MAMMALIAN CELL GENETICS

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This was an extraordinary year. For the 12-month period ending as of this writing, three lines of work culminated in extremely important achievements in diverse areas of molecular biology. First, our studies of *RAS* oncogene function have led to the discovery that *RAS* activates a protein kinase cascade, conserved in evolution from yeast to metazoans. This activation is probably mediated through a direct interaction with a MAP protein kinase kinase kinase (MAPKKK). In mammalian cells, one such MAPKKK upon which *RAS* acts is likely to be the RAF oncoprotein. In *Schizosaccharomyces pombe*, a fission yeast, it is the byr2 kinase. The methodologies we have employed in these studies, cross species complementation and the S. Fields and O.-K. Song genetic system to detect protein/protein interactions, are likely to yield yet additional yeast and mammalian elements that operate in this pathway. Second, we have developed a major new genetic tool that detects the differences between two like DNA populations. This tool (RDA) enables us to discover probes for pathogenic genomes from infected tissues, to clone probes corresponding to the genetic lesions from neoplastic cells and from organisms suffering spontaneous genetic disease, and to clone probes linked to inherited disease loci. Third, in collaboration with Clark Still and his colleagues in the Department of Chemistry at Columbia University, we have developed the most powerful tool yet for conducting combinatorial chemistry. This tool allows the synthesis of extremely large numbers of chemical compounds, the selection of the few with desirable characteristics, and the determination of the structure of those few. In short, the combinatorial methods we have developed enable organic chemists to operate like geneticists. With such methods, the discovery of small molecular agonists and antagonists to desired targets may become as easy as raising monoclonal antibodies.

Regulation of RAS

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RAS genes encode GTP/GDP-binding proteins, found in yeasts and metazoans. They were first discovered as the oncogenes of acutely transforming retroviruses and then rediscovered as mutated alleles of the first human oncogenes. Wild-type RAS proteins participate in physiologically normal signal transduction pathways that regulate growth and differentiation in a wide variety of cell types. Signaling from RAS occurs in its GTP-bound state, and normal RAS protein slowly hydrolyzes GTP. The wild-type proteins are regulated by at least two types of molecules: factors that accelerate guanine nucleotide exchange on RAS and factors that accelerate guanine nucleotide triphosphate hydrolysis by RAS. CDC25 of the yeast *Saccharomyces cerevisiae* was the first protein of the first class to be discovered (Camonis et al., *EMBO J. 5:* 375 [1986]; Broek et al., *Cell 48:* 789 [1987]). Mammalian GAP was the first protein of the second class to be discovered (Trahey and McCormick, *Science 238:* 542 [1987]). Both CDC25 and GAP are

representatives of large families of proteins, found in a wide variety of eukaryotic organisms. Mutant, oncogenic RAS proteins are frequently abnormal in their regulation, and in particular in their response to GAP. The object of this section has been to understand the nature of the components and pathways that regulate RAS.

Work in *S. cerevisiae* has indicated the existence of at least three CDC25-like molecules: CDC25 itself, BUD5 (Chant et al., *Cell 65:* 1213 [1991]; Powers et al., *Cell 65:* 1225 [1991]), and SDC25 (Boy-Marcotte et al., *Gene 77:* 21 [1989]; Damak et al., *Mol. Cell. Biol. 11:* 202 [1991]). Previous genetic analysis suggested a role only for CDC25 in the physiological regulation of RAS. The biochemical role of CDC25 was clarified by the discovery of dominant interfering forms of *RAS2*, RAS^{Gly22}, and RAS^{Ala15}, which block CDC25 function (Powers et al., *Mol. Cell. Biol. 9:* 390 [1989]). BUD5 appears likely to regulate BUD1, which is a RAS-like protein (also known as RSR1) involved in polarity selection during bud formation (Bender and Pringle, *Proc. Natl. Acad. Sci. 86:* 9976 [1989]; Chant and Herskowitz, *Cell 65:* 1203 [1991]). The true physiological role of SCD25 is unknown. In high copy, a truncated *SCD25* gene can complement the loss of *CDC25*, but the disruption of *SCD25* has no apparent phenotype.

Molecular analysis of CDC25 has revealed clues about its regulation. The smallest domain of CDC25 needed for viability is a carboxy-terminal portion (from amino acid 1102 to 1541). The entire molecule is 1589 amino acids long. Regulatory regions are found in the amino-terminal domains. A point mutation (S 364 F) activates CDC25, and overexpression of an amino-terminal domain (from amino acid 1 to 239) blocks wild-type CDC25 function. Cells expressing only the minimal catalytic region of CDC25 still exhibit glucose-dependent cAMP regulation (Van Aelst et al., *Eur. J. Biochem. 193:* 675 [1990]; R. Ballester and L. Van Aelst, unpubl.). Therefore, either this minimal region contains the regulatory domains for glucose responsiveness or the pathway that regulates cAMP in response to glucose does not pass through CDC25. One interesting possibility is that glucose regulates adenylyl cyclase through GPA2, a protein that is in the family of the G_α subunits of heterotrimeric G proteins (Nakafuku et al., *Proc. Natl. Acad. Sci. 85:* 1374 [1988]). GPA2 has been shown to affect cAMP levels and is a homolog of gpa2, an *S. pombe* protein that is known to regulate adenylyl cyclase (Isshiki et al., *Genes Dev. 6:* 2455 [1992]). If this hypothesis is correct, then RAS proteins and GPA2 may codominantly regulate adenylyl cyclase. The codominance of RAS proteins and G proteins is a theme to which we will return.

In *S. pombe*, there is a single known RAS homolog, ras1 (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis et al., *EMBO J. 5:* 2963 [1986]). It is regulated by a CDC25-like protein called ste6 (Hughes et al., *Nature 344:* 355 [1990]). Whereas ras1 is required for cell shape, sporulation, and conjugation, ste6 is required only for conjugation. Therefore, other nucleotide exchange regulators of RAS may be found in *S. pombe*. We have obtained direct evidence for this. Upon testing libraries of mutated *ras1* genes, we found several dominant negative *ras1* mutants. One of these ($ras1^{Tyr62}$) induced a *ras1* null phenotype when overexpressed in wild-type cells but had minimal effects when overexpressed in cells containing the activated $ras1^{Val17}$ gene. We therefore presume that $ras1^{Tyr62}$ blocks the activation of ras1 by acting upon a regulatory molecule other than ste6. One candidate for this is ral2. Disruption of *ral2* produces a phenotype that is very similar to the *ras1* null phenotype (Fukui et al., *Mol. Cell. Biol. 9:* 5617 [1989]). However, the primary sequence of ral2 does not resemble that of CDC25-like molecules. Preliminary data, obtained in collaboration with Dan Broek, indicate that RAS molecules with the Tyr-62 mutation bind irreversibly to CDC25-like molecules.

Genes with regions homologous to the catalytic domain of CDC25 have been found in flies (Sos) and mammals (rasGRF, mSos1, mSos2) (Simon et al., *Cell 67:* 701 [1991]; Botwell et al., *Proc. Natl. Acad. Sci. 89:* 6511 [1992]; Shou et al., *Nature 358:* 351 [1992]). Although rasGRF has been shown to catalyze guanine nucleotide exchange on RAS (Jacquet et al., *J. Biol. Chem. 267:* 24181 [1992]; Shou et al., *Nature 358:* 351 [1992]), this had not been shown for the Sos proteins. In collaboration with Pierre Chardin and Dafna Bar-Sagi, we demonstrated that a fragment (amino acids 707–1059) of hSos1, a human homolog of *Drosophila* Sos and of mouse Sos1, complements the loss of CDC25 in *S. cerevisiae* and stimulates nucleotide exchange on RAS in vitro. Extracts from mammalian cells where hSos1 is overexpressed exhibit higher nucleotide exchange activity on RAS. These data demonstrate that hSos1 is a bona fide nucleotide exchange catalyst for RAS. Larger fragments of hSos1 failed to complement a loss-of-function mutation of CDC25, suggesting the exis-

tence of negative regulatory domains. Further studies will center upon the regulation of hSos1 function.

Recently a gene, *SEM5*, has been identified in *Caenorhabditis elegans*, a simple worm, that encodes a protein that appears to act upstream of RAS (Clark et al., *Nature 356:* 340 [1992]). Homologs of this gene have been found in flies and mammals (Lowenstein et al., *Cell 70:* 431 [1992]). In mammals, the gene is called *GRB2*. This gene encodes a protein composed entirely of SH3 and SH2 domains, in the order SH3/SH2/SH3. SH2 domains, which are found on a wide variety of molecules involved in signal transduction, bind certain peptide motifs containing phosphotyrosine (Pawson and Gish, *Cell 71:* 359 [1992]). SH3 domains are also found on a wide variety of signal transduction molecules, and bind certain peptide motifs, but these motifs are less well characterized (Ren et al., *Science 259:* 1157 [1993]). Two of the human homologs of CDC25, hSos1 and hSos2, contain peptides in their carboxy-terminal domains that are candidate SH3-binding motifs. We therefore reasoned that the Sos proteins might bind to GRB2. To test this, we utilized a genetic assay in *S. cerevisiae* for protein/protein interactions, the two hybrid system of Fields and Song (*Nature 340:* 245 [1989]). These studies indicated that GRB2 interacts with the carboxy-terminal domain of hSos1 (amino acids 1131 to 1333), and this was further demonstrated by direct coprecipitation. Our genetic studies indicated that both SH3 domains are required for interaction.

We have continued our investigations of NF1 in an ongoing collaboration with Francis Collins and his colleagues at the University of Michigan. *NF1* is the locus for Von Recklinghausen's neurofibromatosis, an inherited disease affecting the proliferation and differentiation of neuroepithelium. It encodes a member of the GAP family of GTPase-activating proteins capable of causing the down-regulation of RAS proteins (Xu et al., *Cell 62:* 599 [1990]; Wang et al., *Cell Regul. 2:* 453 [1991]). *NF1* mRNA comes in two alternately spliced forms, one with an insertion in the midst of the region thought to encode the catalytic domain (Anderson et al., *Mol. Cell. Biol. 13:* 487 [1993]). Both spliced forms are expressed in all tissues examined, although in varying ratios. We demonstrated that both forms are catalytically active. Mutational studies of the conserved residues of NF1 have indicated that certain are required for full activity, as judged by genetic assays in *S. cerevisiae* (Gutmann et al., *Oncogene 8:* 761 [1993]). Substitution at residue 1426 abolishes activity, and substitutions at residues 1423 and 1391 merely diminish activity.

Questions remain whether GAP-like molecules have RAS-effector function. The evidence is clear that GAP can down-regulate RAS, but evidence is also accumulating that GAP has some effector activity (Yatani et al., *Cell 61:* 769 [1990]; Martin et al., *Science 255:* 192 [1992]; Schweighoffer et al., *Science 256:* 825 [1992]). We have continued to pursue this issue for the *IRA* genes, which encode GAP-like molecules in *S. cerevisiae* (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]; Tanaka et al., *Mol. Cell. Biol. 10:* 4303 [1990]). Disruption of both *IRA* and *IRA2* in *S. cerevisiae* leads to the same phenotype seen when *RAS2* is activated by mutation. Disruption of *RAS2* in an *ira1*$^-$ *ira2*$^-$ background restores a wild-type phenotype. Thus, the only function we can detect for the IRA proteins is the down-regulation of RAS protein (R. Ballester, unpubl.).

A Conserved Cascade of Protein Kinases: RAS Effector Function

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In the fission yeast *S. pombe*, ras1 participates in pheromone-induced sexual differentiation (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis, *EMBO J. 5:* 2963 [1986]; Nielson et al., *EMBO J. 11:* 1391 [1992]; Neiman et al., *Mol. Biol. Cell 107:* [1993]). *ras1*$^-$ cells are unable to conjugate, and they sporulate inefficiently. *gpa1*, a gene that encodes a protein homologous to the G_α subunits of heteromeric G proteins, is also required for conjugation and sporulation (Obara et al., *Proc. Natl. Acad. Sci. 88:* 5877 [1991]). *gpa1* presumably mediates the signaling from the pheromone receptors. Studies during the past few years have indicated that overexpression of several genes is capable of bypassing the requirement for ras1 for sporulation in *S. pombe*. Among these genes are *byr2* and *byr1*, two genes encoding putative protein kinases and also required for both conjugation and sporulation (Nadin-Davis and Nasim, *EMBO J. 7:* 985 [1988]; Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]). Overexpression of these genes also restored sporulation to *gpa1*$^-$ cells (Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). Epistasis experiments, utilizing sporulation of diploid cells as the observable

phenotype, placed byr1 downstream from byr2, and both downstream from ras1 and gpa1, but these experiments could not order ras1 and gpa1 with respect to each other.

We have succeeded in ordering gpa1 and ras1 by examining expression of *mam2*, the gene encoding the receptor for P-factor pheromone (H.-P. Xu et al., submitted). We have found that mRNA levels for *mam2* depend on components of the pheromone signaling pathway itself. We view this relationship as a rudiment of a sexual arousal response. Disruption of *ras1*, *gpa1*, *byr1*, or *byr2* reduces *mam2* expression 10- to 100-fold in wild-type homothallic cells. By overexpressing these genes, we have confirmed that byr1 and byr2 act downstream from gpa1 and ras1 but also that gpa1 acts downstream from ras1, and ras1 downstream from gpa1. In other words, ras1 and gpa1 are codominant. These same studies find no role for spk1 in the regulation of *mam2* (see below).

Work continues to find other elements of *S. pombe* that participate in the sexual differentiation pathway. We have found mutants of diploid *S. pombe* that sporulate in the absence of ras1 and have demonstrated that one of these mutants is recessive. The mutant locus encodes a product that appears to act upstream of byr2. Hence, the mutant locus might encode a protein that normally functions to block sexual differentiation, perhaps by acting on the pathway between gpa1 and byr2 or between ras1 and byr2. In a similar approach, we have constructed *S. pombe* strains containing a *mam2* promoter-reporter gene (*ADE2*) that we are using to look for new mutations in the sexual differentiation pathway.

In collaboration with Aaron Nieman in Ira Herskowitz's laboratory at the University of California, San Francisco, and Brian Stevenson in the laboratory of George Sprague, University of Oregon, we showed that the byr2 and byr1 kinases are each structurally and functionally related to the STE11 and STE7 kinases of *S. cerevisiae*, respectively (Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). STE11 and STE7 are themselves required for the pheromone-induced sexual differentiation pathways in the budding yeast, with STE11 acting upstream of STE7 (Teague et al., *Proc. Natl. Acad. Sci. 83:* 7371 [1986]; Rhodes et al., *Genes Dev. 4:* 1862 [1990]; Cairns et al., *Genes Dev. 6:* 1305 [1992]; Gartner et al., *Genes Dev. 6:* 1280 [1992]; Stevenson et al., *Genes Dev. 6:* 1293 [1992]). Our studies of cross species complementation suggest that byr1 and byr2 cooperate in *S. cerevisiae*, and hence probably directly interact. Furthermore, byr2 appears to restore pheromone responsiveness to STE11-deficient cells, indicating that the regulation of byr2 by upstream elements has been conserved.

A pair of protein kinases, FUS3 and KSS1, found in *S. cerevisiae*, also participate in the sexual differentiation pathway, probably downstream from STE11 and STE7 (Gartner et al., *Genes Dev. 6:* 1280 [1992]; Errede et al., *Nature 362:* 261 [1993]). They are homologous to the spk1 protein kinase of *S. pombe*, likewise required for sexual differentiation in that organism (Toda et al., *Genes Dev. 5:* 60 [1991]; Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). In our collaborative studies, we demonstrated that spk1 and the FUS3/KSS1 kinases share function. Most significantly, these kinases are structurally related to the MAP/ERK protein kinases of vertebrate cells (Boulton et al., *Cell 65:* 663 [1991]; Toda et al., *Genes Dev. 5:* 60 [1991]). The latter kinases are activated, in a RAS-dependent fashion, by a variety of extracellular factors (DeVries-Smits et al., *Nature 357:* 602 [1992]; Robbins et al., *Proc. Natl. Acad. Sci. 89:* 1624 [1992]; Thomas et al., *Cell 68:* 1031 [1992]; Wood et al., *Cell 68:* 1041 [1992]). This suggested a possible conserved kinase cascade between yeasts and vertebrates. Consistent with this, we demonstrated that ERK2, a member of the MAP kinase family, could partially replace spk1 function in *S. pombe* (Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). Significantly, a MAP kinase kinase, called MEK, was found by others that is homologous to STE7 and byr1 (Crews et al., *Science 258:* 478 [1992]) and, even more recently, a MEK kinase (MEKK) that is homologous to STE11 and byr2 (Lange-Carter et al., *Science 260:* 315 [1993]).

The dependence of agonist-induced MAP kinase activation upon RAS was demonstrated by other investigators who showed that interfering forms of RAS block MAP kinase activation (DeVries-Smits et al., *Nature 257:* 602 [1992]; Robbins et al., *Proc. Natl. Acad. Sci. 89:* 1624 [1992]; Thomas et al., *Cell 68:* 1031 [1992]; Wood et al., *Cell 68:* 1041 [1992]). Moreover, scrape loading cells with mutant, activated RAS leads to MAP kinase activation (Leevers and Marshall, *EMBO J. 11:* 569 [1992]). In collaboration with Ellen Shibuya of Joan Ruderman's laboratory at Harvard Medical School, we showed that this interaction could be observed in cell-free *Xenopus* oocyte extracts (Shibuya et al., *Proc. Natl. Acad. Sci. 89:* 9831 [1992]). Addition of activated RAS protein results in a slow (1–3 hr) activation of MAP kinase.

Mutant RAS that lacks the site for carboxy-terminal processing failed to activate MAP kinase. The slow kinetics of the response to activated RAS probably reflects the slow processing and maturation of RAS.

In vertebrates, the RAF oncoprotein is a candidate downstream effector of RAS. RAF can overcome growth defects in cells in which RAS function is blocked (Smith et al., *Nature 320:* 540 [1986]; Cai et al., *Mol. Cell. Biol. 10:* 5314 [1990]). Interfering forms of RAF block RAS function (Kolch et al., *Nature 349:* 426 [1991]). Moreover, RAF is capable of phosphorylating MEK, a MAP kinase kinase (Dent et al., *Science 257:* 1404 [1992]; Howe et al., *Cell 71:* 335 [1992]; Kyriakis et al., *Nature 358:* 417 [1992]), and RAF becomes hyperphosphorylated in response to RAS activation (Morrison et al., *Proc. Natl. Acad. Sci. 85:* 8855 [1988]; Wood et al., *Cell 68:* 1041 [1992]). To begin our investigation of the relationship between RAS and RAF, we expressed RAF in *S. pombe*. We observed inhibition of sexual conjugation by RAF and partial suppression of the phenotypes of activated RAS. No phenotype was observed when we expressed an "activated" RAF, lacking the amino-terminal, putative regulatory domain. These experiments suggested that interactions with upstream elements regulating RAF may have been conserved.

We decided to test directly whether RAS and RAF could interact. To do this, we employed a genetic system in *S. cerevisiae*, Fields and Song's (*Nature 340:* 245 [1989]) two hybrid system, that detects physical complex formation between proteins fused to the separated activating and binding DNA domains of the transcriptional activator GAL4. These experiments revealed that RAS forms a complex with RAF (L. Van Aelst et al., *Proc. Natl. Acad. Sci.* [in press]). RAS mutated in its effector loop, or in the domain required for GTP binding, would not interact with RAF. Binding was to the amino terminus of RAF, believed to be its regulatory domain. These experiments give further evidence for the hypothesis that RAF is a downstream effector of RAS and suggest that RAF is an immediate downstream effector. At present, we cannot rule out the possibility that the interactions between RAS and RAF are mediated by a third protein.

We further showed that RAF can form a complex with MEK, again using the two hybrid system. This interaction requires only the catalytic domain of RAF. Moreover, RAS can form a complex with MEK, but only if RAF is also overexpressed. This suggests either that RAF forms a protein bridge between RAS and MEK or, less likely in our view, that RAF alters MEK so that it can bind to RAS. It is now easier to formulate possible hypotheses about how RAS regulates RAF function: RAS might directly activate RAF or RAS might cause RAF to colocalize with RAS, bringing RAF in proximity to other regulators or substrates. These comments bear upon MEK regulation and even MAP kinase regulation by RAS.

Our results with RAF encouraged us to examine complex formation between RAS and byr2. byr2 plays a role in *S. pombe* not unlike the role of RAF in mammalian cells. byr2 appears to mediate signals from RAS and appears to activate byr1 (Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]; Neiman et al., *Mol. Biol. Cell 4:* 107 [1993]), which is homologous to MEK. Again, we found that RAS could form a complex with byr2, and again with its amino-terminal, putative regulatory domain. Although byr2 has an overall similarity to RAF in that it is a large protein kinase with a catalytic carboxy-terminal domain, outside this domain it has no evident primary sequence homology with RAF. Moreover, neither RAF nor byr2 bear any resemblance to the GAP family, proteins known to interact with RAS. Thus, we think that either a small and as yet unrecognized primary sequence motif is responsible for RAS interaction or interaction with RAS requires a conformational motif.

Conjugation and Shape in *S. pombe*

E. Chang, J. Camonis

While attempting to define genes that might act on the RAS pathway, we looked for mutations that produced phenotypes similar to those of *ras1*$^-$ cells. Several mutants were obtained that were both round and sterile, and the loci responsible were identified. These were called *scd1* and *scd2* (for *s*hape and *c*onjugation *d*eficient). Sequence analysis indicates that scd1 is globally homologous to *S. cerevisiae* CDC24 (Miyamoto et al., *Gene 54:* 125 [1987]), whereas scd2 is closely related to *S. cerevisiae* BEM1 (Chenevert et al., *Nature 356:* 77 [1992]). Moreover, *CDC24* can partially complement loss of *scd1* function. CDC24 and BEM1 participate in bud formation

and bud site selection in *S. cerevisiae* (Bender and Pringle, *Mol. Cell. Biol. 11:* 1295 [1991]; Chant et al., *Cell 65:* 1213 [1991]). In *S. cerevisiae*, this process involves at least two members of the RAS superfamily: BUD1 (Bender and Pringle, *Proc. Natl. Acad. Sci. 86:* 9976 [1989]; Chant and Herskowitz, *Cell 65:* 1203 [1991]), also known as RSR1, which is closely related to the RAS proteins, and CDC42, which is a member of the RHO subfamily. We tested the interrelationship of these gene products and have found that *S. pombe ras1* (but not *S. cerevisiae RAS2*) can suppress defects in *S. cerevisiae CDC24*, and a member of the mammalian RHO subfamily, CDC42Hs (Shinjo et al., *Proc. Natl. Acad. Sci. 87:* 9853 [1990]), which can suppress the loss of *CDC42* in *S. cerevisiae*, can suppress the morphological defects of *ras1*$^-$ *S. pombe*. We derive two hypothéses from these observations. The first is that common molecular mechanisms can be adapted to different morphogenic programs (budding in *S. cerevisiae*, shape and conjugation in *S. pombe*). The second is that ras1 in *S. pombe* may have a role that is homologous to both *S. cerevisiae* RAS and *S. cerevisiae* BUD1. In other words, whereas *S. cerevisiae* has evolved two diverged forms of RAS, one specialized for regulating morphogenesis (budding) and one specialized for signal transduction (through the cAMP-dependent protein kinases), *S. pombe* has a single form of RAS capable of regulating both morphogenesis (cell shape and conjugation) and signal transduction (the MAP protein kinase cascade).

The Function of CAP and Related Proteins

K. O'Neill

We previously described the adenylyl-cyclase-associated protein (CAP) required for RAS-stimulated adenylyl cyclase activity in *S. cerevisiae* (Field et al., *Cell 61:* 319 [1990]; Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]). The *S. pombe* homolog, cap, is also associated with adenylyl cyclase and appears to be required for its proper regulation (Kawamukai et al., *Mol. Cell. Biol. 3:* 167 [1992]), although the regulator of adenylyl cyclase in *S. pombe* does not appear to be ras1 (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis and Nasim, *Mol. Cell. Biol. 10:* 549 [1990]; Kawamukai et al., *Cell Regul. 2:* 155 [1991]). In *S. pombe*, the *gpa2* gene product, a G_α-like protein, appears to be required for adenylyl cyclase regulation (Isshiki et al., *Genes Dev. 6:* 2455 [1992]). CAP proteins are bifunctional proteins, as we have described in previous years (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]; Kawamukai et al., *Mol. Cell. Biol. 3:* 167 [1992]). The amino terminus is associated with adenylyl cyclase and the carboxyl terminus is associated with morphological functions.

Recently, in collaboration with Makoto Kawamukai, formerly from this lab but now returned to Shimane University, in Japan, we have succeeded in cloning a human homolog of CAP by complementation in *S. pombe* of the loss of carboxy-terminal cap function. This gene encodes a protein that is the homolog of a porcine protein, called ASP-56, isolated from platelets based on its actin-binding properties (Gie-selmann and Mann, *FEBS Lett. 298:* 149 [1992]). In collaboration with Jeff Field, we have shown that both the human and *S. cerevisiae* CAP proteins can bind to *S. cerevisiae* actin. We previously noted that loss of carboxy-terminal CAP function in *S. cerevisiae* could be suppressed by overexpression of profilin, an actin- and phospholipid-binding protein (Vojtek et al., *Cell 66:* 497 [1991]). This leads us to postulate that the carboxyl terminus of CAP and profilin have redundant function. CAP homologs have now been found in worms and hydra, and a second human CAP, CAP2, has been found (collaborations with M. Chalfie, Columbia University, New York, and C. Schaller, University of Hamburg, Germany). The carboxy-terminal portion is the only portion that appears to be so conserved that it functions in cross species complementation. However, the amino-terminal primary structure is at least partially conserved. This raises our hopes that the amino-terminal domains bind adenylyl cyclase in other species, and we are hopeful of discovering a homolog of the yeast form of this enzyme in mammals.

Analysis of the Mechanism of Tumor-promoting Phorbol Esters and Protein Kinases C

C. Nicolette

In previous years, we identified a number of human cDNAs that, when expressed in *S. cerevisiae*, could

block the phenotypes induced by activated RAS (Colicelli et al., *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). One of these, called JC310, has proven to be of particular interest because its overexpression in mammalian cells has a striking phenotype. Analysis indicates that expression of JC310 blocks the action of the tumor-promoting phorbol esters. Phorbol esters activate protein kinases C (PKC), which are thought to mediate the action of the phorbol esters. In particular, phorbol esters induce DNA synthesis in quiescent cells, induce morphological change, induce phosphorylation of PKC substrates, induce phosphorylation of MAP kinase, and induce *fos* expression (Kikkawa and Nishizuka, *The Enzymes*, Academic Press [1986]; Ono et al., *J. Biol. Chem. 263:* 6927 [1988]).

Cells selected for overexpression of JC310 are resistant to phorbol-ester-induced DNA replication, *fos* expression, and morphologic change. However, such cells do undergo the changes in protein phosphorylation induced by the phorbol esters. Cells transiently expressing JC310 and a *fos-CAT* reporter construct do not respond to phorbol esters by induction of CAT (chloramphenicol acetyltransferase). Expression of JC310 appears to have no effect on the cellular response to RAS, growth factors that are mediated through tyrosine kinase receptors, or serum. We have not yet tested factors that act through G proteins. JC310 localizes to the nucleus.

These results require some rethinking about phorbol-ester- and RAS-induced signaling. First, our results throw into question the cooperative, interdependent relationship between RAS and PKCs (Yu et al., *Cell 52:* 63 [1988]; Gauthier-Rouviere et al., *EMBO J. 9:* 171 [1990]). Moreover, JC310 blocks phorbol esters without apparently blocking the activation of PKC, and hence phorbol esters might induce effects through a previously uncharacterized route. Alternately, JC310 blocks nuclear signaling from the PKC. The further implication is that nuclear signaling from phorbol esters proceeds through a "private line" of communication with the nucleus that is not shared by serum, RAS, or growth factors acting via tyrosine kinase receptors. Detailed analysis of the regulation of the *fos* promoter are consistent with this hypothesis (Gilman, *Genes Dev. 2:* 394 [1988]; Cai et al., *Mol. Cell. Biol. 10:* 5314 [1990]). Finally, the failure of cells that overexpress JC310 to respond to the morphological effects of phorbol esters brings into question the belief that these rapid effects are mediated by the purely cytoplasmic effects of activating PKC.

Characterization of Mammalian cAMP Phosphodiesterases

T. Michaeli, G. Bolger

Several years ago, we reported that mammalian genes encoding cAMP phosphodiesterases (PDE) could be cloned by selecting from mammalian cDNA libraries those genetic elements that could block the effects of activated RAS (*RAS2*^{Val19}) in *S. cerevisiae* (Colicelli et al., *Proc. Natl. Acad. Sci. 86:* 3599 [1989]). Our first PDEs were homologous to the *Drosophila dunce* gene involved in learning and memory in fruit flies. We now know that there are four such genes in humans and that they encode high affinity, cAMP-specific PDEs that are rolipram-sensitive. Sequence analysis indicates that they share two regions of conserved sequence upstream of the catalytic region (UCR1 and UCR2) and some of the same splice junctions with the *Drosophila dunce* PDE (G. Bodger et al., submitted).

We have also identified a new human PDE, representing a novel family of PDEs, class VII, that we have called HCP. HCP is a very high affinity (K_m ~0.1 μM) cAMP-specific PDE that is insensitive to both milrinone and rolipram. Its expression is especially high in skeletal muscle, but we find it is also expressed in the heart and brain. HCP was cloned by suppression of the phenotype of a *pde1*$^-$ *pde2*$^+$ *S. cerevisiae* strain. This latter strain is a more sensitive host strain for screening PDEs than is the *RAS2*^{Val19} strain (T. Michaeli et al., *J. Biol. Chem.*, in press.)

Difference Cloning

N. Lisitsyn, N. Lisitsyn, H.-P. Xu

In 1981, it was apparent that gene transfer was detecting mainly mutant *RAS* genes in tumor cells and that an unbiased method was required for the detection of genetic lesions in tumors. In 1985, we began in earnest to develop a "difference cloning" methodology that would enable us to detect the differences between two complex genomes. Our early efforts culminated in a method that could achieve 100–1000-fold enrichment of "difference" sequences (i.e., "target" found in the "tester" genome, but not present in the "driver" genome) by reiterated subtractive methods (Wieland et al., *Proc. Natl. Acad. Sci.*

87: 2720 [1990]). We also realized that the second-order kinetics of DNA reannealing could be harnessed to achieve even greater enrichment, but we could not combine the subtractive and kinetic steps. Hence, we failed to achieve the levels of enrichment required for the difference analysis of complex genomes.

In 1991, Nikolai Lisitsyn arrived at Cold Spring Harbor to continue studies that he had begun on this problem in Moscow. He brought with him an elegant technique, based on the polymerase chain reaction (PCR), to amplify small quantities of homoduplex DNA in the presence of large quantities of unannealed DNA or heteroduplex DNA. While here, we engineered this technique into a powerful difference analysis method that utilized subtractive and kinetic enrichment together in each repetitive step. In the analysis of complex genomes, we found it was necessary first to sample the genomes, reducing their complexity in a reproducible way. We achieved this by "whole genome" PCR in which the DNA is cleaved, ligated to template oligonucleotide linkers, and then amplified by PCR. Only small fragments amplify well and hence the resulting "amplicon" has a smaller overall complexity than the starting material. Through multiple samplings, much of one genome can thus be scanned for differences with another genome. The details of the method, which we call representational difference analysis (or RDA), were recently published (Lisitsyn et al., *Science 259:* 946 [1993]). We demonstrated that RDA could be used to clone polymorphisms between two individuals and to detect viral genomes added in single copy to one genome. The applications of this method, which are myriad, are discussed here.

First, RDA may be used to search for pathogenic organisms in infected tissue (tester) that are not present in uninfected tissue from the same individual (driver) or in the pooled uninfected tissue of the patient's parents. We have demonstrated this method in reconstruction experiments but have not yet succeeded in using it to discover new pathogens in diseased tissue. We have found it necessary to extract high-molecular-weight DNA from the infected tissue, or else artifacts may arise. Three types of problems can be anticipated: when the pathogenic organism does not have a DNA phase, when the infecting organism has created a host reaction but is no longer present, and when other foreign organisms are present that are not related to the pathogenic process.

Second, RDA may be used for analysis of genetic lesions in tumors. This may proceed in either of two ways. When essentially pure tumor cells (uncontaminated by stroma) can be obtained, DNA can be extracted and used as driver against normal DNA as tester. The "target" sequences will represent restriction enzyme fragment polymorphisms that are missing in the tumor due to loss of heterozygosity or sequences that are absent in the tumor due to homozygous deletion. Such events may mark the loss of function of a tumor suppressor gene. There are three problems in applying the method in this manner. First, pairs of pure tumor cell populations and their normal counterparts are hard to come by. Second, the tumor cell source is usually a cell line, and many genetic changes may have occurred during the establishment and maintenance of the cell line. Third, loss of heterozygosity may be so prevalent that probes for these events will greatly outnumber probes for the more interesting lesions resulting from homozygous deletions. We have applied RDA in this manner in several instances and have consistently found probes that reflect loss of heterozygosity.

Alternatively, normal DNA can be taken as driver, with the tumor taken as tester. When applied this way, RDA yields probes for sequences present in tumors that are absent in normal DNA. Such sequences could arise by chromosomal rearrangements that fortuitously create small DNA restriction endonuclease fragments containing sequences not present in amplicons from normal DNA. Thus, this method has the potential to detect probes for translocations, deletions, inversions, and insertions. In addition, we have found that sequences amplified in the tumor cell can also be detected this way. If viral sequences are present in tumor cells, they will also be detected. Rarely, point mutations will create new restriction fragments that can be cloned. There are several advantages to applying RDA to tumors in the above-described manner. Material can be obtained from the patient, at all stages, by biopsy, and the contamination by normal stroma will be well tolerated. Lesions of many sorts should be identifiable and will not be confounded by loss of heterozygosity. The only disadvantage we can anticipate is that some tumor cells may have so many lesions of biological insignificance, present as a consequence of errors of DNA replication or repair, that the significant lesions cannot be found. In that event, it might be necessary to examine model animal systems.

Third, RDA can be applied to both inherited genetic disease and sporadic (or spontaneous) genetic dis-

ease. In the first case, we utilize RDA to find polymorphic markers linked to the disease. This method has been successfully tested in model systems of inherited disease in mice, carried out in collaboration with Eric Lander's group at the Massachusetts Institute of Technology. The method works because progeny with the desired phenotypes can be selected from crosses between polymorphic strains of animals, and their DNA can be used as tester (or driver) against DNA from the strain lacking the trait as driver (or tester). Similar studies with human families can be undertaken, but we have not yet done so.

In the case of sporadic disease, we can anticipate that genetic rearrangement may be responsible for a portion of spontaneous lesions. Such a situation is formally akin to genetic rearrangements in tumor cells. The affected child's DNA is used as tester, and pooled parental DNA is used as driver. We expect to be able to use RDA to isolate probes for genetic rearrangements causing a wide variety of diseases, including mental retardation, severe developmental defects, autism, and juvenile schizophrenias. Molecular approaches to such diseases were previously not generally available.

Combinatorial Chemical Libraries

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The diversity of life is achieved by combinatorial methods: any codon can follow any codon; any amino acid the previous one. A small protein of 200 residues has 20^{200} possible sequences, a number greater than the number of atoms in the universe. From this diversity, proteins and nucleic acid sequences are selected in the course of evolution. Something like this, although not as grand in scale, can now be achieved in the laboratory by synthetic means. Oligopeptides of great diversity (in the millions or even in the billions) can be generated, either chemically or through genetic engineering (Smith, *Science 228:* 1315 [1985]; Scott and Smith, *Science 249:* 386 [1990]; Houghten et al., *Nature 354:* 84 [1991]). The structure of those few peptides selected for some characteristic (usually binding to a receptor) can then be determined. Chemists can generate many equally diverse libraries of organic molecules, but determining the structure of those few compounds that have desired properties has until now not been feasible. In collaboration with Clark Still and his colleagues at Columbia University, we have solved this problem.

Details of the method will be submitted for publication at a later time. In essence, the method entails making compounds on microsphere beads, each bead having a high density of a single compound covalently attached. This method of synthesis has been described previously by other investigators in the generation of vast peptide libraries (Furka et al., *Int. J. Pept. Protein Res. 37:* 487 [1991]; Lam et al., *Nature 354:* 82 [1991]), but whereas the identity of the compounds on a selected bead was previously determined by microsequencing, a method applicable to oligopeptides and oligonucleotides, we perform the synthesis of the compounds on the beads in such a way that the chemical identity of the compound on the bead is readily determined by "reading" the bead. Using this method, we succeeded in synthesizing a peptide library of 10^5 different molecules, selected beads bearing peptides reactive with a monoclonal antibody, and correctly decoded the beads so selected. The method is not restricted to peptides. It is applicable to the discovery of small organic molecules that bind to a given target, inhibit a given enzyme, inhibit the growth of a given microbe, or, more generally, induce a given response in cells. Other uses of the methodology will be in the study of molecular recognition.

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