
9. W. B. Watkins and V. J. Choy, Cell Tissue Res. 180, 491 (1977). Preliminary experiments with double-staining methods have shown that almost all of the ET-positive neurones are simultaneously immunostained by an antiserum against vasopressin and oxytocin, suggesting the colocalization of ET and other neurosecretory hormones in PVN neurones.

10. ET-positive axons from PVN and SON, which passed through the fiber layer of the median eminence and projected to the posterior pituitary, showed varicosities and localized swelling resembling Hering's bodies. In the posterior pituitary, an accumulation of dot-like immunoreactive products for ET was concentrated around the small vessels.

11. For RIA with ETc antiserum, standard ET-1 or unknown samples in 100 μl of RIA buffer [0.05M PBS (pH 7.4), 0.1% bovine serum albumin, 0.1% Triton X-100, 0.15M NaCl, 0.025M EDTA · 2 Na, 0.05% NaN3, and Trasylol (5000 kallikrein inactivator units per milliliter)] were previously incubated with diluted ETc antiserum (200 μl, 1:250) for 12 hours at 4°C. Then each standard or sample was incubated with [125I]labelled ET-1 (50 μl) (approximately 15,000 cpm) for 24 hours and then with diluted goat antibodies to rabbit immunoglobulin G (fiqg) (500 μl, 1:200) for 30 minutes at 4°C. After centrifugation, the radioactivity in each precipitate was determined. The median inhibitory concentration (IC50) of tracer binding by ET-1 was observed at 600 fmol per tube. Cross-reactivity of this antiserum with ET-2 or ET-3 is almost the same as with ET-1.


By contrast, ET-like immunoreactivity in PVN and SON neurones was almost unchanged.


19. A. Gaisd, S. J. Genson, J. M. Polak, unpublished data. Only background levels of the labeling were obtained after poor tissue treatment with ribonuclease.

20. We thank L. L. Iversen for valuable criticism and encouragement and T. Yamaji for the generous gift of neurophysins. Supported in part by a grant from Scientific Research on Priority Areas and by a grant from the Ministry of Education, Science, and Culture of Japan.

21 July 1989; accepted 7 November 1989

**Mutations of the Adenylly Cyclase Gene That Block RAS Function in Saccharomyces cerevisiae**

**JEFFREY FIELD, HAO-PENG XU, TAMAR MICHAELI, ROYMARIE BALLESTER, PHILIP SASS,* MICHAEL WIGLER, JOHN COLICELLI**

The interaction between RAS proteins and adenylly cyclase was studied by using dominant interfering mutations of adenylly cyclase from the yeast *Saccharomyces cerevisiae*. RAS proteins activate adenylly cyclase in this organism. A plasmid expressing a catalytically inactive adenylly cyclase was found to interfere dominantly with this activation. The interfering region maps to the leucine-rich repeat region of adenylly cyclase, which is homologous to domains present in several other proteins and is thought to participate in protein-protein interactions.

**D**ominant interfering mutations can be useful for investigating interactions between components of signal transduction systems (1). Such a mutation can inhibit signal outputs by causing the production of a partially functional protein. The protein is functional in that it binds to an appropriate target, but nonfunctional in that the binding event is not productive. Thus, the mutant molecule sequesters its partner and prevents it from interacting with other components of the system. The net result is a diminished output from the signaling system.

RAS proteins activate adenylly cyclase in *Saccharomyces cerevisiae* (2, 3), and several dominant interfering mutations of RAS have been found (4–7). One of these mutant proteins (7) appears to act by sequestering an "upstream" component, the CDC25 gene product, which is thought to be an activator of yeast RAS. Feig and Cooper (5) isolated a similar mammalian ras mutation that appears to interfere with activation of mammalian Ras. Michaeli et al. (6) demonstrated that mutant RAS proteins that fail to translocate from the cytoplasm to the membrane also

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

*Present address: Lederle Laboratory, Pearl River, NY 10965.
interfere with RAS function in yeast; such mutants appear to sequester a "downstream" component of the RAS pathway. We now describe dominant interfering mutations of the adenyl cyclase gene CYR1.

To genetically screen for interfering forms of adenyl cyclase, we made use of the heat shock–sensitive phenotype of cells containing the yeast gene RAS2val19. This gene contains a mutation equivalent to that of mammalian H-rasval12, one of the oncogenic forms of H-ras. Yeast with this mutation display several abnormal phenotypes, including failure to arrest in the G1 phase of the cell cycle, failure to sporulate, sensitivity to starvation, and sensitivity to heat shock (2, 8). Because increased expression of adenine 3',5'-monophosphate (cAMP) phosphodiesterase genes can suppress these phenotypes (9, 10), we expected that interfering forms of adenyl cyclase might do likewise. We therefore randomly mutagenized a plasmid that expresses CYR1 under the control of the strong promoter of the alcohol dehydrogenase gene ADH-I (11). The plasmid was passed through a mutator strain of Escherichia coli (12), and the resulting "library" of mutant plasmids was transformed into a S. cerevisiae strain, TK161-R2V, containing RAS2val19. Transformants were scored for heat-shock sensitivity. In two screens of separate libraries of about 1000 transformants each, we isolated 10 and 13 plasmids that conferred heat-shock resistance on the RAS2val19 strain (13).

The ease with which interfering mutations could be isolated suggested that many different mutations in the adenyl cyclase gene might give rise to interfering plasmids. The most obvious class of mutations that might be thought to be effective would be those lacking catalytic function, so we tested directly if such mutations would be interfering. The catalytic region of adenyl cyclase is in the COOH-terminal 417 amino acids (14). We constructed a plasmid that had this region deleted and found it to be interfering (15). Further interfering constructs were made by introducing a frameshift mutation at the NcoI restriction site located at the codon for amino acid 1608, just upstream of the catalytic region. We assayed for heat-shock sensitivity and found that RAS2val19 cells containing either a marker plasmid or a plasmid expressing large amounts of full-length adenyl cyclase did not recover from heat shock, but cells containing a plasmid expressing the defective adenyl cyclase with the frameshift mutation did recover (Fig. 1). Thus, the presence of an abundance of a catalytically inactive form of adenyl cyclase appeared to inhibit the activity of the remaining wild-type protein.

To define the smallest region of adenyl cyclase that possesses interfering activity, we constructed a series of deletions that contained the frameshift mutation at the NcoI site (Fig. 2). The first series consisted of deletions from the 5' end of the gene. Molecules with deletions up to the codon for amino acid 605 retained full interfering activity. A molecule with a deletion up to amino acid codon 733 was partially active and those with deletions beyond codon 733 were not active (Fig. 2, group I). In the next series we began with the mutant gene deleted through the codon for amino acid 605 and made further deletions beginning from the NcoI site toward the 5' end. Molecules deleted from the NcoI site toward codon 1302, and thus encoding proteins consisting of amino acids 606 to 1301, were fully interfering (Fig. 2, group II). In the third series, deletions within the region between the codons for amino acids 606 and 1301 were made. Molecules with these mutations were not active (Fig. 2, group III).

The region of adenyl cyclase between amino acids 606 and 1301 contains 26 copies of a 23–amino acid repeating unit (from amino acid position 733 to 1301) that is rich in aliphatic (especially leucine) residues and is punctuated by one residue each of proline and asparagine (Fig. 2). Expression of this repeat region is the probable cause of interference. Deletion of amino acids 606 through 733 has little effect on interference, but deletions within the repeat...
abolish interference. This repeated consensus sequence is also found in tandem repeats present in several other proteins. These include the Chaotin (15) and Toli (16) gene products of Drosophila melanogaster, the porcine ribonuclease inhibitor (17), the a (18) and b (19) chains of the human serum glycoprotein I b, the human serum 4-glyco- protein (20), and the lutfop-choriogonadotropin receptor (21). All these proteins are thought to bind other proteins. The glycoprotein l b a chain (also known as the platelet receptor for the von Willebrand factor) and the luttop-choriogonadotropin receptor are both thought to require the leucine-rich repeats for protein binding (18, 21). The repeat region of adenyl cyclase does not form a “leucine zipper” according to the model of Landschulz et al. (22) because the leucines are not present at every seventh position on an a helix. The leucine-rich repeats may, however, form an analogous structure. Trans-dominant mutations in leucine-zipper proteins have also been reported. These mutations result in partially functional proteins capable of interfering with their appropriate targets (23).

We have postulated that interference by the truncated adenyl cyclase occurs by sequestering a functioning component of the signal transduction pathway. To test if this component is the RAS protein itself, we investigated if overexpression of RAS2val19 could overcome the interference of defective adenyl cyclase. We examined four pairwise combinations of plasmids transformed into a RAS2val19 strain: these plasmids included one expressing RAS2val19, one expressing an interfering cyclase, and two control plasmids carrying the appropriate auxotrophic markers. Transformed strains were tested for heat-shock sensitivity. The two control plasmids together had no effect on heat-shock sensitivity, whereas the interfering adenyl cyclase fully suppressed heat-shock sensitivity. The RAS2val19 plasmid had no effect on heat-shock sensitivity. However, the RAS2val19 plasmid largely overcome the interference caused by the truncated adenyl cyclase (Fig. 3). Cells that contained the interfering adenyl cyclase and overexpressed the RAS2val19 protein were largely heat shock-sensitive.

This last result suggested that interfering adenyl cyclase acts by sequestering RAS2val19 protein. To test this further, we determined if active RAS protein was required for interference. Yeast strains that lack both RAS1 and RAS2 and also both genes encoding the AMP phosphodiesterases, PDE1 and PDE2, are heat shock-sensitive (10). This phenotype presumably reflects the large amounts of intracellular cAMP, which is produced by basal levels of adenyl cyclase activity in the absence of RAS proteins and accumulates because of the absence of cAMP hydrolysis. The heat-shock sensitivity of a ras1 ras2 pde1 pde2 strain was not affected by the interfering adenyl cyclase plasmid (Fig. 4). As a positive control in this experiment, a plasmid expressing PDE2 was shown to suppress the heat-shock sensitivity in the RAS2val19 strain TK161-R2V. Thus, interference was not observed in the absence of RAS proteins.

Our results suggest that expression of the repeat region of adenyl cyclase competes for RAS function. The simplest hypothesis consistent with our data is that this region of adenyl cyclase forms a complex that sequesters RAS. There are several ways to envision this occurring. (i) RAS proteins might interact directly with the repeat region. (ii) Functional adenyl cyclase might form an ineffective complex with the repeat region. (iii) Other proteins might combine with the repeat region to form a complex capable of binding RAS proteins. We have found that other proteins do complex with yeast adenyl cyclase (24).

We have demonstrated that, in yeast, a defective protein target for RAS action can be used to block the RAS signaling pathway. This observation may serve as the basis of a genetic assay for the identification of other targets for RAS action. Therefore, we have searched libraries expressing mammalian cDNAs in yeast for genes suppressing heat-shock sensitivity (25).

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21. 23.R.

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pACYR was constructed by first cloning the 4-kb Hind III fragment of pEF-CYR1 (24), encoding the COOH-terminal region of adenylyl cyclase, into the Hind III restriction site of YEp13 to yield the plasmid pYCYR. The 4.5-kb Bam HI-Xho I fragment of pEF-CYR1, containing the ADH-I promoter and region encoding the NH2-terminal region of adenylyl cyclase, was then cloned into pACYR, which had been digested with Bam HI and Xho I, to create pACYR. pACYR differs from pEF-CYR1 only in the vector used to carry adenylyl cyclase. pEF-CYR1 carries the TR1I auxotrophic marker, whereas pACYR carries LEU2. Both plasmids use the yeast ADH-I promoter to express an adenylyl cyclase fusion protein. This protein has a small peptide epitope fused to the NH2-terminus of adenylyl cyclase. The epitope is recognized by monoclonal antibody 12C5A5, which can be used to monitor and purify the fusion protein (24). The codons deleted are indicated by means of the numbering system of Kataoka et al. (14). In some cases two regions were deleted. Precise deletions were made by reconstructing the parent plasmid with combinations of restriction endonuclease fragments and the desired DNA products of polymerase chain reactions [R. K. Saki et al., Science 239, 487 (1988)]. Detail of the deletions are described elsewhere (J. Colicelli, J. Field, R. Ballister, N. Chester, M. Wigler, in preparation). Each construct was first tested for function by complementation in adenylyl cyclase-deficient or Ras-deficient strains. Constructs were tested by immunoprecipitation of adenylyl cyclase activity with monoclonal antibody 12C5A5. In some cases the enzyme was additionally tested by protein immunoblots or by affinity purification, in each case with the use of monoclonal antibody. After it was established that the deleted plasmids produced a catalytically functional adenylyl cyclase molecule, a frameshift mutation was introduced at the Nco I restriction site. This was performed by digestion with Nco I, filling in E47 with Klenow fragment, followed by ligation. This procedure was monitored by the creation of an Nsi I site formed by the ligation of the filled-in ends of the Nco I site.

28. We thank P. Bird for help in preparing the manus-

cript. Supported by NIH grant CA 39829, the Pfzer Biomedical Research Award, and the Ameri-

can Cancer Society. T.M. is supported by the Da-

non Runyon-Walter Cancer Fund and Fellow-

ship DRG-923. J.F. is supported by the Anna Fuller Fund and NIH. I.C. is supported by Life Sciences Research Foundation. M.W. is an Ameri-

can Cancer Society Professor.

24 August 1989; accepted 14 November 1989

Two Distinct Transcription Factors That Bind the Immunoglobulin Enhancer \(\mu E5/\kappa E2\) Motif

Paula Henthorn, Megerditch Kiledjian, Tom Kadesch

Activity of the immunoglobulin heavy and \(\kappa\) light chain gene enhancers depends on a complex interplay of ubiquitous and developmentally regulated proteins. Two complementory DNAs were isolated that encode proteins, denoted ITF-1 and ITF-2, that are expressed in a variety of cells types and bind the \(\mu E5/\kappa E2\) motif found in both heavy and \(\kappa\) light chain enhancers. The complementary DNAs are the products of distinct genes, yet both ITF-1 and ITF-2 are structurally and functionally similar. The two proteins interact with one another through their putative helix-loop-helix motifs and each possesses a distinct domain that dictates transcription activation.

The Immunoglobulin Heavy chain (IgH) enhancer activates transcription of rearranged heavy chain genes (1). In transfection experiments it stimulates transcription from a variety of promoters, but only in cells of the lymphoid lineage (primarily B cells). This activity is mediated through several protein binding sites. Four of these sites, \(\mu E1, \mu E2, \mu E3\) and \(\mu E4\), were defined initially by in vivo footprinting (2). Two others, octa (3) and \(\mu EBP-E\) (4), were first defined in vitro. Deletions or mutations of these sites generally reduce overall enhancer activity (5–7). However, mutation analyses also suggest that the sequence motifs identified by DNA binding assays do not account for all of the IgH enhancer activity (5, 6). In particular, a deletion that destroys both the \(\mu E1\) and \(\mu E2\) sites and removes the 24 bp between these two sites has a more deleterious effect in B cells than clustered point mutations that simultaneously destroy these two motifs (6). This deletion results in an increase in enhancer activity in mouse L cells, suggesting that the region between \(\mu E1\) and \(\mu E2\) may comprise a negative regulatory element as well (6, 8). An examination of the nucleotide sequence between \(\mu E1\) and \(\mu E2\) reveals an additional E-related motif, referred to as \(\mu E5\) (9). Although this site closely resembles the \(\kappa\) light chain enhancer \(\kappa E2\) site, protein binding to the \(\mu E5\) site has not been detected with crude nuclear extracts (5, 10).

To study the function of this region of the enhancer in more detail, we have isolated cDNAs that encode its cognate DNA binding proteins. We used an oligonucleotide bearing both the \(\mu E5\) and \(\mu E2\) sites to screen a B cell–derived Agt11 cDNA library (11). We analyzed in detail two phage iso-
lates, denoted E2-2 and E2-5, that had binding activity for the oligonucleotide. A series of experiments employing mutant oligonucleotides confirmed that the DNA binding activity encoded by each phage was specific for the \(\mu E5\) motif within the oligonucleotide, as well as for a \(\kappa E2\) motif carried on a different oligonucleotide (12).