8 U/mg of acceptor membranes).

The degree of stimulation by guanine nucleotides in reconstituted membranes was higher than that observed in the wild-type membranes prior to extraction. This may be the result of a relative excess of RAS proteins or some other component provided by the membranes over catalytic subunit reconstituted from the extract. When the amount of extract was held constant while the quantity of acceptor membranes was varied, the fold stimulation by GppNHp was proportional to the amount of membranes added (Fig. 7). Both the amount of activity associated with the membranes and the fold stimulation by GppNHp increased as the ratio of membranes to extract increased.

Enzyme activity appearing in the cytosol fraction after cell disruption could also be reconstituted into membranes. With these fractions, it was sufficient to simply incubate the soluble enzyme together with the membranes. After the incubation period, the membranes were collected by centrifugation and assayed for adenylate cyclase activity. As with the NaCl extracts, the cytosolic extract was able to restore guanine nucleotide-sensitive adenylate cyclase activity to the TC41-1 acceptor membranes (not shown).

Effect of cAMP on reconstitution. Our initial experiments had been based on the assumption that some intrinsic difference between the soluble and membrane-bound forms of adenylate cyclase was responsible for the differences in localization. However, another possible model presumes only a single form of adenylate cyclase but a limiting number of binding sites on the membrane. In this case, the proportion of enzyme in the cytosolic fraction would depend on the abundance of membrane-binding sites. The availability of a reconstitution system allowed us to directly examine changes in the capacity of the membrane to accept soluble adenylate cyclase.

In order to determine whether cAMP has any feedback effects on membrane association, we conducted a reconstitution experiment with acceptor membranes prepared from a *cyr1* mutant grown at several different concentrations of cAMP (Fig. 8A). The yeast cells were first grown to late log phase in YPD medium containing 1 mM cAMP in order to obtain a sufficient quantity of cAMP-dependent cells. The cells were then washed with sterile water to remove the remaining cAMP, and they were then diluted into fresh medium containing the indicated concentration of cAMP.

IBMX, an inhibitor of cAMP phosphodiesterase activity, was also added in order to prevent intracellular degradation of the cAMP and to allow a more effective increase in intracellular cAMP levels. Increasing concentrations of cAMP decreased the capacity of the membranes to bind adenylate cyclase in the subsequent reconstitution assay. The results in Fig. 8B indicate that this effect was due to a decrease in binding capacity rather than a loss of affinity. Membranes from cells grown with low concentrations of cAMP (100 μ M) were able to reconstitute adenylate cyclase activity was a capacity similar to the results shown in Fig. 6 (approximately 2.2 U/mg of acceptor membranes), while membranes grown with high cAMP (5 mM) had a greatly decreased capacity to bind adenylate cyclase (approximately 0.36 U/mg of acceptor membranes). Activity that failed to bind to the membranes prepared from the 5 mM cAMP-grown cells was fully capable of being reconstituted into membranes prepared from cells grown at lower cAMP concentrations (not shown).

Yeast cells carrying mutations in the regulatory subunit of the cAMP-dependent protein kinase, encoded by the *bcy1* gene, produce constitutively activated cAMP-dependent protein kinase activity (25). Because yeast strains with the *bcy1* mutation resemble yeast strains growing at constantly elevated cAMP levels, membranes from *bcy1* strains might also be less effective in reconstituting adenylate cyclase activity. We compared the ability of membranes prepared from a *bcy1* strain (GC118-8) to reconstitute activity with that of membranes from TC41-1 (Fig. 9A). The TC41-1 strain has a wild-type *BCY1* gene, while the *CYR1* gene has been deleted from both strains, so that neither strain produces adenylate cyclase activity of its own. Although the strains were grown under identical conditions, the *bcy1* strain membranes were much less effective in reconstituting adenylate cyclase activity than the control TC41-1 membranes.

FIG. 6. Effect of 381G extract concentration on reconstitution. Strain 381G extract (94 U/ml) was prepared as described in the legend to Fig. 5. Samples containing various amounts of extract (as indicated) were mixed with 150 μ l of TC41-1 membranes (4.5 mg), diluted with 10 ml of reconstitution buffer, and incubated for 30 min at 4°C. The mixture was centrifuged and assayed as described in the legend to Fig. 5. Values plotted represent specific binding, obtained by subtracting the activity of control samples containing no TC41-1 membranes and corresponding volumes of 381G (inset, dashed line) from the total activity obtained with acceptor membranes present (inset, solid line).

In the experiment shown in Fig. 9B, the capacity of the GC118-8 membranes to reconstitute adenylate cyclase was approximately half of that seen with the TC41-1 membranes.

Binding of adenylate cyclase to the membrane appears to be independent of *RAS* proteins. When cells of a *ras1*, 2 mutant strain were disrupted, enzyme activity partitioned primarily with the membranes (not shown). Thus, *RAS* proteins are not necessary for membrane attachment of adenylate cyclase. On the other hand, it is possible that changes in membrane binding affect the amount of adenylate cyclase that can interact with *RAS* proteins; enzyme released from the membrane would be inaccessible to *RAS* proteins and thus inactive.

Localization of adenylate cyclase activity during growth. We examined the localization of yeast adenylate cyclase at various stages of growth in order to determine whether culture density plays a part in the ability of the membrane to bind adenylate cyclase. At low cell density, cAMP levels are presumed to be high; as cells progress through log phase growth towards stationary phase, intracellular cAMP levels fall (8). Thus, harvesting cells at different densities, as shown in Fig. 10, is similar to the in vitro manipulation of cAMP levels in the reconstitution experiment shown in Fig. 8A. Cells were harvested at various densities, disrupted with glass beads, and separated into membrane and cytosol fractions. As wild-type cells reached increasing density, activity in the cytosol decreased, while the amount of activity associated with the membranes steadily increased. This was reflected in the decline of the ratio of supernatant activity to pellet activity (Fig. 10A). Cells at lower density, where cAMP levels are higher, had a much higher proportion of activity in the soluble fraction.

The results shown in Fig. 10B indicate that at least part of this change was mediated by the cAMP-protein kinase

system. Because yeast cells carrying the *bcy1* mutation have constitutively elevated cAMP-dependent protein kinase activity, they are unable to sense changes in intracellular cAMP levels. Therefore, *bcy1* strains should lack the ability to regulate membrane binding of adenylate cyclase in response to cAMP. Cells fractions prepared from a *bcy1* strain (AM53-1A) should resemble those of yeast strains growing at constantly elevated cAMP levels. As demonstrated in Fig. 10B, the *bcy1* cells yielded a relatively high and constant proportion of adenylate cyclase activity in the soluble fraction regardless of culture density.

Role of the repeated leucine-rich motif. In postulating that the *CYR1* protein binds to a protein on the membrane, it is also reasonable to postulate that the *CYR1* protein has a domain that recognizes this protein. We have constructed a set of in-frame deletion mutations in the *CYR1* coding region to try to produce mutant *CYR1* proteins that fail to bind to the membrane (Fig. 11). These constructions are based on pAC2, a YEp24 derivative carrying the *CYR1* gene (4). These mutations span a region of the *CYR1* gene that contains a repeating leucine-rich motif (13) in the middle of the open reading frame. This region is indicated by the shaded area in Fig. 11. Deletions at the amino end of the protein (pAC202 and pAC203) produced a distribution of activity between pellet and supernatant that approximated that of the wild-type enzyme. The deletions produced by pAC103 and pAC104 increased the fraction of adenylate cyclase found in the supernatant fraction. The protein encoded by pAC103 retained only 2 of the 20 leucine-rich repeats and was still partially bound to the membrane. These repeats were entirely absent in the protein encoded by pAC104, and this enzyme was found almost entirely in the supernatant. None of the altered enzymes were stimulated by GppNHp. Reconstitution experiments with the pAC103

FIG. 7. Effect of membrane concentration on reconstitution. Membranes from wild-type yeast strain 381G were extracted as described in the legend to Fig. 5. Samples of the extract were then mixed with the indicated quantity of membranes prepared from the *cyr1* mutant (TC41-1) and dialyzed against reconstitution buffer for 4 h at 4°C. The mixture was centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge, and the pellet was suspended in 750 μ l of dialysis buffer. (A) Samples were assayed for adenylate cyclase activity in the presence of GDP β S (cross-hatched bars) or GppNHp (light bars). (B) The ratio of activity with GppNHp to activity with GDP β S.

and pAC104 enzymes indicate that in the presence of saturating amounts of acceptor membranes, the pAC103 enzyme can be fully reconstituted, while the pAC104-encoded protein was not able to bind to the membranes at all (not shown). Although there are important limitations to the conclusions that can be drawn from this type of experiment (see below), the simplest interpretation is that the region containing the repeats is involved in membrane attachment.

DISCUSSION

Our results support a model containing three features. First, adenylate cyclase is a soluble protein that is indirectly anchored to the membrane through an unidentified component. Second, the capacity of the membrane to bind adenylate cyclase is affected by cAMP and may regulate interactions between the *CYR1* and *RAS* proteins. Finally, the leucine-rich repeats reported by Kataoka et al. (13) may function as a domain that binds to the unidentified component on the plasma membrane. These features are described in more detail below.

Soluble adenylate cyclase. The following observations indicate that adenylate cyclase is not an intrinsic membrane protein.

FIG. 8. Effect of cAMP treatment on the capacity of TC41-1 membranes to reconstitute adenylate cyclase activity. TC41-1 cells were grown overnight in YPD containing 1 mM cAMP. The cells were washed in sterile water and diluted 1:10 into fresh YPD containing the indicated concentration of cAMP and 100 μ M IBMX. The cells were grown for an additional 2 h, and membranes were then harvested as described in Materials and Methods. (A) Membranes (15 mg per assay) from each point were used in reconstitution assays as described in the legend to Fig. 7. Samples were assayed for adenylate cyclase activity in the presence of GDP β S (cross-hatched bars), GppNHp (light bars), or Mn^{2+} (dark bars). Activity is expressed as totals for each reconstitution. (B) Membranes (2.75 mg per data point) from the 100 μ M cAMP (solid diamonds) and 5 mM cAMP (open squares) points described above were used to reconstitute adenylate cyclase activity with various volumes of 381G extract as described in the legend to Fig. 6. Values plotted represent specific binding; corresponding control samples containing no TC41-1 membranes were assayed, and the values obtained were subtracted from the experimental data as described for Fig. 6.

(i) Both soluble and membrane-bound activities are found when cells are disrupted. Soluble adenylate cyclase activity made up a substantial fraction of the total in all cases examined. Although, in the experiments shown, we used a microcentrifuge to separate pellet and supernatant fractions, we have found that both the cytosolic activity and the activity in NaCl extracts remain soluble after ultracentrifugation for 1 h at 100,000 $\times g$. Thus, these activities are not likely to represent unsedimented particulate adenylate cyclase. Additionally, these activities migrate in the included volume on Sepharose 4B.

FIG. 9. Capacity of *bcy1* mutant membranes to reconstitute adenylate cyclase activity. Membranes were prepared from TC41-1, a *cyr1* mutant strain, and GC118-8, a *bcy1 cyr1* mutant strain as described in Materials and Methods. The membranes were used in reconstitution assays as described in the legend to Fig. 8. (A) Reconstitution of guanine nucleotide-sensitive activity. Samples were assayed for adenylate cyclase activity in the presence of GDP β S (cross-hatched bars), GppNHp (light bars), or Mn²⁺ (dark bars). (B) Reconstitution with various volumes of 381G extract as indicated in TC41-1 (solid diamonds) and GC118-8 (open squares). Values plotted represent specific binding; corresponding control samples containing no TC41-1 membranes were assayed, and the values obtained were subtracted from the experimental data as described for Fig. 6.

(ii) Activity remaining bound to the membrane can be removed by the addition of NaCl. Adenylate cyclase is anchored to the membrane through interactions that can be disrupted at high ionic strength. In addition to the experiments shown, we have successfully used other salts, including KCl, NH₄Cl, and MgSO₄, to remove activity from the membrane. Although most of the activity was removed by 0.5 M NaCl, a significant amount of activity remained in the pellet. We have not been able to solubilize this activity either by increasing the concentration of NaCl or by disrupting the bilayer with detergents. Thus, the resistance of NaCl extraction cannot be simply explained by proposing that the enzyme is embedded in a lipid bilayer. The remaining insoluble enzyme must be associated with some insoluble element by covalent modification or by some other mechanism that is not disrupted by detergent or high ionic strength. Incubation at 30°C with dithiothreitol had no effect on release.

FIG. 10. Effect of culture density on localization of adenylate cyclase activity. Yeast cells were grown to stationary phase (S) or to the indicated density (OD₆₀₀) in YPD medium, the cells were disrupted, and membrane and cytosol fractions were prepared as described in Materials and Methods. Membrane and cytosol fractions were assayed for adenylate cyclase activity in the presence of manganese. Activities are expressed as the ratio of supernatant to pellet activity. (A) 381G (wild type). (B) AM53-1A (*bcy1*).

(iii) Addition of NaCl also appears to dissociate the enzyme from a large complex in detergent extracts. Addition of NaCl to detergent extracts produced the dramatic size change demonstrated in Fig. 1. This appears to correspond to the dissociation from the membrane in that a dissociation event must also be occurring in the detergent extract when NaCl is added. After NaCl was added, detergent was no longer required to maintain the solubility of the enzyme. Although we cannot positively identify the target for adenylate cyclase as a protein, these observations are consistent with dissociation of a soluble enzyme from an integral membrane protein. The limited number of binding sites on the membrane and the decrease in reconstitution observed after heat treatment of the acceptor membranes support the idea that adenylate cyclase associates with a protein on the membrane.

Although markedly reduced, the size of the adenylate cyclase complex at high ionic strength is still much greater than can be accounted for by a single *CYR1* polypeptide. It seems likely that even after release from the membrane, this complex also includes the 70-kDa protein reported by Field et al. (7) to be associated with a *CYR1* fusion protein. This 70-kDa protein was immunopurified along with the *CYR1* protein from extracts containing taurocholate and 0.5 M NaCl and is presumably also associated with adenylate cyclase in our extracts as well.

(iv) A 205-kDa *CYR1* protein can be immunoprecipitated from both soluble and particulate fractions with anti-*CYR1* antibodies. The most prominent *CYR1* protein identified by immunoprecipitation was a 205-kDa protein identified in both the cytosolic and membrane fractions. This indicates that the full-length *CYR1* protein can be found in the soluble

FIG. 11. Expression of *CYR1* genes with in-frame internal deletions. Plasmids expressing the indicated portions of the *CYR1* coding region were prepared as described in the Materials and Methods section. Haploid *cyr1-1* yeast cells (NW23-9C) were transformed with the plasmids, and URA⁺ colonies were grown and disrupted as described in the text. Adenylate cyclase activity in supernatant and pellet fractions was measured with manganese ATP. Activity is expressed as percentage of the total.

fraction as well as associated with the membrane. In this experiment, adenylate cyclase was overexpressed by using a multicopy plasmid to improve the sensitivity of the immunoprecipitation. This produced about a 5- to 10-fold increase in activity. The activity in these cells appears to be identical to that observed in wild-type cells in terms of *RAS* coupling, solubility, and hydrodynamic properties. In cells carrying this plasmid, the distribution of adenylate cyclase activity was shifted slightly towards the cytosolic fraction (see below); however, the cytosolic enzyme produced by these cells remained qualitatively indistinguishable from that found in cells carrying only a single copy of the *CYR1* gene.

(v) Soluble activity can be functionally reconstituted onto membranes, regaining sensitivity to stimulation by guanine nucleotides. Both the cytosolic activity and adenylate cyclase extracted from membranes with NaCl could be functionally reconstituted into membranes. This indicates that the soluble adenylate cyclase has not been permanently modified in some way that prevents the enzyme from reassociating with the membrane.

Regulation of membrane binding. The model can be further developed by suggesting that association of adenylate cyclase with the membrane is limited by the availability of sites on the membrane and that this may be used as a mechanism for regulating the interactions between adenylate cyclase and *RAS* proteins. The evidence for this point comes from two observations.

(i) Not all of the adenylate cyclase activity in the cell appears to be associated with the membrane. Whenever we examined cytosolic fractions, we found substantial levels of adenylate cyclase activity, yet the soluble enzyme appears to be capable of membrane association in reconstitution experiments. It appears that binding of soluble adenylate cyclase onto membranes lacking adenylate cyclase is a saturable process limited by the number of binding sites (Fig. 6). Under normal conditions, the amount of activity that could be reconstituted into the TC41-1 membranes remained relatively constant, averaging about 2 U/mg of acceptor membranes. This is within the range of the specific activity of wild-type membranes prepared from the otherwise isogenic *CYR1*⁺ strain. Additionally, the concentration of enzyme extract needed to produce half-maximal binding was approximately 1.8 U/ml. If one assumes a turnover number of 1,000/min, this corresponds to an enzyme concentration

of 1.8 pM. Such an assumption is not unrealistic in light of the turnover number of 1,200/min reported for the purified mammalian adenylate cyclase and the reported similarities between the catalytic domains of the yeast and mammalian enzymes (15, 23). Regardless of the actual affinity for the membranes, complete reconstitution took place with enzyme that had been diluted well beyond the level produced during cell disruption. Thus, although it could be argued that soluble enzyme was observed as an artifact of cytosol dilution during cell disruption, reconstitution back to the membranes occurred at even greater dilutions. Together, these arguments indicate that binding to the membrane is limited by the number of sites that are available.

(ii) cAMP has a negative effect on the ability of membranes to bind adenylate cyclase. Membranes prepared from cells grown at high levels of cAMP were less effective in binding adenylate cyclase than membranes grown at lower cAMP levels. This was the result of an apparent decrease in the number of binding sites. Activity that failed to bind to membranes prepared from cells grown at high cAMP was fully capable of reconstitution when added to other TC41-1 membranes.

Additional evidence indicating that cAMP has a negative influence on membrane binding comes from strains with altered cAMP metabolism. The *bcy1* mutation results in constitutive activation of the target for cAMP, the cAMP-dependent protein kinases. Thus, these mutants resemble cells growing at elevated cAMP levels. Like membranes from cells grown at high cAMP levels, *bcy1* mutant membranes were relatively ineffective in binding adenylate cyclase activity. This reflected an apparent decrease in the number of sites available.

Overexpression of adenylate cyclase tended to reduce the fraction of activity associated with the membrane. The yeast strain expressing *CYR1* on the multicopy plasmid pAC2 had about 50% of its total activity associated with the membrane, while the isogenic wild-type strain had about 60 to 70% of the total activity associated with the membrane. On the other hand, strains lacking *RAS* proteins produced very low levels of cAMP. These strains tended to have a smaller proportion of adenylate cyclase activity in the soluble fraction than strains producing more activity (not shown). In strains expressing *CYR1* at high levels with a *GAL10* promoter, most of the activity was in the particulate fraction, but this

activity was not extractable with detergents or NaCl, nor was it responsive to guanine nucleotide stimulation.

In our experiments, the effects of cAMP were directed at the membranes rather than at the *CYR1* protein itself. The experiments described demonstrate an effect on the capacity of the membranes to bind adenylate cyclase; we have not yet determined what effect cAMP has on the ability of the *CYR1* protein to bind to the membrane. The effects of cAMP did not seem to be directed at stimulation by *RAS* proteins. Indeed, in the reconstitution experiment shown in Fig. 8A, at the higher cAMP points, although the total amount of enzyme reconstituted into the membranes was reduced, the fold stimulation by GppNHp was actually increased. The degree of stimulation by GppNHp observed in the reconstitution experiments was also uniformly higher than that observed in the native membranes. Stimulation by GppNHp increased as the ratio of acceptor membranes to extract increased (Fig. 7). These observations may indicate that the availability of *RAS* proteins normally limits the response to adenylate cyclase to guanine nucleotides.

It is tempting to propose a model in which the *CYR1* protein is bound reversibly to the membrane in a manner that can be regulated in accordance with the needs of the cell. The response to cAMP suggests the possibility of a feedback loop. Feedback control of cAMP production has been reported previously (19). Inhibition of *RAS2* activity by protein kinase A has also been reported (20). In addition, there are consensus sequences for cAMP-dependent protein kinase phosphorylation on all of the other identified components of the system, *CYR1*, *CDC25*, and *IRA1* (2, 3, 13, 24). Dissociation of adenylate cyclase from the plane of the membrane could be another feedback mechanism to prevent stimulation by *RAS* proteins and decrease the production of cAMP.

We also observed changes in the proportion of activity in the cytosol fraction during growth (Fig. 10). As cells increased in density, the proportion of adenylate cyclase in the supernatant decreased. Although there are many possible explanations for this effect, the shift from supernatant to pellet is consistent with the observed regulation of binding capacity by cAMP. Cells carrying the *bcy1* mutation failed to shift adenylate cyclase activity from the soluble fraction to the pellet fraction during growth (Fig. 10). These cells are presumably insensitive to changes in cAMP and would not be expected to regulate membrane binding in response to cAMP levels. It should be noted that the *bcy1* mutant cells stopped growing at a much lower density than the wild-type cells. The *bcy1* cells stopped growing at a density of approximately 5 OD₆₆₀ units, while the wild-type cells reached approximately 20 OD₆₆₀ units before they stopped growing.

The size of the complex in detergent solution at low ionic strength, where the enzyme is presumably associated with the protein(s) that anchors it to the membrane, suggests a very large complex. This is consistent with the large number of inputs that must feed into the adenylate cyclase system. The regulation of cAMP levels and cAMP-dependent protein kinase activity appears to be pivotal in the decision to undergo mitosis and meiosis (11, 17). Additionally, cAMP levels are modulated by glucose (6) as well as during the cell cycle (27), and proper regulation of adenylate cyclase activity is required for the appropriate response to mating factors, nitrogen starvation, and heat shock (14, 18, 26). A key to understanding the regulation of this system will be identifying the proteins that make up this complex.

Binding domain. It is reasonable to postulate that binding of adenylate cyclase to its target on the membrane occurs via

a specific binding domain on the *CYR1* protein. Our experiments with in-frame deletions indicate that the loss of the leucine-rich repeats roughly correlates with loss of membrane binding. This must be viewed cautiously, however. The interpretation is complicated by the effects that adenylate cyclase activity may have on its own localization; both pAC103 and pAC104 express activated enzyme, and all of the mutant *CYR1* proteins are uncoupled from stimulation by *RAS* proteins. The resulting changes in intracellular cAMP concentrations might contribute to the reduction in membrane binding observed with these constructions. Reconstitution experiments with the pAC103 and pAC104 enzymes addressed this limitation. The pAC104 enzyme did not bind to the membranes at all, in agreement with the results shown in Fig. 11. On the other hand, the enzyme encoded by pAC103, which retains two of the repeats, was reconstituted into the membranes. Qualitatively, the pAC103 enzyme and the wild-type enzyme bound to the membrane equally well.

Another obvious limitation to interpretation is intrinsic to deletion experiments; we do not know how the deletions alter structure. It is possible that our deletions alter protein structure at sites distant from the leucine-rich repeats. This limitation has been addressed by the recent work of Field et al. (7a). In these experiments, overexpression of the leucine-rich repeats of the *CYR1* protein suppressed the heat shock sensitivity of the *RAS2*^{val19} mutation, suggesting that the truncated *CYR1* proteins were interfering with the coupling between *RAS2* and the wild-type adenylate cyclase also present in the cells. The authors propose that this may be due to sequestration of the mutant *RAS2* protein by the overexpressed leucine-rich repeats. An alternative explanation is that the mutant *CYR1* proteins are interfering with the ability of the wild-type enzyme to bind to the membrane, uncoupling adenylate cyclase from *RAS*.

The region of leucine-rich repeats remains an attractive candidate for a domain that interacts with a target protein on the membrane. Positive identification of this domain, as well as identification of the protein(s) that it binds to, will be important steps to understanding the regulation of this system.

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LITERATURE CITED

1. Barbacid, M. 1987. *ras* genes. Annu. Rev. Biochem. 56:779-827.
2. Broek, D., T. Toda, T. Michaeli, L. Levin, C. Birchmeier, M. Zoller, S. Powers, and M. Wigler. 1987. The *S. cerevisiae* *CDC25* gene product regulates the *RAS*/adenylate cyclase pathway. Cell 48:789-799.
3. Camonis, J. H., M. Kalekine, G. Bernard, H. Garreau, E. Boy-Marcotte, and M. Jacquet. 1986. Characterization, cloning and sequence analysis of the *CDC25* gene which controls the cAMP level of *Saccharomyces cerevisiae*. EMBO J. 5:375-380.
4. Casperson, J. F., N. Walker, and H. R. Bourne. 1985. Isolation of the gene encoding adenylate cyclase in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 82:5060-5063.
5. Casperson, J. F., N. Walker, A. R. Brasier, and H. R. Bourne. 1983. A guanine nucleotide-sensitive adenylate cyclase in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 258:7911-7914.
6. Eraso, P., and J. P. Gancedo. 1985. Use of glucose analogues to

- study the mechanism of glucose-mediated cAMP increase in yeast. *FEBS Lett.* **191**:51–54.
7. Field, J., J. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler. 1988. Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell. Biol.* **8**:2159–2165.
 - 7a. Field, J., X. Hao-Peng, T. Michaeli, R. Ballester, P. Sass, M. Wigler, and J. Colicelli. 1990. Mutations of the adenylyl cyclase gene that block Ras function in *Saccharomyces cerevisiae*. *Science* **247**:464–467.
 8. François, J., P. Eraso, and C. Gancedo. 1987. Changes in the concentration of cAMP, fructose 2,6-biphosphate and related metabolites and enzymes in *Saccharomyces cerevisiae* during growth on glucose. *Eur. J. Biochem.* **164**:369–373.
 9. Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649.
 - 9a. Hartwell, L. 1980. Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormone. *J. Cell Biol.* **85**:811–822.
 10. Heideman, W., G. F. Caspersen, and H. R. Bourne. 1987. Adenylyl cyclase in yeast: hydrodynamic properties and activation by trypsin. *J. Biol. Chem.* **262**:7087–7091.
 - 10a. Heideman, W., G. F. Caspersen, and H. R. Bourne. 1990. Adenylyl cyclase in yeast: antibodies and mutations identify a regulatory domain. *J. Cell. Biochem.* **42**:229–242.
 11. Ishikawa, T., I. Uno, and K. Matsumoto. 1986. The role of cAMP in controlling yeast cell division. *BioEssays* **4**:52–56.
 12. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**:163–168.
 13. Kataoka, T., D. Broek, and M. Wigler. 1985. DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylyl cyclase. *Cell* **43**:493–505.
 14. Kataoka, T., S. Powers, C. McGill, O. Fasano, J. Strathern, J. Broach, and M. Wigler. 1984. Genetic analysis of yeast *RAS1* and *RAS2* genes. *Cell* **37**:437–445.
 15. Krupinski, J., F. Coussen, H. A. Bakalyar, W. Tang, P. G. Feinstein, K. Orth, C. Slaughter, R. Reed, and A. G. Gilman. 1989. Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. *Science* **244**:1558–1564.
 - 15a. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 16. Lowry, O. H., N. S. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
 17. Matsumoto, K., I. Uno, and T. Ishikawa. 1983. Initiation of meiosis in yeast mutants defective in adenylyl cyclase and cyclic AMP-dependent protein kinase. *Cell* **32**:417–423.
 18. Matsumoto, K., I. Uno, and T. Ishikawa. 1985. Genetic analysis of the role of cAMP in yeast. *Yeast* **1**:15–24.
 19. Nikawa, J., S. Cameron, T. Toda, K. M. Ferguson, and M. Wigler. 1987. Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. *Genes Dev.* **1**:931–937.
 20. Resnick, R. J., and E. Racker. 1988. Phosphorylation of the *RAS2* gene product by protein kinase A inhibits the activation of yeast adenylyl cyclase. *Proc. Natl. Acad. Sci. USA* **85**:2474–2478.
 21. Robinson, L. C., J. B. Gibbs, M. S. Marshall, I. S. Signal, and K. Tatchall. 1987. *CDC25*: a component of the RAS-adenylyl cyclase pathway in *Saccharomyces cerevisiae*. *Science* **235**:1218–1221.
 22. Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylyl cyclase assay. *Anal. Biochem.* **58**:541–548.
 23. Smigel, M. D. 1986. Purification of the catalyst of adenylyl cyclase. *J. Biol. Chem.* **261**:1976–1982.
 24. Tanaka, K., K. Matsumoto, and A. Toh-e. 1989. *IRA1*, an inhibitory regulator of the RAS-cyclic AMP pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**:757–768.
 25. Toda, T., S. Cameron, T. Sass, M. Zoller, and M. Wigler. 1987. Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**:1371–1377.
 26. Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler. 1985. In yeast, RAS proteins are controlling elements of adenylyl cyclase. *Cell* **40**:27–36.
 27. Watson, C. D., and D. R. Berry. 1977. Fluctuations in cAMP levels during the cell cycle of *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **1**:175–178.