GENETICS OF CELL PROLIFERATION

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The focus of our group remains largely on the signal transduction pathways involved in growth control, with a special emphasis on pathways involving RAS proteins. The RAS proteins were first discovered as the products of retroviral oncogenes. They are low-molecular-weight GTP- and GDP-binding proteins. We and other investigators have found mutant RAS proteins in a large number of human tumor cells. Closely related proteins are found in yeast, and we have attempted to explore the function of these proteins both in the budding yeast *Saccharomyces cerevisiae* and in the fission yeast *Schizosaccharomyces pombe*. RAS function is best understood in *S. cerevisiae*, where we identified its major effector as adenylyl cyclase. However, the mechanism by which RAS activates adenylyl cyclase remains largely unknown. A new component of the RAS/adenylyl cyclase system has been discovered, called CAP, which may integrate this signaling system with remodeling of the cellular cytoskeleton. In *S. pombe*, where little is understood about RAS function, we have identified several genes that appear to encode

components of the RAS signaling system. We use both yeasts to explore the function of mammalian RAS and putative RAS-related proteins, such as the product of the neurofibromatosis locus, NF1, and we have developed genetic screens to clone novel mammalian cDNAs which encode proteins that function in signal transduction pathways. Among these proteins are candidates for mammalian RAS effectors. This work has also led to the cloning of genes encoding cAMP phosphodiesterases, enzymes of considerable physiological importance. Finally, we continue work aimed at developing methods for genomic difference cloning that will enable us to discover recessive oncogenes and novel pathogens.

S. cerevisiae Adenylyl Cyclase

J. Field, J. Colicelli, T. Michaeli, K. Ferguson, R. Ballester, D. Young, N. Chester, H.-P. Xu, G. Heisermann, M. Wigler

The *CYR1* gene encodes adenylyl cyclase, the one known target for RAS protein action (Kataoka et al., *Cell 43:* 493 [1985]; Toda et al., *Cell 40:* 27 [1985]). This large (220-kD) protein has two distinct domains: a 40-kD carboxy-terminal catalytic domain and a 60-kD leucine-rich domain with a repeating motif, located in the center of the molecule. In the past year, we completed two related studies of adenylyl cyclase/RAS interactions. In the first study, a series of in-frame deletion and insertion mutations were made in *CYR1*, and the encoded product was tested for RAS responsiveness (Colicelli et al., *Mol. Cell. Biol. 10:* 2539 [1990]). Most of the amino-terminal region, to within 100 amino acids of the leucine-rich repeat domain, is not required for retention of RAS responsiveness. Large deletions or small insertions within the repeat domain destroy RAS responsiveness. Large deletions in the region between the repeat and the catalytic domain also destroy RAS responsiveness, but small insertions are tolerated throughout this region. We conclude from these data that the structure of the leucine-rich repeat domain is very sensitive to perturbation and is critical for RAS responsiveness. The latter conclusion is confirmed by the second study. We discovered that overexpression of catalytically defective adenylyl cyclase molecules inhibits RAS function in yeast (Field et al., *Science 247:* 464 [1990]). Truncated genes encoding fragments of adenylyl cyclase were tested for this effect. The smallest fragment capable of inhibiting RAS function was precisely the leucine-rich repeat domain. These studies further emphasize the importance of this domain in RAS responsiveness. Future studies will focus on determining what other proteins bind to the leucine-rich repeat.

S. cerevisiae CAP

J. Field, A. Vojtek, J. Gerst, K. Ferguson, M. Kawamunkai, M. Riggs, L. Rodgers, M. Wigler

To explore the mechanism by which RAS stimulates adenylyl cyclase, we designed an epitope fusion-immunoaffinity protocol to purify the adenylyl cyclase complex from yeast (Field et al., *Mol. Cell. Biol. 8:* 2159 [1988]; *Cell 51:* 219 [1990]). The complex is RAS-responsive and contains at least one other protein, with an apparent mobility of 70 kD, which we have called CAP. Antibodies raised to CAP were used to screen expression libraries, resulting in the cloning of *CAP*. Sequence and restriction endonuclease analysis indicates that *CAP* is identical to the gene we prevously called *SUPC*. We had previously identified an allele of *SUPC* as a suppressor of the activated $RAS2^{val19}$ allele. Gene disruption indicates that CAP function is required by cells to be fully responsive to RAS. However, cap^- cells have other phenotypic defects. They are round, often enlarged and multinucleated, sensitive to nitrogen deprivation, and fail to grow in rich medium or in the presence of an excess of certain amino acids, such as valine (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]). CAP is in fact a bifunctional protein; the amino terminus is required for full RAS responsiveness and the carboxyl terminus is required for the diverse growth and morphological effects.

To gain insight into the nature of the carboxyl terminus, we have sought genes on multicopy plasmids that suppress the phenotype that results from the loss of CAP. One strong suppressor has been found so far: *PFY*, the gene encoding profilin (A. Vojtek et al., in prep.). Profilins are low-molecular-weight proteins that can block actin polymerization by binding the actin monomer (Lassing and Lindberg, *Nature 314:* 472 [1985]). This binding is released by interaction with lipid micelles containing phosphatidyl inositol phosphates.

The surprising connection between profilin and CAP led us to examine the phenotypes of pfy^- and

cap⁻ cells more closely. These studies were performed in collaboration with Susan Brown and Brian Haare at the University of Michigan. *pfy*⁻ cells look like *cap*⁻ cells. They are round and enlarged. They also grow less well in rich medium. *cap*⁻ cells, like *pfy*⁻ cells, have no discernible actin cables and bud randomly from their surface. Overexpression of *PFY* in *cap*⁻ cells restores most of *cap*⁻ phenotypic defects, save one: Such cells grow in rich medium, survive a nitrogen starvation, are smaller, bud asymmetrically from one pole, and have actin cables, but they are not RAS-responsive. These results suggest that the carboxyl terminus of CAP is required for proper profilin function and that the phenotype which results from its loss is mainly due to loss of profilin function.

Some biochemical insight into CAP emerges from these experiments. In our collaboration with the group at the University of Michigan, we have found mutant *PFY* genes that can suppress a *pfy*⁻ cell but not a *cap*⁻ cell. These mutant genes encode proteins that apparently bind actin with affinities equal to those of wild-type profilins, so some function beside actin binding is required for suppression of *cap*⁻ cells, perhaps phospholipid binding. To test this, we expressed form I and form II *Acanthamoeba* profilins in *pfy*⁻ and *cap*⁻ yeasts. Form II profilin binds phosphatidyl inositol phosphates with high affinity and suppresses both *cap*⁻ and *pfy*⁻ cells. However, form I profilin, which binds phospholipids with lower affinity, suppresses *pfy*⁻ cells but not *cap*⁻ cells. These results suggest that CAP effects profilin function, and hence the cellular cytoskeleton, by effecting the metabolism of phosphatidyl inositol phosphates.

We do not yet know if CAP is a regulatory molecule, and if so, what regulates it. However, it physically connects two major pathways: (1) the cAMP pathway, presumably involved in growth signaling, and (2) a pathway in phospholipid metabolism effecting the remodeling of the cell. CAP is therefore a prime candidate for integrating these pathways. Its proximity to adenylyl cyclase means it is proximal to RAS, and although we have no evidence yet that CAP is controlled by RAS, we note that the types of changes in mammalian cells induced by oncogenic RAS, namely, morphological transformation with its dissolution of actin cables and alterations in phospholipid metabolism, resemble the effects of altered CAP function in yeast.

Future studies will be directed to finding CAP homologs in other organisms, exploring the precise biochemical function of CAP, and looking for further genetic clues into its interactions with other proteins.

Mammalian cDNAs Selected for Interfering with RAS Function in *S. cerevisiae*

J. Colicelli, C. Nicolette, E. Chang, L. Rodgers, M. Riggs, M. Wigler

The observation that dysfunctional forms of adenylyl cyclase interfere with RAS function inspired the design of a genetic screen for mammalian cDNAs that encode products which can interact with yeast RAS proteins. Cells expressing the *RAS2*^{val19} gene are heat-shock-sensitive, one of the phenotypic hallmarks of a constitutively activated RAS pathway. The same phenotype results from overexpressing adenylyl cyclase or the catalytic subunits of the cAMP-dependent protein kinase, encoded by the *TPK* genes, or from the disruption of the cAMP phosphodiesterases, encoded by the *PDE1* and *PDE2* genes. We designed a high-copy yeast shuttle vector that can express mammalian cDNA inserts from a strong yeast promoter. cDNA libraries constructed in this vector were screened for clones that could suppress the heat-shock-sensitive phenotype of *RAS2*^{val19} strains. Several clones were so isolated, and these fell into two sets (Colicelli et al., *Proc. Natl. Acad. Sci. 86:* 3599 [1989]; *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). The first set of clones could also suppress the heat-shock-sensitive phenotype of *pde*⁻ strains. These cDNAs encode cAMP phosphodiesterases (see below). The second set failed to suppress *pde*⁻ phenotypes and hence contained candidates encoding proteins that interact with RAS proteins.

Three genes were represented among this second set: *JC99, JC265,* and *JC310*. Sequence analysis revealed no relationship between these genes and genes in the known data banks; however, *JC99* and *JC265* encode proteins that are clearly related to each other and presumably are members of a family. Further genetic analysis in *S. cerevisiae* indicates that all three genes can interfere with the activity of *RAS1* and *RAS2* and the mammalian Ha-*ras* genes expressed in *S. cerevisiae*. The mechanism of action is not known.

Future studies will aim at expressing products of the genes in mammalian cells, *Xenopus* oocytes, and the yeast *S. pombe* in an attempt to understand

whether they interact with RAS proteins directly and to determine if they are effectors of RAS action.

Function of the Von Recklinghausen Neurofibromatosis Gene Product, NF1

R. Ballester, M. Wigler

The gene for the Von Recklinghausen neurofibromatosis locus *NF1* was recently identified (Cawthon et al., *Cell 62:* 193 [1990]; Viskochil et al., *Cell 62:* 187 [1990]; Wallace et al., *Science 249:* 181 [1990]). People afflicted with this disease have widespread disorders of the proliferation and differentiation of tissue of neuroectodermal origin. The partial sequence of the *NF1* cDNA was determined and found to encode a protein with sequence similarities to the mammalian GAP and yeast IRA proteins (Buchberg et al., *Nature 347:* 291 [1990]; Xu et al., *Cell 62:* 599 [1990]). GAP (*G*TPase *a*ctivation *p*rotein) is a factor that accelerates GTP hydrolysis by RAS and is capable of down-regulating wild-type RAS proteins (Trahey and McCormick, *Science 238:* 542 [1987]; Ballester et al., *Cell 29:* 681 [1989]; Zhang et al., *Nature 246:* 754 [1990]). Oncogenic RAS proteins are generally resistant to GAP. The IRA proteins are structurally related proteins with similar functions (Tanaka et al., *Cell 60:* 803 [1990]). They are encoded by the *IRA1* and *IRA2* loci, and disruption of either loci leads to a heat-shock-sensitive phenotype, the result of activation of the RAS pathway. The NF1 protein bears about the same relatedness to GAP as do the IRA proteins, but NF1 shows a greater global similarity to the IRA proteins. In collaboration with F. Collins and D. Marchuk at the University of Michigan, we have begun to examine the function of the human NF1 protein when expressed in yeast (Ballester et al., *Cell 63:* 851 [1990]). Expression of NF1 can restore heat-shock resistance to yeast defective in IRA function, as we have previously demonstrated for GAP, and can down-regulate mammalian Ha-ras when they are co-expressed in yeast. Unlike GAP, NF1 appears to be capable of interfering with the function of the activated Ha-ras^{val12} mutant protein in yeast. These results indicate that NF1 can interact with a wide range of RAS proteins. We directly demonstrated that NF1 possesses GAP-like activity: Extracts of yeast cells expressing NF1 accelerate the GTP hydrolysis of purified Ha-ras, but not of Ha-ras^{val12}, proteins. In the future, we intend to examine whether NF1 shares other functions with the IRA proteins.

S. pombe Adenylyl Cyclase

D. Young, M. Kawamukai, K. Ferguson, M. Wigler

We have begun studying *S. pombe* as an experimental system to explore RAS function. *S. pombe* contains a single *ras1* gene encoding a RAS homolog (Fukui and Kaziro, *EMBO J. 4:* 687 [1985]). *ras1* does not regulate *S. pombe* adenylyl cyclase (Fukui et al., *Cell 44:* 239 [1986]). In search of clues to RAS function, we have begun to explore the difference between the regulation of adenylyl cyclase in the two organisms. First, we cloned and sequenced the *S. pombe cyr1* gene (Young et al., *Proc. Natl. Acad. Sci. 86:* 7989 [1989]). It encodes a large protein with about 30% sequence identity to *S. cerevisiae* CYR1 overall. The homology between the catalytic domains is stronger. Like *S. cerevisiae CYR1*, the *S. pombe* gene encodes a leucine-rich repeat domain, and the catalytic domain is carboxy-terminal. Thus, the overall structures of the two yeast cyclases are very similar and differ radically from the structures of the mammalian adenylyl cyclases that have been discovered recently (Krupinski et al., *Science 244:* 1558 [1989]).

Disruption of *S. pombe cyr1* produces a phenotype we call hypersexed (Kawamukai et al., *Cell Regul. 2:* 155 [1991]). *cyr1*$^-$ cells are perfectly viable, but mate prematurely in rich medium, unlike wild-type cells that mate upon starvation. Overexpression of *cyr1* leads to a partial sterile phenotype. Overexpression of the repeat domain encoded by *cyr1* also leads to a hypersexed phenotype, suggesting that expression of this fragment of adenylyl cyclase interferes with the function of the wild-type protein, much as we have found in our studies of the *S. cerevisiae* cyclase. This study suggests that both molecules share a conserved regulatory mechanism. Other evidence points to the conclusion that *S. pombe* cyr1 is regulated.

Full-length cyr1 molecules can be purified from *S. pombe*, using the epitope addition immunoaffinity method, and these preparations have high levels of enzymatic activity when assayed in the presence of Mn^{++} ion. The ratio of Mn^{++} to Mg^{++} activities is much higher for *S. pombe* cyr1 than it is for *S. cerevisiae* CYR1. We see no stimulation of activity in the presence of guanine nucleotides. We have clear evidence that a protein antigenically related to *S.*

cerevisiae CAP is associated with *S. pombe* cyr1, and we are in the midst of characterizing this molecule.

An *S. pombe* Kinase in the ras1 Pathway

Y. Wang, H.-P. Xu, M. Riggs, L. Rodgers, M. Wigler

We have sought genes in *S. pombe* that encode components of the ras1 signaling pathway. *ras1*$^-$ cells are viable but fail to sporulate or conjugate and are round in shape rather than cylindrically elongated (Fukui et al., *Cell 44:* 329 [1986]). We selected for *S. pombe* genes present on high-copy shuttle vectors that could restore conjugation to cells expressing a dominant interfering *S. cerevisiae RAS2*^{ala15} gene. In this way, we isolated *byr2* (*by*pass of *r*as, no. 2, also known as *sir1*) (Wang et al., *Cell Biol.* [1991] in press). *byr2* can also suppress the sporulation defects of *ras1*$^-$/*ras1*$^-$ cells but not the cell-shape abnormalities or the conjugation defects of a *ras1*$^-$ cell. *byr2* has the potential to encode a serine/threonine kinase, with a carboxy-terminal catalytic domain. In size and structure, byr2 resembles the protein kinases C, the raf kinases, and the cGMP-dependent protein kinases, but it has no particularly close relationship to any individual kinases. In its genetic properties, *byr2* most closely resembles *byr1*, an *S. pombe* gene encoding another protein kinase that was identified previously (Nadin-Davis and Nasim, *EMBO J. 7:* 985 [1988]). Expression of *byr1* can likewise suppress the sporulation defects of *ras1*$^-$/*ras1*$^-$ diploids. Like *byr1*$^-$ cells, *byr2*$^-$ cells are perfectly viable and of normal shape but are absolutely defective in conjugation and sporulation. *byr1* and *byr2* are thus both good candidates for genes encoding downstream components of the ras1 signaling pathways. Since overexpression of *byr1* can complement the sporulation defects of *byr2*$^-$ cells, but not vice versa, we can place the site of action of byr1 downstream from byr2.

A Putative Nucleic-acid-binding Protein in the ras1 Pathway

H.-P. Xu, Y. Wang, M. Riggs, L. Rodgers, M. Wigler

We directly selected for *S. pombe* genes that on high-copy shuttle vectors are capable of suppressing the sporulation defects of *ras1*$^-$/*ras1*$^-$ diploids. One of these genes, called *byr3* (also known as *prs* and *HP18*), was found to encode a protein comprised almost in its entirety of a zinc finger repeat motif (H.-P. Xu, Ph.D. Thesis [1990]). This metal-binding motif is commonly found in proteins that bind double- or single-stranded DNA, or RNA. The motif structure of byr3 resembles most closely the metal-binding motif of CNBP, a putative sterol regulatory element that binds single-stranded DNA (Rajavashisth et al., *Science 245:* 640 [1989]). On the basis of its structure, we imagine that *byr3* encodes an inhibitor of gene transcription.

The genetic characterization of *byr3* is as follows. High-copy expression of *byr3* suppresses the sporulation defects of *ras1*$^-$/*ras1*$^-$ diploids, but not the shape abnormalities or conjugation defects of *ras1*$^-$ cells, and none of the phenotypic defects of *byr2*$^-$ or *byr1*$^-$ cells. *byr3*$^-$ cells are perfectly viable and normally shaped, but they are partially sterile. This defect is restored by high-copy wild-type *ras1* or *byr2*, but not by *byr1*. This result indicates again that the byr2 kinase lies closer to the root of the ras1 signaling pathway than does the byr1 kinase. This result further suggests that byr2 and byr1 have common but perhaps parallel functions and that byr1 acts at least partly through byr3. Further studies will focus on resolving this model and delineating the role, if any, that byr3 plays in transcriptional regulation.

GAP-like Molecules in *S. pombe*

Y. Wang, H.-P. Xu, M. Riggs, L. Rodgers, M. Wigler

We have sought genes that on high-copy shuttle vectors are capable of inhibiting the phenotype of *S. pombe* cells expressing *ras1*^{val17}, the activated allele of *ras1*. Such cells are partially sterile, highly agglutinable, and develop long exaggerated conjugation tubes. One gene was isolated that we called *sar1* (*s*uppressor of *a*ctivated *r*as) (Wang et al., *Mol. Cell. Biol.* [1991] in press). Upon DNA sequencing, it eventually became clear that *sar1* encoded another member of the family of proteins that included the GAP, IRA, and NF1 proteins. The homology with other members of this family resides strictly in the "catalytic" domain, and sar1 is closest in sequence to NF1.

Genetic evidence indicates that *sar1* downregulates *ras1* in *S. pombe*. *sar1*$^-$ cells have the same

phenotype as *ras1*^{val17} cells, and *ras1*$^{-}$ mutations are epistatic to *sar1*$^{-}$; i.e., *ras1*$^{-}$ *sar1*$^{-}$ double mutants have the same phenotype as *ras1*$^{-}$ cells. Expression of sar1, like expression of GAP and NF1, can complement yeast defective in the *IRA* genes.

Many investigators have speculated that GAP-like molecules encode RAS effectors. There is some evidence for this in mammalian cells (Tatani et al., *Cell 61:* 769 [1990]), but no evidence of this for the *S. cerevisiae* IRA proteins. There is also no support for this hypothesis from studies of sar1 function in *S. pombe*.

An RNase in the Regulation of *S. pombe* Sexual Function

H.-P. Xu, Y. Wang, M. Riggs, L. Rodgers, M. Wigler

Disruption of ras1 function leads to failure to conjugate and sporulate. We therefore selected for genes on high-copy plasmids that caused failure to sporulate. One such gene was isolated and called *hcs* (*h*igh copy *s*terile) (Xu et al., *Nucleic Acids Res. 18:* 5304 [1990]). Disruption of *hcs* causes lethality. Overexpression causes no diminution in growth rate or change in shape, but it renders cells unable to sporulate or conjugate. Sequence analysis reveals the *hcs* can encode 363 amino acids with 24% sequence identity to *Escherichia coli* RNase III. RNase III is a double-strand-specific RNase involved in processing of ribosomal RNA and mRNAs in *E. coli* (Nashimoto and Uchida, *Mol. Gen. Genet. 201:* 25 [1985]; Portier et al., *EMBO J. 6:* 2165 [1987]; Takiff et al., *J. Bacteriol. 171:* 2581 [1989]). This work suggests that some control of the pathways regulating sexual differentiation may be exerted at the level of RNA stability or availability and implicates regulation of RNases in the control of gene expression in eukaryotes.

Mammalian cDNAs Selected for RAS Function in *S. pombe*

H.-P. Xu, M. Riggs, L. Rodgers, M. Wigler

It is altogether possible that the function of *ras1* in *S. pombe* resembles its function in mammalian cells. To explore this possibility, we screened mammalian cDNA libraries cloned into *S. pombe* expression vectors for genes capable of inducing sporulation in *ras1*$^{-}$/*ras1*$^{-}$ diploids. Many classes of candidates were obtained. The first class comprised mammalian *RAS* genes, which was expected (Xu et al., *Cell Regul. 1:* 763 [1990]). The second class comprised mammalian *RAP* genes, which was unexpected, including *RAP1A*, *RAP1B*, and *RAP2* (Xu et al., *Cell Regul. 1:* 763 [1990]). The *RAP* genes encode members of the RAS superfamily (Pizon et al., *Oncogene 3:* 201 [1990]). RAP proteins show approximately 50% identity with RAS proteins. RAP1A is also known as Ki-*rev*-1, which was discovered as a weak inhibitor of the morphologic transformation induced by oncogenic *RAS* (Kitayama et al., *Cell 56:* 77 [1989]). These mammalian *RAP* genes can induce sporulation in *ras1*$^{-}$/*ras1*$^{-}$ diploid *S. pombe* and can restore normal cell shape to *ras1*$^{-}$ haploid cells, but they fail to restore conjugal competence to *ras1*$^{-}$ cells. We next explored the function of mammalian *RAP1A* in *S. cerevisiae*. In that yeast, expression of *RAP1A* was not able to provide RAS-like functions. Indeed, expression of *RAP1A* weakly antagonized the phenotype of the activated *RAS2*^{val19} allele. These studies indicate that mammalian RAP can interact with RAS targets, sometimes productively and sometimes nonproductively.

The third class of cDNAs comprise two genes known as *ATG16* and *ATG29* (H.-P. Xu, Ph.D. Thesis [1990]). In virtually all its genetic aspects, *ATG29* resembles a weaker form of *ATG16*. In high copy, both genes can induce sporulation in *ras1*$^{-}$/*ras1*$^{-}$ diploid yeast. They fail to correct shape and conjugation defects of *ras1*$^{-}$ cells, but both can correct the conjugation defects of *byr3*$^{-}$ yeast. Neither can correct the phenotypic defects of *byr1*$^{-}$ or *byr2*$^{-}$ yeast. These results suggest that ATG16 and ATG19 can each carry out a portion of ras1 function lying upstream of the byr1 and byr2 kinases and also suggest that they operate on the same pathway. Strikingly, both *ATG16* and *ATG29* can induce haploid sporulation in cells containing the activated *ras1*^{val17} allele. Haploid sporulation is a very rare event in wild-type yeast. This result again strongly suggests the relatedness of the function of these gene products, which can evidently act synergistically with *ras1*^{val17}. Finally, coexpression of *ATG16* and *ATG29* can induce haploid sporulation in wild-type *S. pombe*. Thus, their gene products can cooperate. Future studies will focus on the characterization of the *ATG16* and *ATG29* gene products and their function in mammalian cells.

Mammalian cAMP Phosphodiesterase Genes

J. Colicelli, T. Michaeli, G. Bolger, M. Riggs, L. Rodgers, M. Wigler

In the course of selecting for mammalian cDNAs that could suppress the phenotype of $RAS2^{val19}$ cells, a rat cAMP phosphodiesterase (PDE) gene was cloned (Colicelli et al., *Proc. Natl Acad. Sci. 86:* 3599 [1989]) and named rat *DPD* because it was homologous to the D*rosophila melanogaster d*unce *p*hosphodiesterase. A great variety of cAMP phosphodiesterases are expressed in mammalian tissues and are of considerable importance, since cAMP regulates many physiological processes. A better understanding of this complex family of enzymes will emerge once the genes encoding them are cloned and characterized. Approaching this problem through biochemical purification and sequencing has been a slow process, and yeast selection for cDNAs encoding PDEs provides a way to accelerate this discovery.

By screening a cDNA library made from a human cell line, we have discovered three different human PDE genes. Two of these are closely related and encode rolipram-inhibitable, high affinity, cAMP-specific PDEs. These two genes are homologous to the rat *DPD*. We discovered other members of this family by using the rat *DPD* as a probe. Using degenerate oligonucleotide probes to conserved regions as primers in polymerase chain reactions, we have concluded that there are probably only four genes that comprise this family. The third human PDE cDNA cloned by complementation in yeast has biochemical properties that suggest it represents a previously undiscovered family of PDEs. We plan to characterize the human PDE families further.

Genomic Difference Cloning

I. Wieland, G. Asouline, K. O'Neill, M. Wigler

We have been in the process of developing a method of "genomic difference cloning." This method allows the identification by cloning a DNA sequence present in one genome that is absent in an otherwise similar or identical genome (Wieland et al., *Proc. Natl. Acad. Sci. 87:* 2720 [1990]). Such differences arise in important situations: when an individual or a tissue is infected with a DNA-based pathogen or when a neoplastic cell has lost sequences from both alleles of a recessive oncogene. The outline of one method for difference cloning was described in last year's Annual Report and was published this past year. This method is not yet powerful enough to be used routinely for our purposes. We have concentrated this year on the incremental improvement of our methodologies.

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