THE UNIVERSITY OF CALGARY

The Cyclic AMP Signal Transduction Pathway in Fission Yeast *Schizosaccharomyces Pombe*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Cyclic AMP Signal Transduction Pathway in Fission Yeast *Schizosaccharomyces Pombe*" submitted by Jiahua Li in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The cyclic AMP signaling pathway plays an important role in controlling sexual development in the fission yeast *Schizosaccharomyces pombe*. This thesis presents the cloning and characterization of components of the cAMP pathway, including *pka1* encoding the catalytic subunit of *S. pombe* cAMP-dependent protein kinase. The upstream positive regulator for *S. pombe* adenylyl cyclase has not been identified. Although RAS proteins regulate adenylyl cyclase activity in the budding yeast *S. cerevisiae*, Ras does not regulate adenylyl cyclase in *S. pombe*. By using the yeast two hybrid system to screen an *S. pombe* cDNA library, the *S. pombe* homolog of 14-3-3 was identified as a protein capable of interacting with the C-terminal domain of *S. pombe* adenylyl cyclase. Future studies will be conducted to determine the role of 14-3-3 protein in the regulation of adenylyl cyclase in *S. pombe*.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
aa	amino acid
ADH	alcohol dehydrogenase
AMP	adenosine-3',5'-monophosphate
ATP	adenosine 5'-triphosphate
bp	base pairs
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
cAMP	cyclic AMP
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal alkaline phosphatase
C-terminal	carboxyl-terminal
D. discoideum	*Dictyostelium discoideum*
D. melanogaster	*Drosophila melanogaster*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	disodium ethylenediaminetetra-acetate
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
HA-epitope	hemagglutinin-epitope
IPTG	isopropyl-β-D-thio-galactopyranoside
kb	kilo base(s)

kD	kilo Dalton(s)
mg	milligram(s)
ml	millilitre(s)
M	moles per liter
Mb	mega base(s)
NBT	nitro blue tetrazolium
NLS	nuclear localization signal
NP-40	nonidet P-40
nt	nucleotides
N-terminal	amino-terminal
O.D.	optical density
pfu	plaque forming units
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline
Tris	Tris (hydroxymethyl) aminomethane
µg	microgram(s)
µl	microlitre(s)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1. INTRODUCTION

I. Life cycle and mating-type system of fission yeast *S. pombe*

Yeasts are among the most tractable eucaryotes for biological research at the molecular level, because of their low complexity and their highly developed formal genetics. Although less studied than the budding yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe* is proving increasingly attractive as an experimental system for investigating problems of eucaryotic cell and molecular biology.

S. pombe is a simple unicellular eucaryote with a genome size of 14 megabases (Mb). It has a typical eucaryotic cell cycle with discrete G1, S, G2, and M phases. In normal minimal or complex media the generation time is between 2 and 4 hours; G2 is about 0.7 of a cell cycle, and the remaining phases are each of about 0.1 of a cell cycle length.

Normally *S. pombe* cells are haploid and may be of two mating types known as h^+ and h^-. Under conditions of nutrient limitation, haploid cells of opposite mating types mate in pairs, forming diploid zygotes which are heterozygous at the mating type locus (h^+/h^-). The zygotes usually undergo meiosis immediately to form four haploid spores. Thus, normally the haploid phase predominates. However, under appropriate conditions and with low frequency a zygote may grow out to produce vegetatively dividing diploid cells. Upon entering the stationary growth phase these diploids undergo azygotic meiosis to form haploid spores (Gutz et al., 1974).

Isolates of *S. pombe* from the wild are homothallic (h^{90}) strains; that is, they can switch their mating type between h^+ and h^- every other generation. This means that a single cell gives rise to a colony containing h^+ and h^- cells which then can mate with one another when nutritional conditions become limiting. Mutations and rearrangements at the mating-type locus give rise to h^+ and h^- strains which either can not switch or switch rarely and are called heterothallic strains.

The mating-type region of fission yeast consists of three components, *mat1*, *mat2-P* and *mat3-M*. Cell-type is determined by the alternate allele present at *mat1*, either P in an h^+ or M in an h^- cell. *mat1-P* and *mat1-M* each encode two genes: *Pc*, *Pi* and *Mc*, *Mi*. *Pc* and *Mc* are necessary and sufficient for mating and confer an h^+ or h^- mating type, respectively. All four genes are required for meiotic competence in an h^+/h^- diploid. The transcription of each *mat* gene is strongly influenced by nutritional conditions and full induction is observed only in nitrogen-free medium.

II. Yeast mating response pathways

1. The *S. cerevisiae* pheromone response pathway

Pheromone response in yeast *S. cerevisiae* starts with extracellular peptide mating factors ("a"-factor and "α"-factor) binding to integral membrane protein receptors (for review, see Marsh et al., 1991). Cells of "a" mating type produce an "α"-factor receptor, encoded by *STE2*; and cells of "α" mating type produce an "a"-factor receptor, encoded by *STE3*. Both receptors are coupled to the same heterotrimeric G protein, Gαβγ. The α, β, and γ subunits of the G protein are encoded by

GPA1/SCG1, *STE4*, and *STE18*. Stimulation of the receptor causes a switch from the GDP-bound state of Gα to the GTP-bound state, which leads to release of the βγ subunit of the G protein (Dietzel et al., 1987; Miyajima et al., 1987). Gβγ activates downstream components of the signaling pathway. A group of protein kinases function downstream of the G protein. This pheromone-response module is composed of the *STE11*, *STE7*, and *FUS3/KSS1* gene products (Cairns et al., 1992; Gartner et al., 1992; Stevenson et al., 1992; Zhou et al., 1993). The kinase modules are highly conserved in eucaryotic organisms: FUS3 and KSS1 are structurally related to mammalian MAP/ERK kinase; STE7 is structurally related to MAP kinase kinase; and STE11 is structurally related to the mammalian MEK kinase, a MAP kinase kinase activator. STE11 phosphorylates and activates STE7 (Neiman and Herskowitz, 1994). STE7, in turn, phosphorylates and activates FUS3 and KSS1 (Gartner et al., 1992; Errede et al., 1993). The activated FUS3 and KSS1 activate the transcription factor, STE12, by phosphorylating it (Elion et al., 1993). STE12 activates transcription of several genes coding for components of the pheromone response pathway and genes necessary for cell fusion (Dolan et al., 1990). The product of *STE20* seems to function between the G protein and STE11 (Leberer et al., 1992). The precise role of STE20 is unknown. STE5 is another component of the pheromone-response pathway. Recent studies suggest that STE5 is a scaffolding protein that facilitates interactions between components of the pheromone-responsive mitogen-activated protein kinase module. STE5 forms a complex with the STE7, STE11, and FUS3 protein kinases and is required for efficient

interaction between STE7 and STE11 (Marcus et al., 1994; Choi et al., 1994). STE11, STE7 and FUS3 or KSS1 appear to associate independently with STE5 and bind to different regions. The precise functional role of STE5 remains to be determined. The pheromone signaling pathway is similar in "a" and "α" cells except for the receptors.

2. Comparison of *S. pombe* and *S. cerevisiae* mating response pathways

In *S. pombe*, as in *S. cerevisiae*, cells of opposite mating type conjugate in response to mating factors (Fukui et al., 1986a). Cells of the "*h*+" mating type produce P-factor and cells of the "*h*-" mating type produce M-factor. *mam2* encodes P-factor receptor, a protein with seven transmembrane spanning domains homologous to the STE2 mating pheromone receptor of *S. cerevisiae* (Kitamura et al., 1991). *map3*, the counterpart of *mam2* in h^+ cells, encodes M-factor receptor.

The *S. cerevisiae* and *S. pombe* mating response pathways are highly analogous. Byr1 and Byr2 protein kinases are required for both conjugation and sporulation of *S. pombe* (Wang et al., 1991; Nadin-Davis et al., 1988). *byr1* or *byr2* mutants are viable but are absolutely defective in conjugation and sporulation. Epistasis experiments indicate that Byr1 lies downstream of Byr2 while both Byr1 and Byr2 act downstream of Ras1, and Gpa1, the G-protein α-subunit that mediates the occupancy of mating-factor receptors. The Byr2 and Byr1 protein kinases of the *S. pombe* pathway are structurally related to the STE11 and STE7 protein kinases, respectively, of the *S. cerevisiae* pathway.

The *S. pombe* Byr1 and Byr2 pair of kinases can complement a *S. cerevisiae STE7⁻STE11⁻* double mutant (Neiman et al., 1993), indicating that Byr2 and Byr1 are not only structurally but also functionally related to STE11 and STE7, respectively, and that Byr2 and Byr1 comprise a functional unit. A third putative kinase, Spk1, is also required for conjugation and sporulation in *S. pombe* (Toda et al., 1991). Spk1 is structually and functionally homologous to the FUS3 and KSS1 kinases, which function downstream of STE11 and STE7 in *S. cerevisiae*. A STE5 homolog in *S. pombe* has not been reported.

There are several major differences between the pheromone signaling pathways of the two yeasts. (1) In *S. cerevisiae*, haploid cells of opposite mating types conjugate under rich growth conditions, in contrast, starvation is a prerequisite for mating in *S. pombe*. (2) Unlike *S. cerevisiae*, *S. pombe* cells typically undergo immediate meiosis and sporulation after conjugation. This process requires many of the same signal transduction components as does the mating response. (3) The G proteins of the two yeasts offer a striking functional contrast. The Gα subunit of *S. cerevisiae* inhibits sexual differentiation, and the Gβγ subunits mediate the response. In *S. pombe*, it is the Gα subunit, encoded by *gpa1*, that appears to mediate the response and thereby induce sexual differentiation (Obara et al., 1991). Disruption of *gpa1* is not lethal but confers sterility and sporulation deficiency on *S. pombe* cells. (4) The *RAS* genes of *S. cerevisiae* are required for vegetative growth but do not participate in conjugation. In contrast, the *S. pombe ras1* gene, a homolog of the mammalian and *S. cerevisiae RAS* genes, is required for both conjugation and sporulation (Fukui et al., 1986b).

Although *ras1* disruption does not affect growth rate in *S. pombe*, it results in the complete inability to mate, and a *ras1*$^-$/*ras1*$^-$ diploid sporulates very poorly. It has been observed that *S. pombe* Ras1 and Gpa1 provide independent inputs into the MAP kinase module to stimulate the pheromone response pathway (Xu et al., 1994). Expression of *mam2* is dependent on Ras1, Gpa1, Byr1 and Byr2. Overexpression of *gpa1* induces *mam2* expression in cells that lack *ras1*, while Ras1 is required to maintain *mam2* expression in h$^-$ cells that lack *gpa1*. Results from yeast two hybrid experiments (Van Aelst et al., 1993) and from biochemical studies (Masuda et al., 1995) showed that *S. pombe* Byr2 protein associates with human and *S. cerevisiae* Ras proteins suggesting that Byr2 is an immediate downstream target of Ras1 in *S. pombe*.

III. Cyclic AMP plays an important role in the regulation of sexual development in *S. pombe*

In the budding yeast *S. cerevisiae*, cAMP plays a role in growth control and the sensing of nutrient conditions. Attenuation of the cAMP pathway in *S. cerevisiae* strongly inhibits cell growth (Kataoka et al, 1984; Matsumoto et al, 1982). Activation of the cAMP pathway prevents cells from arresting in the G1 phase of the cell cycle upon nutrient starvation, and leads to phenotypes including sensitivity to heat-shock treatment or nitrogen starvation (Cameron et al, 1988; Kataoka et al, 1984).

However, the evidence indicates that cAMP is involved in the control of sexual development in *S. pombe*. (1) Addition of cAMP to the

medium has been shown to inhibit mating and meiosis (Calleja et al., 1980), and to repress expression of genes required for sexual development (Watanabe et al., 1988; Sugimoto et al., 1990). (2) *S. pombe* cells that lack the *cyr1* gene for adenylyl cyclase and do not have a measurable amount of cAMP are highly derepressed for sexual development. The *cyr1* disruptants show a tendency to enter the sexual reproduction pathway under rich nutritional conditions (Macda et al., 1990; Kawamukai ei al., 1991). (3) Nitrogen starvation lowers the level of intracellular cAMP by about 50% in *S. pombe*. Nitrogen starvation is a prerequisite of sexual development in *S. pombe* and a number of genes involved in sexual development are transcriptionally activated in response to nitrogen starvation, including each of the four mating type genes, *mam2* encoding P-factor receptor, *mei* which is required for premeiotic DNA synthesis, *gpa1* encoding G-protein α-subunit, and *ste11* encoding a transcription factor that positively regulates transcription of genes necessary for sexual development. Thus, it appears that nitrogen starvation results in a decrease in the intracellular cAMP level which, in turn, triggers initiation of sexual development in fission yeast. Indeed the cAMP level was observed to be reduced by approximately 50% when *S. pombe* cells initiate sexual development under physiological conditions (Mochizuki & Yamamoto, 1992).

IV. The cyclic AMP pathway in *S. cerevisiae*

In *S. cerevisiae*, activation of the cAMP signaling pathway involves RAS proteins (Figure 1). Two RAS genes (*RAS1* and *RAS2*) have been

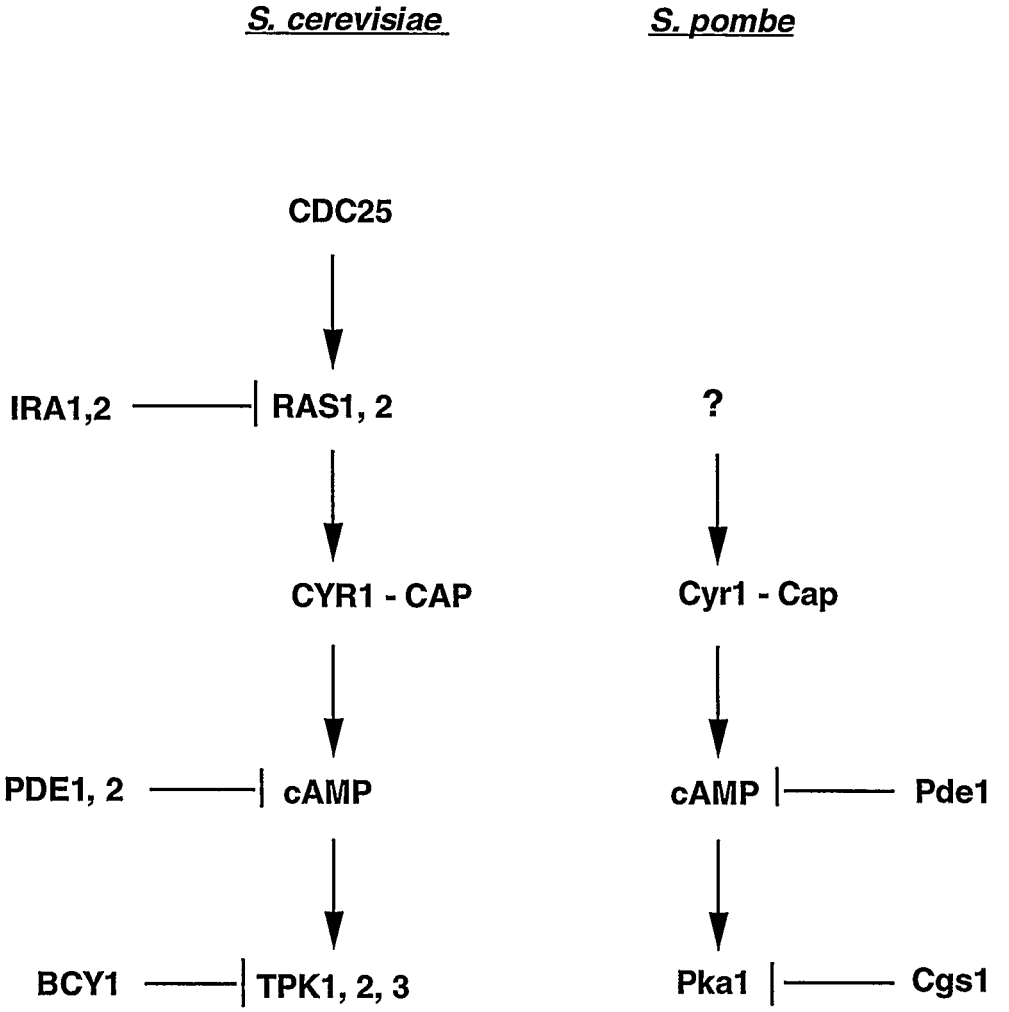

Figure 1. Schematic diagrams of proposed cAMP pathways in *S. cerevisiae* and *S. pombe*

isolated and at least one functional RAS gene is required for the vegetative growth of *S. cerevisiae* (Kataoka et al., 1984). In this yeast, RAS is activated by CDC25, a guanine nucleotide exchanging factor (Broek et al, 1987; Jones et al, 1991). RAS activates adenylyl cyclase, which is encoded by *CYR1* (Broek et al, 1985; Toda et al, 1985). It was also shown that at least one other protein, the adenylyl cyclase-associated protein (CAP) may be involved in the regulation of adenylyl cyclase by RAS (Field et al, 1990a). Adenylyl cyclase activity in membranes from cells that lack CAP is not stimulated by RAS proteins *in vitro*. Adenylyl cyclase catalyzes the conversion of ATP into the second messenger cAMP. There are three genes, *TPK1, TPK2 and TPK3* encoding the catalytic subunits of cAMP-dependent protein kinases (Toda et al, 1987). The redundency of the *TPK* genes in *S. cerevisiae* may reflect the fact that the activity of cAMP-dependent protein kinases is indispensable for cell proliferation in this yeast.

V. *S. pombe* adenylyl cyclase

Little was known about the cAMP signaling pathway in *S. pombe* until recent years. The *S. pombe* adenylyl cyclase gene, *cyr1*, was cloned by cross-hybridization with the *S. cerevisiae* adenylyl cyclase gene (Young et al., 1989; Yamawaki-Kataoka et al., 1989). The gene contains an open reading frame that is 5097-bp long and encodes a protein of 1692-amino acid residues in length. The C-terminal region of this protein has strong homology with the catalytic domain of the *S. cerevisiae* adenylyl cyclase. Expression of the C-terminal region of the *S. pombe* adenylyl cyclase can suppress a temperature-sensitive

mutation in the *S. cerevisiae* adenylyl cyclase gene. An *S. cerevisiae* strain that lacks its endogenous adenylyl cyclase gene and expresses the C-terminal region of *S. pombe* adenylyl cyclase protein has measurable adenylyl cyclase activity (Young et al., 1989).

The function and regulation of *S. pombe* adenylyl cyclase were analyzed by disrupting and by overexpressing this gene (Kawamukai et al., 1991). *S. pombe* strains lacking the *cyr1* gene have no measurable cAMP or adenylyl cyclase activity, yet grow at near normal rates. Thus, loss of adenylyl cyclase in *S. pombe* does not produce the dramatic inhibition of growth seen upon the loss of adenylyl cyclase in *S. cerevisiae*. Such *cyr1* disrupted *S. pombe* cells conjugate and sporulate in a rich medium that inhibits conjugation and sporulation in wild-type strains, and this prematuare sexual activity is inhibited by the addition of exogenous cAMP. Strains that overexpress adenylyl cyclase have higher levels of adenylyl cyclase activity than do normal cells and are relatively sterile upon nutrient limitation compared with wild-type cells and have an elongated morphology.

VI. Regulation of adenylyl cyclase

In *S. cerevisiae*, adenylyl cyclase is regulated by RAS1 and RAS2, which are homologs of mammalian Ras oncoproteins. Genetic studies showed that intracellular cAMP levels are significantly elevated in *S. cerevisiae* strains containing an activating mutation in *RAS2*; and cells with disrupted *RAS* are phenotypically similar to cells deficient in adenylyl cyclase (Toda et al., 1985). Biochemical evidence demonstrated that purified *S. cerevisiae* or human RAS activates *S. cerevisiae* adenylyl

cyclase in the presence of guanine nucleotides (Broek et al., 1985). However, the cAMP concentration of *S. pombe* cells was shown not to be affected by the disruption or mutational activation of *ras*, suggesting that adenylyl cyclase is not regulated by Ras in *S. pombe* (Fukui et al., 1986b).

The *S. cerevisiae* adenylyl cyclase is a large protein of 2026 amino acids. This protein has been divided into several functional domains, based both on biochemical and genetic evidence. The carboxyl-terminal 40 kD contains the entire catalytic domain, which retains a Mn^{2+}-dependent cyclase activity (Kataoka et al., 1985), and the most C-terminal portion of this domain is required for activation of the Mg^{2+}-dependent activity by RAS proteins and GTP (Yamawaki-Kataoka et al., 1989). Another 30 kD separates this domain from a large leucine-rich repeat region of about 60 kD that is also required for RAS responsiveness (Colicelli et al., 1990). The function of the 70-kD N-terminal domain is unknown, although a 100-amino acid domain just N-terminal to the leucine-rich repeat is required for optimal RAS responsiveness (Colicelli et al., 1990).

The *S. cerevisivae* and *S. pombe* adenylyl cyclase proteins show strong homology (60%) within their respective catalytic domains (Young et al., 1989). Homology is not as striking outside the catalytic domains perhaps reflecting divergent regulation. A segment corresponding to the N-terminal 620 residues of *S. cerevisiae* adenylyl cyclase appears lost from *S. pombe* cyclase, and the C-terminal 140 residues are not well conserved between the two yeast species. However, both cyclases share a common motif outside their catalytic domain. The *S. cerevisiae*

cyclase contains a 23-amino acid leucine-rich consensus sequence, PXXaXXLXXLXXLXLXXNXaXXa (where a is an aliphatic amino acid and X is any amino acid), that is tandemly repeated 22 times. A similarly tandemly repeated sequence is found in the middle region of *S. pombe* adenylyl cyclase. Very similar motifs are also found tandemly repeated in a variety of eucaryotic proteins, including human glycoproteins (Takahashi et al., 1985; Lopez et al., 1987), *Drosophila* chaoptin (Reinke et al., 1988), and *Toll* gene product (Hashimoto et al., 1988).

The *S. cerevisiae* adenylyl cyclase gene has been analyzed by deletion and insertion mutagenesis to localize regions required for activation by the RAS proteins (Suzuki et al., 1990). The N-terminal 657 amino acids were found to be dispensable for activation. However, almost all 2-amino acid insertions in the middle 600 residues comprising leucine-rich repeats and deletions in the C-terminal 66 residues completely abolished activation by the RAS protein, whereas insertion mutations in the other regions generally had no effect. The structural constraints in the leucine-rich repeat region appear to be quite stringent, since even single 2-amino-acid insertion in this region abolished RAS responsiveness. The fusion containing both the N-terminal 1600 residues and the C-terminal 66 residues of the *S. cerevisiae* cyclase rendered the catalytic domain of the *S. pombe* cyclase, which otherwise does not responde to RAS protein, activatable by the RAS protein. Thus the leucine-rich repeats and the C-terminus of the *S. cerevisiae* adenylyl cyclase appear to be required for RAS responsiveness. The importance of the leucine-rich repeats for RAS

responsiveness has also been suggested by studies showing that overexpression of the leucine-rich repeat appears to block RAS function, and it was suggested that the interference by this truncated adenylyl cyclase occurs by sequestering RAS protein (Field et al., 1990b). Recently, interaction of the leucine-rich region of *S. cerevisiae* adenylyl cyclase with human or *S. cerevisiae* RAS protein was shown by the yeast two hybrid system (Vojtek et al., 1993; unpublished data of our lab).

What regulates adenylyl cyclase in *S. pombe* is not clear. There is no evidence that Ras protein regulates adenylyl cyclase in *S. pombe*. It was proposed that the loss of the ability of *S. pombe* adenylyl cyclase to interact with Ras protein might be brought about by the loss of an interaction site, based on the structural homology with the *S. cerevisiae* adenylyl cyclase (Suzuki et al., 1990). A leucine-rich repeat-like structure does exist in the *S. pombe* cyclase, but it appears much more irregular and has a much lower homology with its counterpart in the *S. cerevisiae* cyclase than that between the catalytic domains of the two cyclases (Yamawaki-Kataoka et al., 1989). Considering the extreme structural vulnerability of the *S. cerevisiae* leucine-rich repeats, the observed extent of homology appears too low to assure the conservation of the ability to interact with Ras protein in the *S. pombe* cyclase. Moreover, very little homology exists in the C-terminal 66 residues between adenylyl cyclases of the two yeast species, which also supports the hypothesis that *S. pombe* adenylyl cyclase may have lost the interaction sites with Ras protein.

VII. *S. pombe* cAMP phosphodiesterase

In addition to adenylyl cyclase, cAMP phosphodiesterase also plays an important role in balancing the cAMP level in *S. pombe* (Figure 1). The *S. pombe pde1/cgs2* gene was cloned by our group and another group (Mochizuki et al, 1992; Matviw et al, 1993). This gene encodes a protein Pde1 that is 24% identical to the *S. cerevisiae* low-affinity cAMP phosphodiesterase. Extracts from *S. cerevisiae* cells which lack the genomic cAMP phosphodiesterase genes but express the *S. pombe* Pde1 exhibit high levels of cAMP phosphodiesterase activity. Deletion of *pde1* results in elevated level of intracellular cAMP and makes *S. pombe* cells partially sterile and meiosis-deficient. Overexpression of *pde1* reduces the cAMP level and can suppress the inhibition of sexual development in *S. pombe.*

S. cerevisiae contains two distinct cAMP phosphodiesterases: PDE1, a low-affinity enzyme, and PDE2, a high-affinity enzyme (Nikawa et al., 1987; Sass et al., 1986). These proteins do not share significant sequence homology with each other. High-copy-number plasmids containing either *PDE1* or *PDE2* can reverse the growth arrest defects of yeast cells carrying the $RAS2^{val19}$ mutation, a mutational activation of *RAS* in *S. cerevisiae. PDE1* or *PDE2* by itself is not an essential gene. *PDE1* and *PDE2* together account for the aggregate cAMP phosphodiesterase activity detectable in *S. cerevisiae.* Disruption of both phosphodiesterases led to a phenotype similar to that induced by the $RAS2^{val19}$ mutation. The presence of two distinct cAMP phosphodiesterases suggests the possibility that they belong to different regulatory pathways. With the exception of the *D. discoideum*

PDE and the *S. pombe* Pde1, the known cAMP phosphodiesterases of other organisms are related to PDE2. These include the *D. melanogaster* dunce protein (Chen et al., 1986) and related mammalian PDEs (Conti et al., 1992). It is not known whether *S. pombe* also contains a PDE2 related enzyme.

VIII. *S. pombe* cAMP-dependent protein kinase

In eucaryotes, cAMP exert its effects by activating a cAMP-dependent protein kinase. The holoenzyme of this kinase is a tetrameric protein consisting of two catalytic subunits and two regulatory subunits. cAMP activates the kinase by dissociating the inactive holoenzyme into two active monomeric catalytic subunits and the dimeric regulatory subunit (Krebs and Benvo, 1979). *cgs1* encodes the regulatory subunit of cAMP-dependent protein kinase (protein kinase A) of *S. pombe*. Cgs1 is homologous to BCY1, the regulatory subunit of cAMP-dependent protein kinase of *S. cerevisiae*. While wild-type cells contain a Pka1 activity dependent on cAMP, *S. pombe* cells defective in *cgs1* have a Pka1 activity that is unregulated by cAMP, and the cells scarcely mate and sporulate (DeVoti et al, 1991). Recently *pka1* encoding the catalytic subunit of cAMP-dependent protein kinase in *S. pombe* was cloned by our group and another group (Maeda et al., 1994; Yu et al., 1994). It contains an uninterrupted open reading frame encoding a 512-amino-acid protein. The C-terminal region (amino acid 200-512) of Pka1 is 51-63% identical to cAMP-dependent protein kinase (Pka) catalytic subunits from other eucaryotes. Introduction of *pka1* into wild-type *S. pombe* cells leads to failure of the cells to mate and

sporulate, and cells have an abnormal elongated morphology similar to that resulting from activation of the cAMP pathway. Disruption of *pka1* slows cell growth but is not lethal. The *pka1*-disrupted cells are derepressed for sexual development in the presence of rich nutrition.

There are three genes, *TPK1*, *TPK2*, *TPK3*, in *S. cerevisiae* encoding the catalytic subunits of the cAMP-dependent protein kinases (Toda et al., 1987). Gene disruption experiments showed that neither single or double disruptions of *TPK* genes have growth-defective phenotypes but at least one *TPK* gene is required for a cell to grow normally. Comparison of the predicted amino acid sequences of the *TPK* genes indicates the existence of conserved and variable domains. The carboxyl-terminal 320 amino acid residues have more than 75% homology to each other and more than 50% homology to the bovine protein kinase A catalytic subunit. The N-terminal regions show no homology to each other and are heterogeneous in length. In contrast to *S. cerevisiae*, *S. pombe* appears to have only one gene that encodes the cAMP-dependent protein kinase. The redundancy of the *TPK* genes in *S. cerevisiae* may reflect that the activity of cAMP-dependent protein kinase is indispensable for cell growth in this organism (Toda et al., 1987). In *S. pombe*, however, although cAMP-dependent protein kinase has a pivotal role in controlling sexual development, its loss does not result in growth arrest.

The major substrate(s) of cAMP-dependent protein kinase in *S. pombe* is yet unclear. It was proposed that the physiological function of *S. pombe* cAMP-dependent protein kinase might be in repression of *ste11*, which encodes a key transcription factor that positively regulates

transcription of genes necessary for sexual development (Sugimoto et al., 1991). It is possible that the Ste11 protein autoregulates its expression, with its activity being down-regulated by phosphorylation by cAMP-dependent protein kinase(Sugimoto et al., 1991). Alternatively *ste11* may be regulated by another transcription factor that is a substrate of cAMP-dependent protein kinase. Which possibility is the case remains to be addressed.

IX. Objectives

The cAMP signaling pathway is important for cell growth in the budding yeast *S. cerevisiae* and plays an important role in controlling sexual development in the fission yeast *S. pombe*. The cAMP pathway has been well addressed in *S. cerevisiae*, however, much less in known for this pathway in *S. pombe*. Progress has been made since 1989 when the adenylyl cyclase gene was cloned. Several components including cAMP phosphodiesterase and adenylyl cyclase-associated protein (Cap) have been identified and cloned. However, many aspects remain to be studied. In particular, the upstream positive regulator for adenylyl cyclase is still unknown. The objective of my research is to further study the cAMP signaling pathway in *S. pombe* and to look for the components that can associate with adenylyl cyclase, therefore providing some information for future work in identifying the upstream regulator of adenylyl cyclase in *S. pombe*.

CHAPTER 2. CLONING AND CHARACTERIZATION OF *S. POMBE PKA1* ENCODING A HOMOLOG OF cAMP-DEPENDENT PROTEIN KINASE

I. Background

To identify components of the cAMP signaling pathway in *S. pombe*, one approach is to identify *S. pombe* genes that complement various mutations in the *S. cerevisiae* RAS/cAMP signaling pathway. Expression of a dominant-interfering $RAS2^{val19ala22}$ in *S. cerevisiae* leads to a temperature sensitive phenotype (Powers et al., 1989). Previous studies suggested that this protein interferes with the activation of normal RAS by binding to CDC25 and blocking CDC25 function (Powers et al., 1989). Overexpression of CDC25, or the CDC25-related protein BUD5 in the presence of wild-type RAS, or components that act downstream from RAS, such as adenylyl cyclase or cAMP-dependent protein kinases, will suppress the temperature sensitive phenotype (Powers et al., 1989; 1991). To identify *S. pombe* cDNA clones that encode proteins related to CDC25 or components of the cAMP pathway, a genetic screen to identify *S. pombe* suppressors of the dominant-interfering $RAS2^{val19ala22}$ mutation in *S. cerevisiae* was conducted in our lab. An *S. pombe* cDNA library constructed in the *S. cerevisiae* high-copy expression vector pADANS was screened for clones that suppress the temperature sensitive phenotype of the *S. cerevisiae* strain RS60-15B which contains the dominant-negative $RAS2^{val19ala22}$ mutant allele. RS60-15B was transformed with the *S. pombe* cDNA library and 282 temperature-resistant colonies were isolated from over 10^5

transformants that were screened for growth at 37 °C. Segregation analysis indicated that only 16 of the 282 colonies have a temperature-resistant phenotype dependent on the presence of a plasmid carrying *S. pombe* cDNA. Plasmids were recovered from these 16 clones and partial DNA sequence analysis revealed that the cDNA clones fell into four classes, termed pSR1, pSR2, pSR3 and pSR10. My research began with the complete DNA sequence analysis of these pSR plasmids.

II. Materials and methods

1. DNA manipulation and cloning

(i) Bacterial cultures

Bacterial cultures were grown in LB media (1.2% tryptone, 0.6% yeast extract, 1% NaCl, pH 7.0) at 37 °C with constant shaking. Ampicillin was added to a final concentration of 100μg/ml when it was required.

(ii) Plasmid DNA extraction

Plasmid DNA was prepared from bacterial cultures by the alkaline lysis method as per a protocol by Sambrook et al. (1989).

(iii) DNA digestion with restriction enzymes

Restriction enzymes were purchased from New England BioLabs or Gibco BRL. Digestion of DNA with restriction enzymes was carried out at 37 °C for 2-3 hours following the manufacturer's instructions.

(iv) Gel electrophoresis of DNA and purification of DNA fragments

DNA fragments were fractionated on 1% agarose gels. Desired fragments were excised from the gels and purified by electroelution as described by Sambrook et al. (1989).

(v) Dephosphorylation of linearized plasmid DNA

To reduce transformation background causing by vector self-ligation and recircularization, plasmid vector linearized by one restriction enzyme digestion was dephosphorylated to remove the 5' phosphates. Briefly, the linearized plasmid DNA was incubated at 37 °C for 1 hour in a total volume of 100 µl containing 1.5 units calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) and 1 X CIP buffer (50 mM Tris-HCl pH 8.3, 1 mM $MgCl_2$, 0.5 mM $ZnCl_2$). At the end of the dephosphorylation reaction, 40 µl of 10 X STE buffer (100 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM EDTA) was added and the CIP was inactivated by heating at 68 °C for 20 minutes. The dephosphorylated plasmid DNA was purified by extraction 4 times with an equal volume of phenol: chloroform (1:1) and once with chloroform. DNA was precipitated and dissolved in TB (10 mM Tris-HCl pH 7.4).

(vi) Ligation of foreign DNA into vectors

Ligation reactions between foreign DNA and vectors were carried out in a 20 µl total volume containing 200-500 ng of DNA, 1 X ligation buffer (50 mM Tris-HCl, 10 mM DTT, 1 mM ATP) and 2 units T4 DNA ligase (Gibco BRL). The ligation mix was incubated at 16 °C overnight and was used to transform competent *E. coli* cells.

(vii) Preparation and transformation of competent *E. coli*

Competent *E. coli* cells were prepared using the calcium chloride method. Briefly, *E. coli* cells were grown to O.D.600=0.6 in LB media and cooled on ice for 10 minutes. Cells were recovered by centrifugation, resuspended in half culture volume of ice-cold 50 mM $CaCl_2$, 10mM Tris-HCl solution and incubated on ice for 1 hour. Cells were collected by centrifugation and resuspended in 1/50 culture volume of ice-cold solution of 50 mM $CaCl_2$, 10 mM Tris-HCl, 20% glycerol, and were stored as aliquots at -70 °C.

For each transformation, 100 µl of competent *E. coli* was added to DNA with 100 µl TCM (50 mM $CaCl_2$, 30 mM $MgCl_2$, 10 mM Tris-HCl), incubated on ice for 45 minutes, heat shocked at 42 °C for 2 minutes and cooled on ice for 2 minutes. 4 ml LB media was added and incubated at 37 °C with shaking for 1 hour followed by plating a 0.5 ml aliquot on LB agar plate containing the appropriate antibiotic.

(viii) Identification of bacterial colonies that contain recombinant plasmids

Bacterial colonies that contain recombinant plasmids were identified by two methods: restriction analysis of small-scale preparations of plasmid DNA or amplification of insert DNA by PCR from mini-scale bacterial lysates.

2. DNA sequence analysis

The nucleotide sequence of DNA was determined by a modified dideoxy chain-termination method (Sanger et al., 1977) using the Taq

DyeDeoxy^{TM} Termination Cycle Sequencing Kit (Applied BioSystems) following the supplier's instructions. The template DNA used for sequence analysis was prepared from bacteria by the alkaline lysis method and purified by polyethylene glycol (PEG) precipitation.

3. Southern hybridization

(i) DNA electrophoresis and transfer

DNA was digested with one or more restriction enzymes and DNA fragments were separated by electrophoresis on a 1% agarose gel. After electrophoresis was completed, the gel was treated with 1% HCl for 8 minutes and then treated three times with several volumes of 0.5 M NaOH, 1.5 M NaCl for 30 minutes with gentle agitation to denature the DNA. The gel was then neutralized by soaking for 30 minutes in several volumes of a solution of 0.5 M Tris, 1.5 M NaCl. The DNA in the gel was capillary transferred to nitrocellulose filter (Schleicher & Schuell) as described by Sambrook et al. (1989) and fixed by baking the filter for 3 hours at 80 °C in a vacuum oven.

(ii) Probe preparation and labelling

DNA probe used in these southern blots was a 1011-bp fragment made by PCR from pSR10 plasmid using PCR primers:

5'-AGACGGTCAGCTATGGATGG-3' (20mer)

5'-TACATCAGCATAAGCGTCAA-3' (20mer)

Gel purified probe DNA fragment was radiolabelled to ^{32}P by nick-translation using a Nick-Translation Kit purchased from Gibco BRL. Manufacturer's instructions were followed.

(iii) Hybridization and autoradiography

The DNA immobilized on the filter was hybridized to the probe as follows: The nitrocellulose filter was pre-hybridized for 2-4 hours at 65 °C in a pre-hybridization solution containing 6 X SSC (1 X SSC is 150 mM NaCl, 15 mM NaCitrate), 0.1% SDS, 1X Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.2% BSA) and 0.05 mg/ml denatured calf thymus DNA. The ^{32}P-labelled probe was denatured for 10 minutes at 98 °C and then added to the pre-hybridization solution. The filter was incubated at 65 °C for 24 hours and washed three times with 2X SSC, 0.1% SDS, and autoradiographed using Kodak X-OMAT film.

4. Screening the library by plaque filter hybridization

(i) Ligation and packaging

DNA was digested with restriction enzymes, agarose gel purified and ligated to λ ZAP II (Stratagene) digested and dephosphorylated arms in a reaction volume of 21 µl containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 3 units T4 DNA ligase. The ligation reaction was carried out at 12 °C overnight and 5 µl from the ligation mix was used for packaging with a Gigapack II Plus packaging kit (Stratagene) following the protocol recommended by Stratagene.

(ii) Infection, transfer and hybridization

Host strain XL1-Blue was grown in NZCYM media (1% Casitone, 0.5% yeast extract, 0.5% NaCl, 0.1% Casamino acids, 10mM $MgSO_4$, 10mM $MgCl_2$) supplemented with 0.2% maltose at 37 °C with shaking.

Packaged λ phage particles were mixed with XL1-Blue cells, and the infected bacteria were plated on NZCYM plate with top-agar (NZCYM + 0.8% agarose). Plates were incubated for 12 hours at 37 °C followed by incubation for 4 hours at 4 °C. Duplicate plaque lifts were performed by placing nitrocellulose filters (Schleicher & Schuell) onto each plate. Phages on the filter were amplified by infecting BB4 cells and incubating for 12 hours at 37 °C. Each filter was then treated for 3 minutes in denaturation buffer (0.5 M NaOH, 1.5 M NaCl), 3 minutes in neutralization buffer (0.5 M Tris, 1.5 M NaCl) and 3 minutes in 4 X SSC. The filters were then pre-hybridized, hybridized with ^{32}P-labelled DNA probe and washed as for Southern analysis. Filters were exposed to X-ray film for appropriate periods of time and putative positive plaques were picked, replated at lower density, and re-screened two more times for purification of the plaque.

(iii) *In vivo* excision

After positive plaques were obtained from the screen, *in vivo* excision of the pBluescript phagemid from the λ ZAP II vector was performed. Briefly, the recombinant λ ZAP II phage stock ($5X10^5$ pfu) was mixed with 500 µl of $O.D._{600}$=1.0 XL1-Blue cells and 1.5 µl of R408 (*f1*) helper phage ($7.5X10^{10}$ pfu/ml) (Stratagene) and incubated for 15 minutes at 37 °C. 5 ml of NZCYM media was added followed by incubation at 37 °C for 3 hours with shaking. The mix was then heated at 70 °C for 20 minutes then spun 5 minutes at 4,000rpm. The supernatant containing the pBluescript phagemid packaged as filamentous phage particle was collected and mixed with XL1-Blue

cells, incubated for 15 minutes at 37 °C and then plated onto LB/ampicillin agar plates. Colonies appearing on the plate contain the pBluescript double stranded phagemid with the cloned DNA insert.

5. Yeast strains and genetics

(i) Strains and media

The genotype of homothallic *S. pombe* strain SP870 is h^{90} *leu1*-32 *ade6*-210 *ura4*-d18. *S. pombe* cells were grown at 30 °C in rich media YEA (0.5% yeast extract, 75 ng/ml adenine, 3% glucose) or minimal media PMA (0.3% phtallic acid, 0.18% Na_2HPO_4, 0.5% NH_4Cl, 2% glucose, 75ng/ml adenine, and vitamin, mineral and salt supplements).

(ii) Yeast transformation

Yeast cells were grown to $O.D._{600}$=1.0, harvested by centrifugation at 3000 rpm for 5 minutes. Cells were washed once with several volumes of LTE (0.1 M Lithium acetate, 10 mM Tris-HCl, 1 mM EDTA), resuspended in 1.5 ml LTE, and incubated at 30 °C with shaking for 60 minutes. Then 0.15 ml of cells were mixed with DNA (2-10 μg) and 0.35 ml 50% PEG4000 in TE (10mM Tris-HCl, 1mM EDTA), incubated at 30 °C for 60 minutes, heat shocked at 42 °C for 5 minutes, and centrifuged at 5000 rpm for 10 seconds. The supernatant was discarded and the cells were resuspended in 400 μl of TE and plated. Colonies usually appeared after 3 days.

III. Results

1. DNA sequence analysis of pSR plasmids

Four plasmid clones (pSR1, pSR2, pSR3 and pSR10) were identified when screening for *S. pombe* suppressors of the dominant-interfering *RAS2^{val19ala22}* mutation in *S. cerevisiae*. To analyze these clones, *S. pombe* cDNA fragments in pSR plasmids were subcloned into pBluescript plasmid. The nucleotide sequence of the cDNAs were determined and compared to other DNA sequences listed in the database. The cDNAs in pSR1 and pSR2 are identical to the *S. pombe ras1* gene. Therefore, expression of *S. pombe* Ras1 can suppress the temperature sensitive phenotype of the *S. cerevisiae* strain containing the dominant interfering *RAS2^{val19ala22}* mutation. Interestingly, pSR3 contains *S. pombe ras1* in the anti-sense orientation. The cDNA in pSR10 encodes a 431-amino acid protein that has 63% identity with the C-terminal 320 amino acid region of *S. cerevisiae* TPK2 and 50% identity with the *Drosophila* protein kinase A catalytic subunit. The gene encoding this *S. pombe* homolog of cAMP-dependent protein kinase catalytic subunit was named *pka1*.

2. Restriction mapping of *pka1*

For isolating clones containing the entire *pka1* sequence, the first step was to get a restriction map of *pka1*. Genomic Southern blot analysis was conducted using a probe that was derived by PCR from pSR10.

The coding sequence for *pka1* in pSR10 is 1293 bp in length coding for the C-terminal 431 amino acids of the protein. The probe contains a 1011-bp fragment homologous to nt 226-1236 of the cDNA sequence in pSR10. Based on the DNA sequence of pSR10, there is a *Eco*RV

restriction site within the probe sequence and it is 905 bp away from the beginning of the probe. One *Hind*III site and one *Bst*EII site were located outside the probe sequence and are 206 bp and 130 bp 5' to the beginning of the probe, respectively. Three genomic Southern blots (Figure 2, 3 and 4) revealed a restriction map of *pka1* (Figure 5). A 7.2-kb *Eco*RI fragment (Figure 2) and a 4.3-kb *Eco*RV fragment (Figure 3 and Figure 4) hybridized to the probe sequence. Double digestion of *S. pombe* DNA with *Eco*RI and *Eco*RV gave a strong signal of a 2.3-kb fragment (Figure 4). Therefore, one *Eco*RI site is located 1395 bp 5' to the beginning of the probe sequence and is 1170 bp 5' to the beginning of the cDNA sequence of *pka1* in pSR10. Since the protein coding sequences of known *pka1* related genes in other organisms are 1053-1194 bp in length, we were pretty sure that the 7.2-kb *Eco*RI fragment contained the entire coding sequence of *S. pombe pka1* gene.

3. Cloning the genomic sequence of *pka1*

To clone the 7.2-kb *Eco*RI fragment bearing the genomic sequence of *pka1*, a library was constructed by inserting *Eco*RI fragments of *S. pombe* genomic DNA into the λ ZAP II vector. The library was screened by plaque filter-hybridization using the same DNA probe as for the Southern blots. Five independent positive plaques were isolated and the recombinant pBluescript phagemids containing *S. pombe* DNA inserts were excised from λ ZAPII vector by an *in vivo* excision method. Restriction analysis revealed that they all contained 7.2-kb *Eco*RI inserts. Two of these clones were used for further DNA sequence

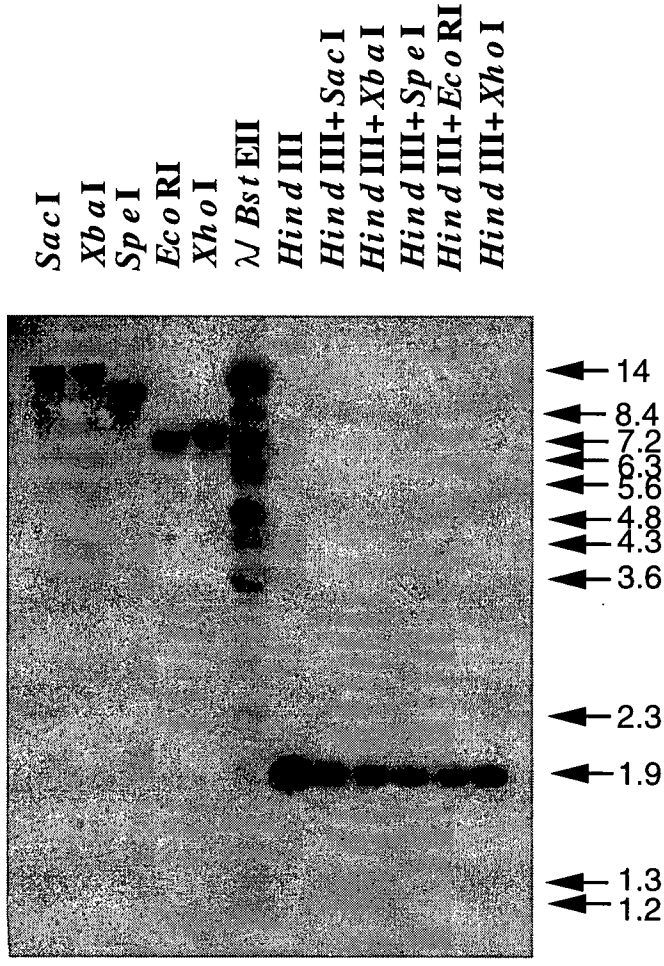

Figure 2. *S. pombe* genomic Southern blot 1

S. pombe genomic DNA digested with different enzymes were electrophoresed on 1% agarose gel. λ DNA *BstE* II digested fragments were also loaded as molecular weight markers. DNA was transferred to nitrocellulose filter and hybridized to ^{32}P-labelled probe DNA and λ DNA. Blot was washed and autoradiographed. The positions of size markers are shown, with sizes given in kilobases.

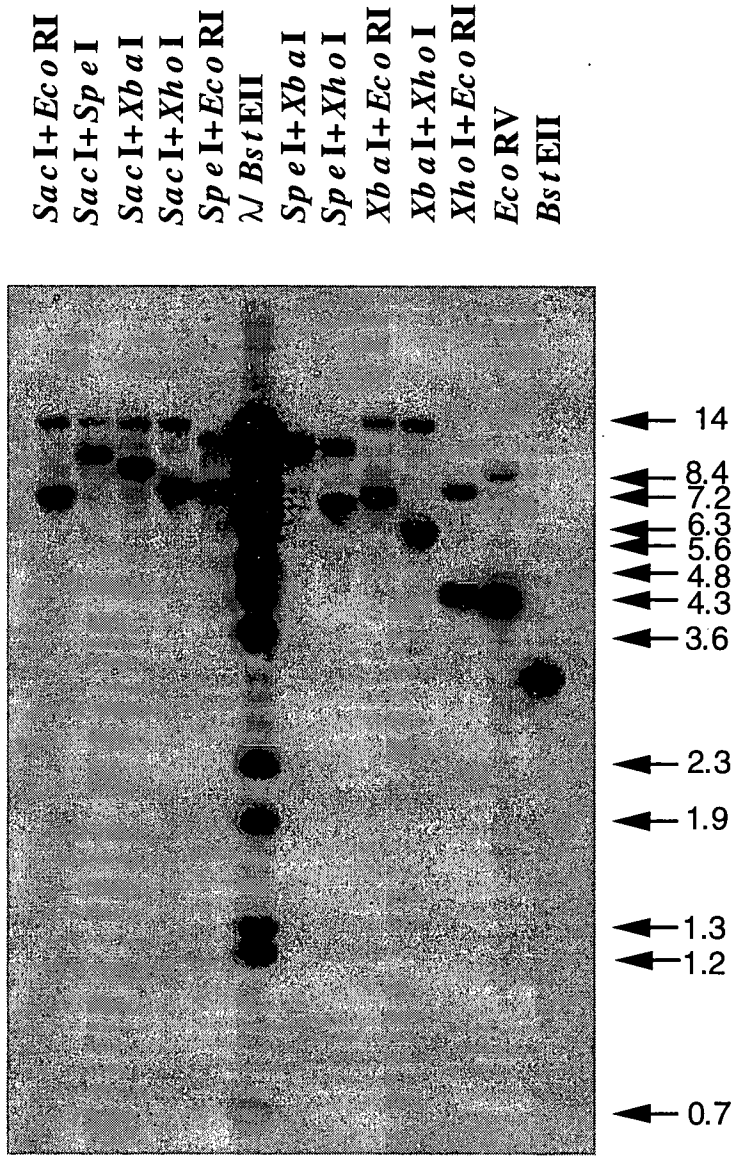

Figure 3. *S. pombe* genomic Southern blot 2

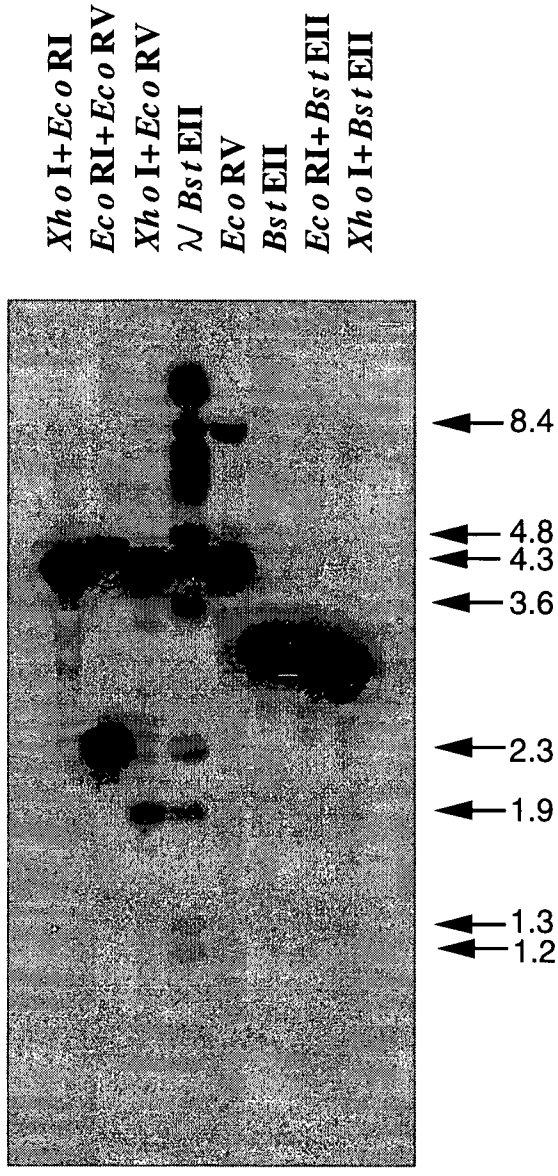

Figure 4. *S. pombe* genomic Southern blot 3

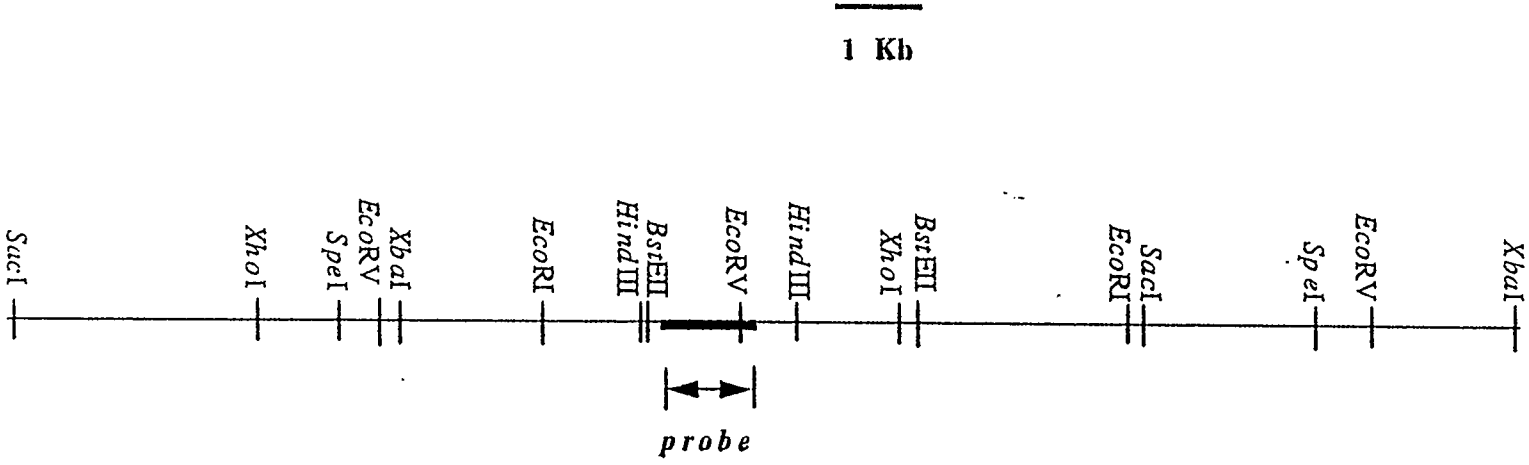

Figure 5. Restriction map of *S. pombe pka1* sequence

Based on the Southern blot results, sites for restriction endonucleases were indicated. The thick bar represents the DNA probe used for Southern blots.

analysis by Gang Yu in our lab to determine the coding region. It appears that *pka1* has a single open reading frame encoding a 512 amino acid protein. The nucleotide sequence of *S. pombe pka1* has been submitted to GenBank (accession No. U08622). The C-terminal region (amino acids 200-512) of *S. pombe* Pka1 is 51-63% identical to cAMP-dependent protein kinase (protein kinase A) catalytic subunits from other organisms (Figure 6). Compared to other Pkas, *S. pombe* Pka1 has a long N-terminal region that is missing in the other proteins. The amino acid sequence of Pka1 reveals a consensus sequence that has been found in the other protein kinases. This sequence is Gly-X-Gly-X-X-Gly (where X is any amino acid), followed 17 residues later by Lys, the putative ATP-binding site. Another consensus sequence, Asp-Phe-Gly and Ala-Pro-Glu is found downstream of the ATP-binding site (Figure 6).

4. Overexpression of Pka1 in *S. pombe* cells leads to a sterile phenotype

To characterize the function of Pka1, a pU-pka plasmid was constructed by cloning the entire coding region of *pka1* gene into the NotI sites of the *S. pombe* expression vector pAAUN. pAAUN contains the *S. pombe ura4* gene and the *adh1* promoter sequence flanking the *Not*I site. *S. pombe* homothallic strain SP870 (*h90*) was transformed with pU-pka or pAAUN. After growing 3 days on minimal media, cells were examined by iodine vapor staining. Strains that have sporulated should appear dark upon iodine staining. As shown in Figure 7B, SP870 (h^{90}) cells containing empty vector pAAUN appear dark indicating that

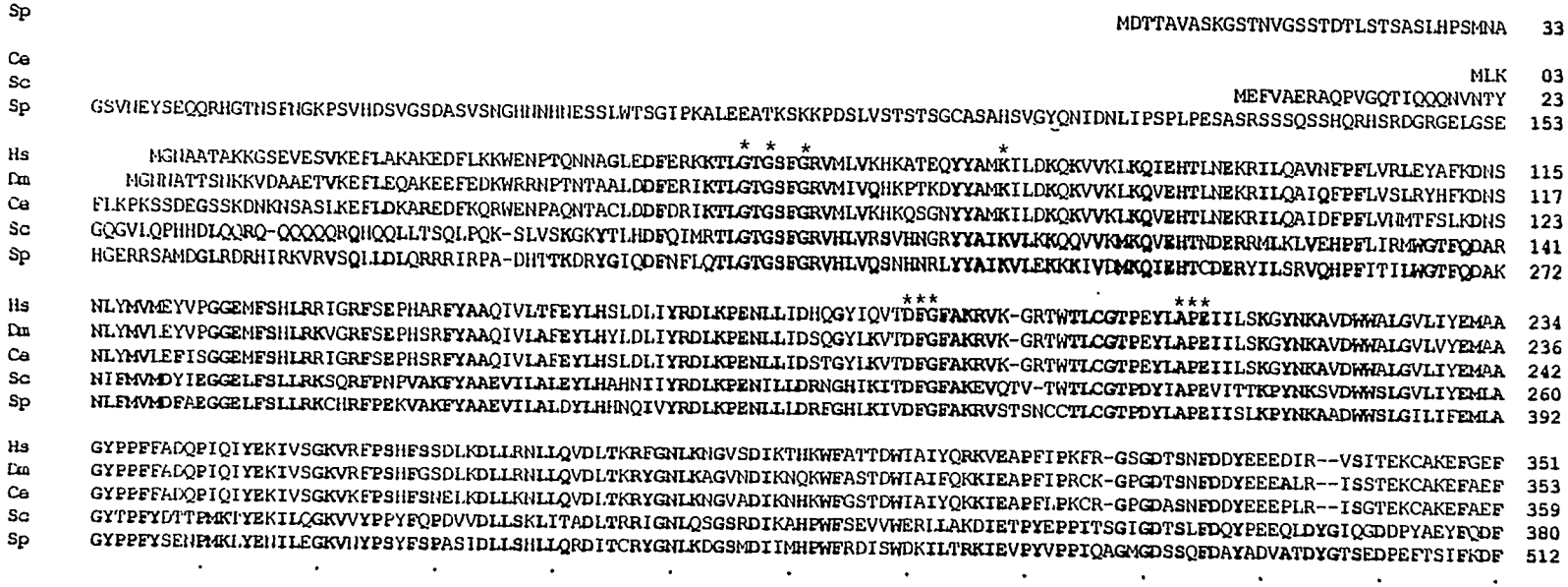

Figure 6. Comparison of *S. pombe* Pka1 with Pka catalytic subunits from other organisms

The sequences of the *S. pombe* (Sp) Pka1, *S. cerevisiae* (Sc) TPK2, *C. elegans* (Ce) Pka, *D. melanogaster* (Dm) Pka and human (Hs) PKA Cβ subunit were aligned. Those aa which are identical between *S. pombe* Pka1 and proteins from other organisms are in bold. The consensus sequences among protein kinases are indicated by *.

this strain has sporulated normally. The sterile strain, SPRU, in which the *ras1* gene has been deleted, did not sporulate and did not stain. SP870 cells containing pALY1, which directs expression of *S. pombe* adenylyl cyclase, were partial sterile as indicated by the intermediate color upon staining. SP870 cells harboring pU-pka, thus overexpressing *S. pombe* Pka1 protein, exhibited a complete sterile phenotype.

The sterile phenotype of *S. pombe* cells overexpressing Pka1 was also observed when cells were examined by microscopy (Figure 8). SP870 (h^{90}) cells containing pAAUN sporulated normally while cells containing pU-pka failed to sporulate and exhibited an abnormal elongated morphology, which is characteristic of *S. pombe* cells in which the cAMP pathway is constitutively activated.

IV. Discussion

The *S. pombe pka1* gene encoding the catalytic subunit of cAMP-dependent protein kinase was identified and cloned. It encodes a protein of 512 amino acids which is 51-63% identical to cAMP-dependent protein kinase catalytic subunits from other organisms. Overexpression of Pka1 can suppress the temperature-sensitive phenotype of *S. cerevisiae* strains containing the dominant-negative $RAS2^{val19ala22}$ mutation, suggesting that it acts downstream from RAS in the *S. cerevisiae* RAS/cAMP pathway. *S. pombe* cells expressing high level of Pka1 are sterile and have an abnormal elongated morphology.

So far four major components of the cAMP signaling pathway have been identified and cloned in *S. pombe*, including adenylyl cyclase (Cyr1), cAMP phosphodiesterase (Pde1), the regulatory subunit of

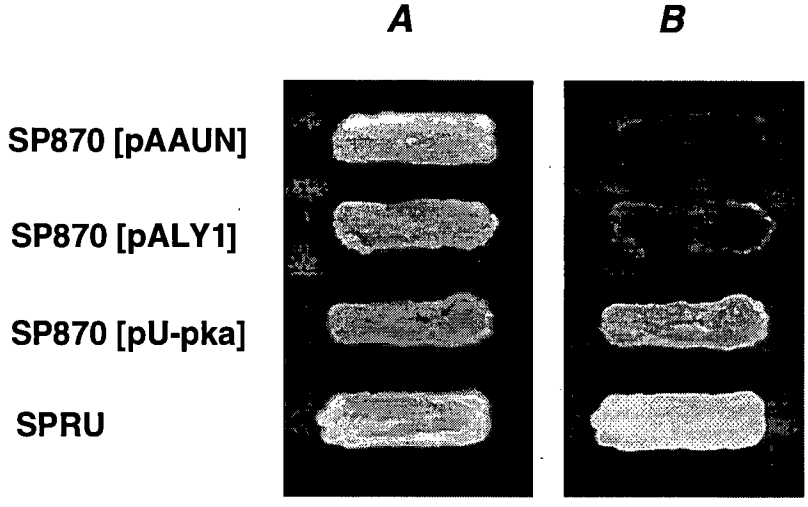

Figure 7. Overexpression of Pka1 inhibits sporulation of *S. pombe*

Patches of *S. pombe* strains containing the designated plasmids were grown on minimal media PMA (+leucine) plates for 3 days (A), and stained with iodine vapor for 2 minutes (B). Strains that have sporulated appeared dark upon iodine staining. Sterile strains did not sporulate and did not stain.

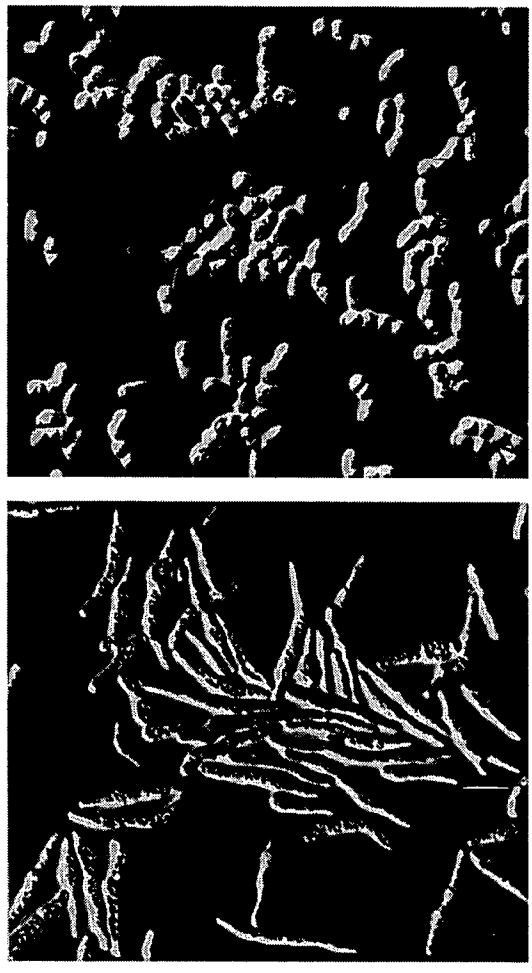

Figure 8. Morphology of *S. pombe* cells overexpressing Pka1

SP870 (h^{90}) cells harboring pAAUN or pU-pka were grown on minimal media PMA (+leucine) plate for 3 days and examined by microscopy.

cAMP-dependent protein kinase (Cgs1) and the catalytic subunit of cAMP-dependent protein kinase (Pka1). Disruption and overexpression of these genes have given consistent results. Any genetic manipulation that increases the intracellular cAMP level, or the activity of cAMP-dependent protein kinase, prevents *S. pombe* cells from initiating sexual development. Such cells are sterile and have an abnormal elongated morphology. Any manipulation that decreases the cAMP level or the activity of cAMP-dependent protein kinase activity derepresses *S. pombe* cells for sexual development. Such cells undergo mating and sporulation in the presence of rich nutrition, bypassing the requirement of nutrient starvation for sexual development. These observations strongly suggest that the role of cAMP signaling pathway in *S. pombe* is to control sexual development in response to nutrient conditions.

There are three *TPK* genes in *S. cerevisiae* encoding the catalytic subunits of cAMP-dependent protein kinases. From the Southern blot results (Figure 2 and 3 and 4), it does not appear that *S. pombe* has other *pka1* related genes. Disruption of *pka1* in *S. pombe* has been studied (Maeda et al., 1994), which also supports the idea that *S. pombe* does not have a functional homolog of *pka1*. Disruption of *pka1* slows cell growth but is not lethal. The *pka1*-disrupted cells undergo mating and sporulation even in the rich media. These phenotypes are similar to those of the *cyr1*-disrupted strain, which has no detectable adenylyl cyclase activity and is highly derepressed for sexual development (Kawamukai et al., 1989). The redundancy of the *TPK* genes in *S. cerevisiae* suggests that the activity of cAMP-dependent

protein kinase is required for cell growth in this yeast. Although the activity of cAMP-dependent protein kinase is important for controling sexual development in *S. pombe*, it is not required for cell proliferation.

The *TPK* genes in *S. cerevisiae* encode proteins of 380-398 amino acids. The *Drosophila* protein kinase A catalytic subunit has 353 amino acid residues in length. Compared to these protein kinases, *S. pombe* Pka1 has a long N-terminal region that is missing in the other proteins. The function of this region is unclear. It does not appear to be required for the function of Pka1. The truncated Pka1 containing the C-terminal 431 amino acids is able to suppress the temperature-sensitive phenotype of *S. cerevisiae* strain containing the dominant-interfering $RAS2^{val19ala22}$ mutation. Overexpression of this truncated Pka1 also leads to a sterile phenotype in *S. pombe* cells (Gang Yu, personal communication). Such cells have an elongated morphology, which is shared with cells overexpressing full length Pka1. Therefore, the C-terminal domain of Pka1 is sufficient for its function. Whether the long N-terminal region is involved in the regulation of Pka1 activity remains to be determined.

The homology of *S. pombe* Pka1 protein with *S. cerevisiae* TPK proteins and other cAMP-dependent protein kinase catalytic subunits, together with the genetic evidence that disruption or overexpression of *pka1* in *S. pombe* cells results in phenotypes similar to those of cells in which the cAMP pathway has been inhibited or activited, strongly suggests that Pka1 is the catalytic subunit of *S. pombe* cAMP-dependent protein kinase. Further biochemical experiments could be performed to confirm this hypothesis. One approach is to overexpress

pka1 and *cgs1* in *S. pombe* cells and to examine whether the kinase activity from the protein preparations is dependent on exogenously added cAMP, and whether *pka1* overexpression results in the induction of kinase activity relative to cells not overexpressing *pka1*.

CHAPTER 3. SEARCHING FOR COMPONENTS INTERACTING WITH ADENYLYL CYCLASE IN *S. POMBE*

I. Background

Although the adenylyl cyclase gene, *cyrl*, in *S. pombe* has been cloned for several years, the regulator for adenylyl cyclase in this yeast is still unknown. Although RAS proteins regulate adenylyl cyclase in *S. cerevisiae*, there is no evidence that Ras activates adenylyl cyclase in *S. pombe*. Disruption or mutational activation of *ras* does not affect the cAMP level in *S. pombe*. How adenylyl cyclase is regulated in *S. pombe* is not clear. However, the genetic evidence suggests that *S. pombe* Gpa2 modulates adenylyl cyclase activity (Isshiki et al, 1992). *gpa2* encodes a 354 amino acid protein homologous to G-protein α-subunit. Gpa2 shares 36.2% identity with *S. pombe* Gpa1 and 42.4% identity with *S. cerevisiae* Gpa2. *S. pombe* Gpa1 was characterized to be involved in the mating pheromone pathway (Obara et al., 1991). Gpa2 seems to have a different function. Disruption of *gpa2* slows cell growth but is not lethal. Cells defective in *gpa2* mate and sporulate readily in nitrogen-rich media, bypassing the requirement of nitrogen starvation for the initiation of sexual development. These phenotypes are reminiscent of those of the *cyr1*-disrupted cells. Addition of cAMP to the media can suppress the phenotypes of the *gpa2*-disrupted cells. The level of cAMP in *gpa2* null mutants is only one-third of the wild-type level. Mutations inhibiting the GTPase activity of Gpa2 cause a slight increase in intracellular cAMP levels and result in leaky sterility. Combination of this type of mutation with a null mutation in *pde1* encoding

phosphodiesterase leads to a remarkable increase in the cAMP level. Finally, cells defective in *gpa2* fail to produce cAMP in response to glucose stimulation. These results suggest that Gpa2 is involved in the regulation of the intracellular cAMP level in accordance with the nutritional conditions, most likely as a positive regulator of adenylyl cyclase. To investigate whether Gpa2 could be the direct activator of adenylyl cyclase, and to identify proteins capable of interacting with cyclase, a yeast two hybrid system approach was applied. The results of these two hybrid experiments will be presented in this chapter.

II. Materials and methods

1. Yeast two hybrid system

The yeast two hybrid system was designed to detect physical interactions between protein domains that are expressed as fusion proteins in *S. cerevisiae* (Fields and Song, 1989; Chien et al., 1991). Interacting fusion proteins combine to form a DNA-binding and transcriptional activation dimer that induces transcription of reporter genes. In the system that I used, which is a modification of the Fields two hybrid system and developed by Dr. S. M. Hollenberg (Vojtek et al., 1993), one hybrid is a fusion between the LexA DNA-binding domain and a protein of interest. The second hybrid is a fusion between a nuclear localized VP16 acidic activation domain or GAL4 activation domain and a second protein of interest. Individually, these hybrids are unable to activate transcription. The two hybrids were coexpressed in an *S. cerevisiae* strain L40 that contains two integrated reporter constructs, the yeast *HIS3* and the bacterial *lacZ*, which contain

binding sites for the LexA protein. Transactivation of the reporter constructs is dependent on the formation of a complex between the proteins fused to LexA DNA binding domain and VP16 or GAL4 activation domain. If the two hybrids are able to interact, then transcriptional activation of *HIS3* and *lacZ* occurs; the yeast strain expressing both hybrid proteins is prototrophic for histidine and contains detectable β-galactosidase activity.

(i) Two hybrid plasmid vectors

The LexA fusion vector BTM116, constructed by Paul Bartel and Stanley Fields, was kindly provided by Anne Vojtek (Fred Hutchinson Cancer Research Center). It carries the *TRP1* gene and has a polylinker downstream of the LexA coding sequence (Figure 9). The pVP16 vector, also provide by Anne Vojtek, carries the *LEU2* gene and expresses a nuclear localized VP16 activation domain under the control of the *S. cerevisiae ADH1* promoter (Figure 10). pGADGH is the parental vector for the *S. pombe* cDNA library (kindly provided by Greg Hannon, Cold Spring Harbor Laboratory). It expresses a nuclear localized GAL4 activation domain under the control of the *ADH1* promoter. It also contains a *LEU2* gene for use as a selectable marker in yeast (Figure 11).

(ii) Strains and media

The *S. cerevisiae* reporter strain L40 (*MATa trp1 leu2 his3 ade2 LYS2:: lexA-HIS3 URA3::lexA-lacZ.*) was provided by Anne Vojtek. Yeast cells were grown at 30 °C in rich media YPD (2% peptone, 1% yeast extract,

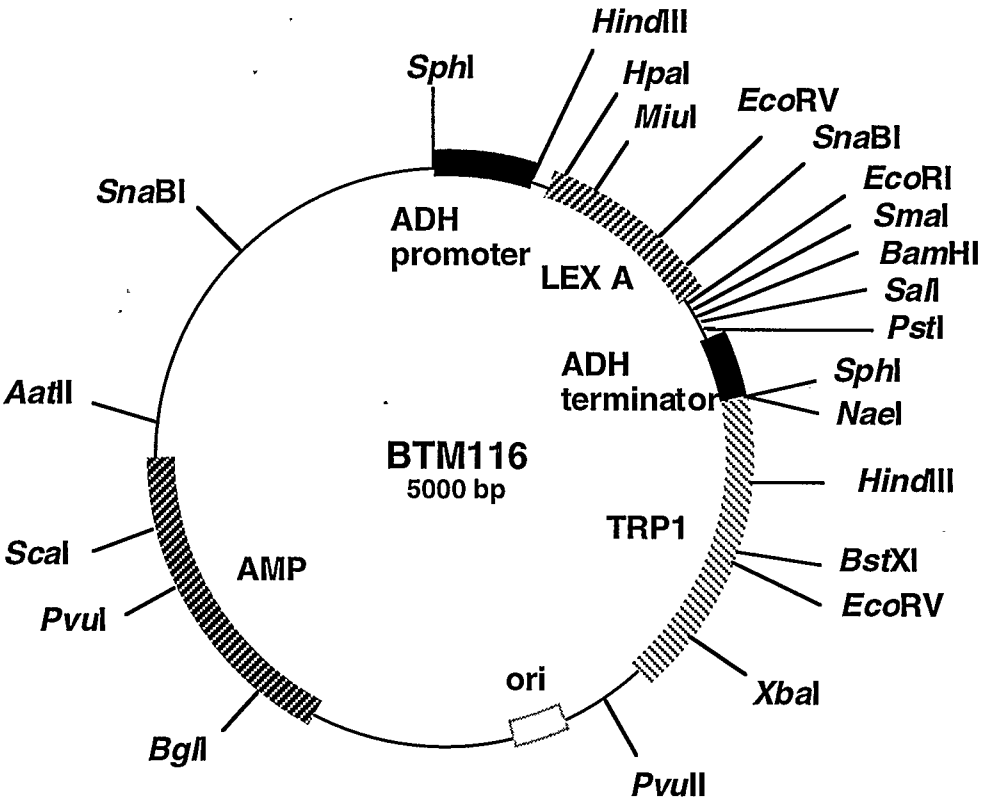

Figure 9. BTM116 plasmid

The LexA fusion vector BTM116, constructed by Paul Bartel and Stanley Fields, carries the *TRP1* gene and has a polylinker downstream of the LexA coding sequence.

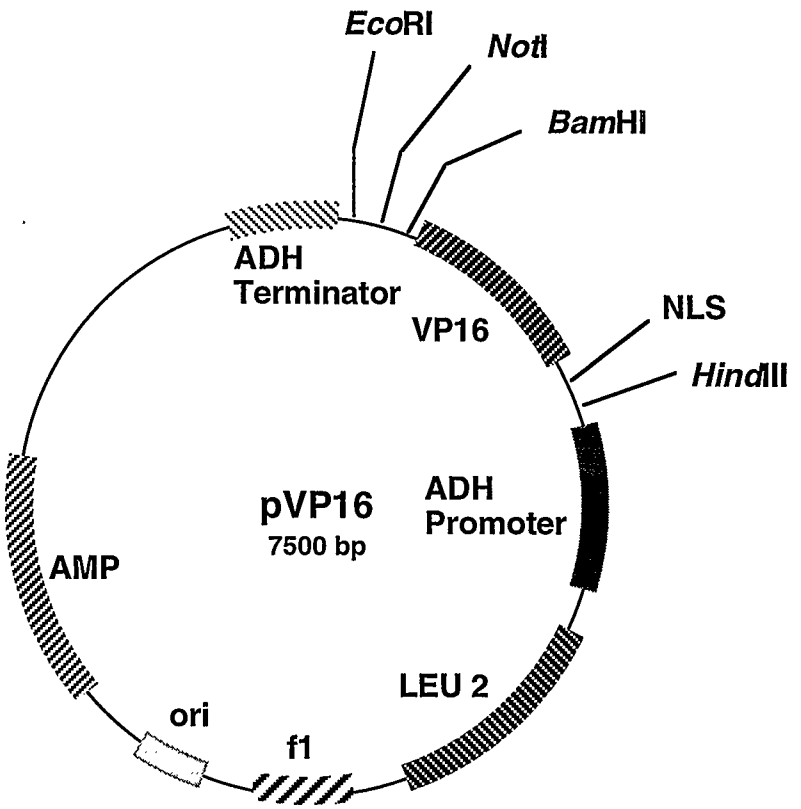

Figure 10. pVP16 plasmid

The pVP16 vector carries the *LEU2* gene and expresses a nuclear localized VP16 activation domain under the control of the *S. cerevisiae ADH1* promoter.

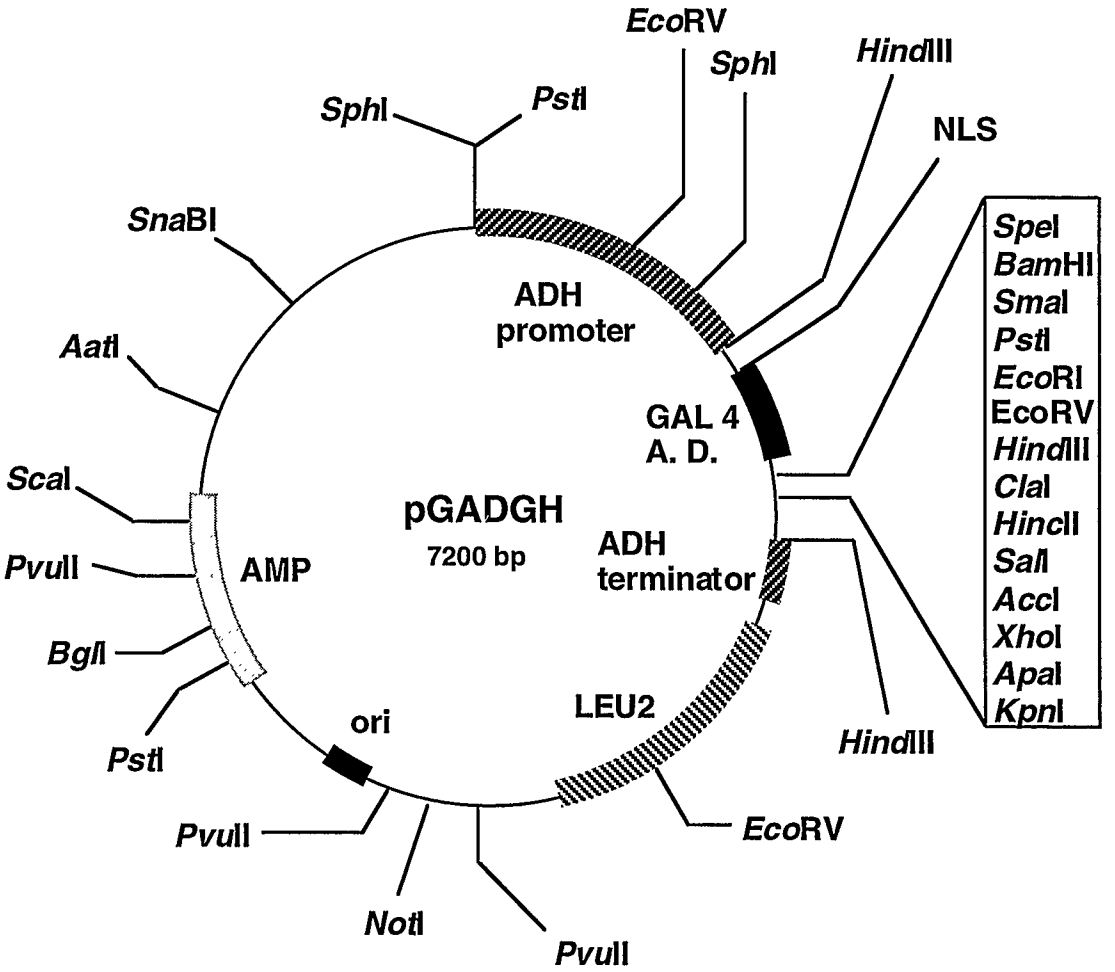

Figure 11. pGADGH plasmid

pGADGH, constructed by Greg Hannon, carries a *LEU2* gene and expresses a nuclear localized GAL4 activation domain under the control of *ADH1* promoter.

2% glucose) or in YC media (0.12% yeast nitrogen base without amino acids and $(NH_4)_2SO_4$, 0.5% ammonium sulfate, 1% succinic acid, 0.6% NaOH, 2% glucose, 0.01% each of Ade, Arg, Cys, Thr, Leu, Lys, Trp, Ura, 0.005% each of Asp, Ile, Met, Phe, Pro, Ser, Tyr, Val and His). YC-TUK media is YC lacking Trp, Ura and Lys, used for maintaining selection for LexA plasmid. YC-TULK media is YC lacking Trp, Ura, Leu and Lys, used for maintaining selection for LexA and VP16 or GAD plasmids. YC-THULK media is YC lacking Trp, His, Ura, Leu and Lys, used for selection of histidine prototrophs. YC-ULK media is YC lacking Ura, Leu and Lys, used for segregation of LexA plasmid and maintaining GAD plasmid.

(iii) β-galactosidase assay

Yeast cells were patched to YC plates, incubated for 3 days at 30°C, then transfered to a nitrocellulose filter (Schleicher & Schuell). The filter was kept at -70°C for 30 to 60 minutes. The filter was then thawed at room temperature and placed on the top of one Whatman 3mm paper that had been pre-wet in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal). The filter was incubated for 1-24 hours at 30°C. Blue coloration after incubation is indicative of β-galactosidase activity.

(iv) Small scale yeast transformation

Small scale yeast transformation was performed by the lithium acetate method as described in chapter 2.

(v) Yeast two hybrid test

The L40 strain of yeast was transformed with plasmids expressing LexA-fusions and was grown on YC-TUK plates for 3 days. Cells expressing a LexA-fusion were further transformed with plasmid expressing a VP16-fusion or GAD-fusion and grown on YC-TULK plates for 3 days. Colonies were picked and tested for β-galactosidase activity and the ability to grow in the absence of histidine (YC-THULK).

(vi) Large scale yeast transformation and library screening

To identify proteins that interact with adenylyl cyclase, a library of hybrid proteins between a nuclear localized GAL4 activation domain and *S. pombe* cDNA fragments (GADGH-cDNA library, provided by Greg Hannon, Cold Spring Harbor Laboratory) was screened.

10 ml cultures of L40 cells containing LexA fusion plasmid was grown in YC-TUK media overnight, diluted to $O.D._{600}$=0.3 in 200 ml YC-TUK media and grown another 4 hours to $O.D._{600}$=0.6. Cells were harvested and washed with 50 ml TE (10 mM Tris-HCl, 1 mM EDTA), resuspended in 4 ml LTE (0.1 M Lithium acetate, 10 mM Tris-HCl, 1 mM EDTA), incubated at room temperature for 10 minutes, and mixed with 300 µl 10 mg/ml denatured salmon sperm DNA and 50 µg of library DNA. 28 ml 40% PEG4000 in LTE was then added and incubated at 30 °C for 30 minutes. Cells were mixed with 3.5 ml DMSO, heat shocked at 42 °C for 8 minutes and cooled to room temperature in water bath. Cells were pelleted, washed with 50 ml TE, resuspended in 200 ml YPD and incubated at 30 °C for 1 hour with shaking. Cells were then pelleted, washed with 50 ml TE and resuspended in 200 ml YC-TULK.

200 µl of cells were plated onto YC-TULK plates to measure primary transformation efficiency. The rest of the cells were grown at 30 °C with shaking for another 16 hours to obtain efficient expression of the *HIS3* reporter gene. After the 16 hours of incubation at 30 °C, cells were harvested and washed twice in 50 ml TE and resuspended in 10 ml TE. 10^{-3} to 10^{-7} of total cells were plated onto YC-TULK to measure transformation amplification. 5% of total cells were plated on YC-THULK plate for ten plates to select for His^+ colonies. After 3-5 days of incubation, His^+ colonies were picked from YC-THULK plates, grown on YC-TULK plates for 3 days and tested for β-galactosidase activity.

For eliminating false positives, His^+ $lacZ^+$ colonies were grown for 3 days in YC-ULK media to enable segregation of LexA plasmid. Loss of LexA plasmid was selected by replica plating to YC-ULK, YC-TULK and YC-HULK plates. Colonies which required Trp for growth were assayed for β-galactosidase activity to confirm loss of LexA plasmid, and these Trp^-Leu^+ isolates were retransformed with plasmid containing LexA-adenylyl cyclase fusion or LexA-lamin. Both transformants were grown on YC-TULK plates and tested for β-galactosidase activity. The transformants that strongly transactivated the *lacZ* reporter construct preferentially with LexA-cyr plasmid were selected as putative true positives. Plasmids were recovered from these clones, retransformed to cells containing LexA-cyr or LexA-lamin, and tested for β-galactosidase activity and the ability to grow in the absence of histidine to confirm further that they are truly positive clones.

2. Plasmids

Plasmids expressing various fusion proteins were constructed by subcloning or by PCR as following: DNA sequences of interest were amplified by PCR using Vent DNA polymerase (New England Biolabs). DNA fragments purified by agarose gel electrophoresis were inserted in frame to plasmid vectors and cloned.

LexA-mcyr contains nt 2520-4701 of *S. pombe cyr1* (encoding aa 307-1033 of adenylyl cyclase) cloned into *Eco*RI site of BTM116.

LexA-ccyr contains nt 4433-6680 of *S. pombe cyr1* (encoding aa 945-1692 of adenylyl cyclase) cloned into BTM116 which had been digested with *Bam*HI and *Pst*I.

VP16-gpa2 contains the entire coding region of *S. pombe gpa2* and was cloned into pVP16 that had been cut with *Bam*HI and *Not*I.

GAD-Ncap contains nt 1-917 of *S. pombe cap* (encoding aa 1-305 of Cap) cloned into GAD424 vector that had been digested with *Bam*HI and *Pst*I.

VP16-Ncap contains nt 1-913 of *S. pombe cap* (encoding aa 1-304 of Cap) cloned into pVP16 digested with *Bam*HI and *Not*I.

LexA-lamin was provided by Anne Vojtek. It expresses human lamin C (aa 66-230) as a fusion to the DNA-binding domain of LexA and was used as a negative control for two hybrid test.

LexA-VP16 was provided by Anne Vojtek. It contains the VP16 acidic activation domain expressed as a fusion to LexA and is a positive control for the L40 yeast strain.

GST-Ncap was constructed by subcloning the *Bam*HI-*Not*I DNA insert from VP16-Ncap into pGEX-5X-1. It expresses amino acid 1-304 of *S. pombe* Cap as a fusion to GST.

GST-14-3-3 was constructed by subcloning the *Eco*RI-*Xho*I cDNA insert from GAD-cDNA10 clone into pGEX-4T-3.

HA-ccyr contains nt 4434-6712 of *S. pombe cyr1* (encoding aa 945-1692 of adenylyl cyclase) cloned into pAD4-HA vector that expresses hemagglutinin-epitope and had been cut with *Sal*I and *Sac*I.

3. Plasmid recovery from yeast

Following the yeast two-hybrid screen, plasmids were recovered from the positive clones by the following method. Yeast cells were picked from cell patches and resuspended in 200 µl BE buffer (0.1 M Tris-HCl pH 9.0, 0.1 M NaCl, 0.5% SDS, 1 mM EDTA). One volume of acid washed glass beads were added to cells and vortexed vigorously for two periods of 1.5 to 2 minutes each, followed by phenol-chloroform extraction 3 times. The aqueous phase was collected and SDS was added to 1%, heated at 65°C for 10 minutes and passed through a sephadex G-50 spin column. DNA was precipitated and resuspended in a small volume of TB (10 mM Tris-HCl pH 7.4).

4. Yeast cell extract preparation

10 ml overnight culture of yeast cells was diluted 1:3 with fresh media and grown till $O.D._{600}$= 1.0. Cells were harvested and washed with 10 ml solution of ice cold 200 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$. Cells were resuspended in 220 µl breaking buffer (200 mM Tris-HCl pH

7.4, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 μg/ml leupeptin, 1 mM PMSF), added to 0.25g glass beads, and vortexed vigorously for 5 periods of 20 seconds each, followed by centrifugation at 10,000 rpm for 5 minutes at 4 °C. Supernatant was recentrifuged at 14,000 rpm for 5 minutes at 4 °C, and protein was stored as aliquots at -70 °C.

5. GST-fusion protein purification

Overnight cultures of *E. coli* transformed with parental or recombinant pGEX plasmid was diluted 1:10 in 300 ml of fresh LB media and grown for 1 hour at 37 °C before adding IPTG to 0.1 mM. After a further 3 to 4 hours of growth, cells were pelleted and resuspended in 1/100 culture volume of ice-cold MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4). Cells were lysed on ice by mild sonication and after adding Triton X-100 to 1%, were subjected to centrifugation at 10,000 rpm for 5 minutes at 4 °C. The supernatant was mixed at room temperature with 1 ml 50% glutathione-agarose beads (Sigma) for 10 minutes (Glutathione-agarose beads were preswollen in MTPBS, washed three times in the same buffer and stored in MTPBS at 4 °C as a 50% solution). Beads were then collected by centrifugation at 1000 rpm for 5 minutes and washed three times with 10 ml MTPBS. Fusion protein was eluted three times with one bead volume of 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione (Sigma) (final pH 7.5, freshly prepared).

6. Protein quantitation

The amount of protein was quantified by Bradford assay using Bradford reagent (Bio-Rad) and following the manufacturer's instructions.

7. SDS-PAGE gel electrophoresis

Proteins were resolved by reducing sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) following the Bio-Rad Mini-protein II Dual Slab Cell Instruction Manual.

8. *In vitro* binding assay

(i) Binding reactions

10 μg of GST, GST-14-3-3 or GST-Ncap protein bound to glutathione-agarose beads was mixed with 70 μg of cell extracts prepared from yeast strains expressing hemagglutinin epitope (HA)-tagged *S. pombe* C-cyclase protein (HA-ccyr), and binding buffer was added so that the total volume was 300 μl containing 150 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4. After 3 hour incubation at 4 °C, the beads were collected and the supernatant sampled (unbound fraction). The beads were washed three times with 0.5 ml washing buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40), and resuspended in 40 μl of SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue) (bound fraction).

(ii) Western blots

Both bound and unbound fractions of protein samples from the above binding assay were boiled for 5 minutes at 95 °C and separated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell). Filters were then incubated at room temperature for 1 hour in blocking solution containing 5% skim milk in TBS (20 mM Tris-HCl pH 7.4, 140 mM NaCl), followed by incubation for 3 hours in TBS containing primary antibodies (monoclonal antibody 12CA5 was used to detect HA-tagged protein; monoclonal antibody GST-12 (Santa Cruz Biotechnology) was used to detect GST and GST fusion proteins). Filters were then washed three times for 10 minutes with TBS, incubated for 1 hour in TBS containing goat anti-mouse IgG conjugated with alkaline phosphatase (Gibco BRL) at a 1:2000 dilution, followed by washing 3 times for 10 minutes with TBS. The immunocoupled alkaline phosphatase was detected by incubating the filter for 5 minutes in 10 ml of alkaline phosphatase buffer (100 mM NaCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 9.5) containing 44 µl of NBT (Gibro-BRL) and 33 µl of BCIP (Gibco-BRL).

III. Results

1. The Leucine-rich repeat region of *S. pombe* adenylyl cyclase does not interact with Gpa2

Genetic evidence suggests that Gpa2 modulates adenylyl cyclase activity in *S. pombe* (Isshiki et al., 1992). To investigate whether Gpa2 could be the direct activator of *S. pombe* adenylyl cyclase, a yeast two

hybrid system approach was used. In *S. cerevisiae*, the leucine-rich region of adenylyl cyclase (amino acids 734-1300) is important for activation of the *S. cerevisiae* adenylyl cyclase by RAS proteins (Suzuki et al., 1990). Experiments with the yeast two hybrid system have shown that a region of amino acids 606-1310 of *S. cerevisiae* adenylyl cyclase, which contains the leucine-rich repeat region, can interact with RAS protein (Vojtek et al., 1993). Based on that, our hypothesis was that the leucine-rich region (amino acids 326-999) of adenylyl cyclase may associate with Gpa2. The DNA sequence encoding amino acids 307-1033 of *S. pombe* adenylyl cyclase was amplified by PCR and fused to the LexA DNA-binding domain (LexA-mcyr). The full length coding sequence of gpa2 was amplified from a *S. pombe* cDNA library by PCR and fused to the VP16 acidic activation domain (VP16-gpa2). Plasmids containing two independent LexA-mcyr clones and two independent VP16-gpa2 clones were subjected to partial DNA sequence analysis to ensure that the DNA inserts had been cloned in frame to the LexA DNA binding domain and VP16 activation domain, respectively. Yeast strains were cotransformed with LexA-mcyr and VP16-gpa2 and tested for histidine prototrophy and β-galactosidase activity. As shown in figure 12, yeast cells containing LexA-mcyr and VP16-gpa2 were unable to activate the *HIS3* and *lacZ* reporter genes and, therefore, were unable to grow in the absence of histidine and were white when tested for β-galactosidase activity. Therefore, *S. pombe* Gpa2 protein does not interact with leucine-rich repeat region of adenylyl cyclase in the yeast two hybrid system.

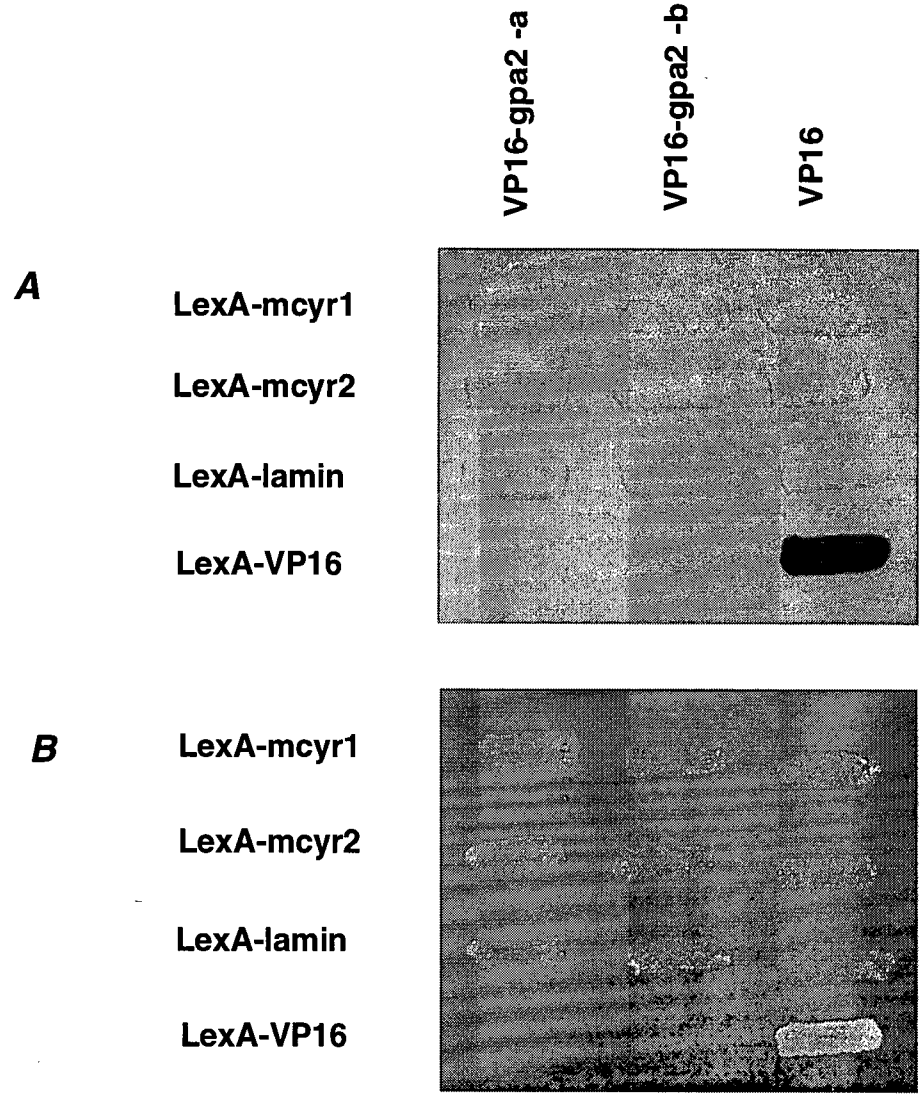

Figure 12. No interactions between Gpa2 and the leucine-rich repeat region of adenylyl cyclase (mcyr). (A) β-galactosidase assay; (B) Growth of cells in the absence of histidine

The *S. cerevisiae* reporter strain L40 was cotransformed with various combinations of LexA-fusion plasmids and VP16-fusion plasmids. Cells were grown on YC-TULK plates for 3 days, patched and tested for β-galactosidase activity (panel A); or replica plated onto THULK plate, grown 3 days and photographed (panel B). Two independent LexA-mcyr clones (LexA-mcyr1 and LexA-mcyr2) and two independent VP16-gpa2 clones (VP16-gpa2-a and VP16-gpa2-b) were tested.

2. Searching for proteins that interact with the leucine-rich region of adenylyl cyclase by yeast two-hybrid system

The yeast two hybrid tests showed that Gpa2 does not interact with the leucine-rich repeat region of adenylyl cyclase, so, although genetic evidence suggests that Gpa2 modulates adenylyl cyclase activity, Gpa2 may not regulate adenylyl cyclase directly. To identify proteins that interact with the same leucine-rich repeat region of adenylyl cyclase, an *S. pombe* cDNA library was screened by the yeast two hybrid system. The *S. cerevisiae* reporter strain, containing LexA-mcyr, was transformed with the library of *S. pombe* cDNA fragments expressed as fusions to the GAL4 activation domain (GADGH-cDNA). Approximately $2.2x10^6$ yeast transformants were screened (see "Materials and methods" for details). Thirty-four clones were initially selected as being positive for both growth on histidine-negative plates and for β-galactosidase activity. However, after segregation of the LexA plasmid, they still retain the ability to grow in the absence of histidine or show β-galactosidase activity. Therefore, none of the clones turned out to be true positives for interaction with the leucine-rich repeat region of adenylyl cyclase.

3. Searching for proteins that interact with the C-terminal domain of adenylyl cyclase by yeast two-bybrid system

No clones scored positive when screened for interaction with the leucine-rich repeat region of adenylyl cyclase. One possibility is that this region alone may not be sufficient for interacting with the regulator of cyclase. In *S. cerevisiae*, the leucine-rich repeat region and

the C-terminus are required for RAS responsiveness. Deletion of the C-terminal 66 residues would abolish the activation of adenylyl cyclase by RAS protein in *S. cerevisiae* (Suzuki et al., 1990). It is possible that the C-terminal domain of *S. pombe* adenylyl cyclase is involved in its regulation. To attempt to identify proteins interacting with the C-terminal domain of adenylyl cyclase, the *S. pombe* cDNA library was screened using C-cyclase as bait. DNA sequence encoding amino acids 945-1692 of *S. pombe* adenylyl cyclase was fused to the LexA-DNA binding domain (LexA-ccyr). To test that this plasmid clone expresses C-cyclase protein, it was cotransformed into the yeast reporter strain L40 with another plasmid GAD-Ncap which expresses the N-terminal domain of *S. pombe* Cap as a fusion to the GAL4-activation domain. Previous experiments showed that *S. pombe* Cap can be coimmunoprecipitated with adenylyl cyclase (Kawamukai et al., 1992) and the yeast two hybrid data in our lab specified that the N-terminal domain of *S. pombe* Cap interacts with the C-terminal domain of cyclase. As seen in figure 13, cells containing LexA-ccyr and GAD-Ncap can grow on histidine-negative plates and have detectable β-galactosidase activity. This LexA-ccyr clone was used in a subsequent screen where it was cotransformed with the GADGH-cDNA library into the *S. cerevisiae* reporter strain L40. Approximately $5x10^5$ yeast transformants were screened and fifteen clones were selected as being positive both for growth in the absence of histidine and for β-galactosidase activity. After segregation of the LexA plasmid and retransformation with LexA-ccyr or LexA-lamin, ten clones were identified that transactivated the *HIS3* and *lacZ* reporter constructs

with LexA-ccyr plasmid but not with LexA-lamin, and therefore were selected as positive clones. Plasmids were recovered from these clones, retransformed into cells containing LexA-ccyr or LexA-lamin and retested to confirmed that they were true positive clones (Figure 13). DNA sequence analysis of the 10 clones revealed that three unique clones had been identified. One of them, GAD-cDNA10, contains a DNA sequence that is identical to the *S. pombe rad24* gene, which encodes the *S. pombe* homolog of mammalian 14-3-3. The other two clones, GAD-cDNA1 and GAD-cDNA13, have no apparent homology with other DNA sequences in the database.

4. Association of *S. pombe* 14-3-3 with the C-terminal domain of adenylyl cyclase *in vitro*

S. pombe rad24 gene has been cloned and it encodes a protein of 271 amino acids that is homologous to human and *S. cerevisiae* 14-3-3 proteins (Ford et al., 1994). The GAD-cDNA10 clone identified in the screen encodes amino acids 71-271 of *S. pombe* 14-3-3 and lacks the N-terminal region. The identification of 14-3-3 in the yeast two hybrid screen indicated that 14-3-3 and C-cyclase form a complex in *S. cerevisiae*. To test whether these two proteins interact *in vitro*, the DNA sequence homologous to *rad24* was subcloned from the pGADGH vector into pGEX-4T-3 (GST-14-3-3). It expresses 14-3-3 as a bacterial fusion with glutathione S-transferase (GST). C-cyclase was expressed in *S. cerevisiae* as a hemagglutinin epitope-tagged protein (HA-ccyr). A fusion between the N-terminal domain of *S. pombe* Cap and GST (GST-Ncap)

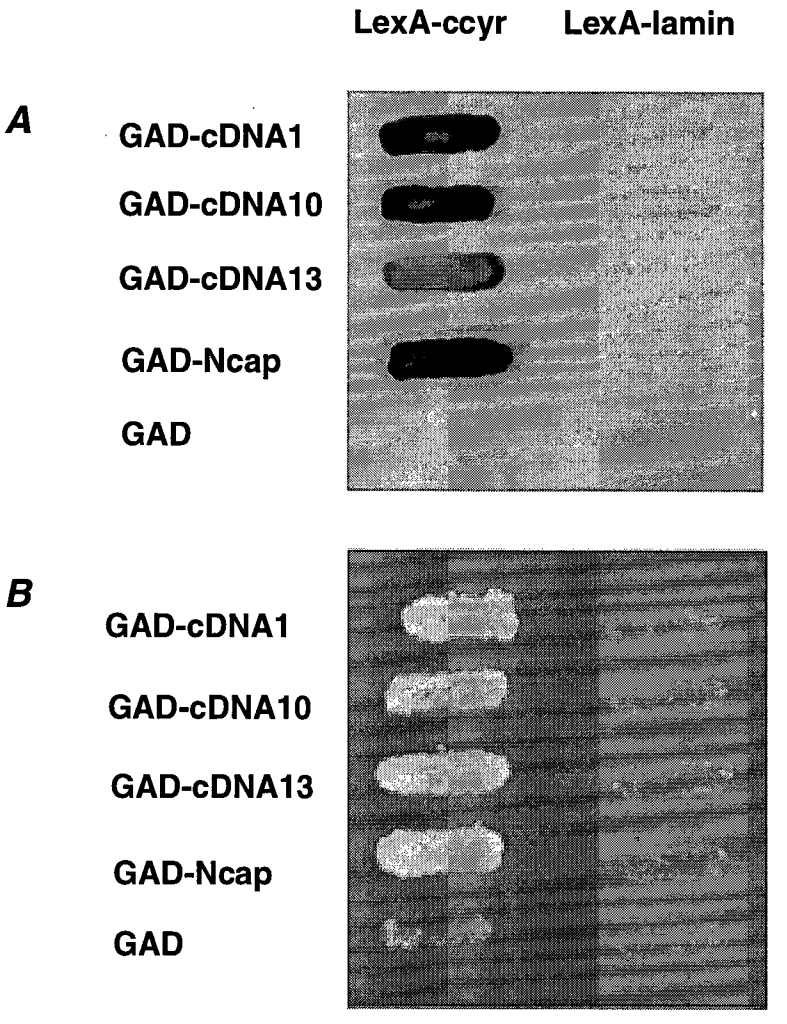

Figure 13. Three unique cDNA clones (GAD-cDNA1, GAD-cDNA 10, GAD-cDNA 13) were identified encoding proteins associated with C-terminal domain of adenylyl cyclase (ccyr). (A) β-galactosidase assay; (B) Growth of cells in the absence of histidine

Different GAD-fusion plasmids were transformed to L40 strain containing LexA-ccyr or LexA-lamin. Cells were grown on YC-TULK plates for 3 days, patched and tested for β-galactosidase activity (panel A); or replica plated onto THULK plate, grown 3 days and photographed (panel B).

was also included in the *in vitro* binding assay. The purified GST, GST-Ncap or GST-14-3-3 fusion protein that had been immobilized on glutathione-agarose beads was incubated with cell lysates prepared from yeast strains expressing HA-ccyr or containing a control vector. Following washing, bound GST-fusion proteins, and any associated proteins, were sampled and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibody against hemagglutinin-epitope (HA-epitope). GST-14-3-3 and GST-Ncap, but not GST alone, bind specifically to HA-ccyr *in vitro* (Figure 14A). Immunoblot analysis with antibody against GST confirmed that all the reactions contained about equal amounts of GST, GST-14-3-3 or GST-Ncap (Figure 14B).

IV. Discussion

In *S. cerevisiae,* adenylyl cyclase is activated by RAS proteins. The leucine-rich repeat region of *S. cerevisiae* adenylyl cyclase interacts with RAS proteins and is important for its activation by RAS proteins. Genetic analysis indicates that *S. pombe* Gpa2 protein, which is a G protein α-subunit, regulates adenylyl cyclase activity in *S. pombe.* However, our results from yeast two hybrid experiments (Figure 12) showed that Gpa2 does not interact with the middle domain (amino acids 307-1033)of *S. pombe* adenylyl cyclase containing the leucine-rich repeat region. Interpretation of this result could be that Gpa2 does not activate adenylyl cyclase directly and there are other components functioning between Gpa2 and adenylyl cyclase; or that the leucine-rich repeat region of *S. pombe* adenylyl cyclase alone is not sufficient to

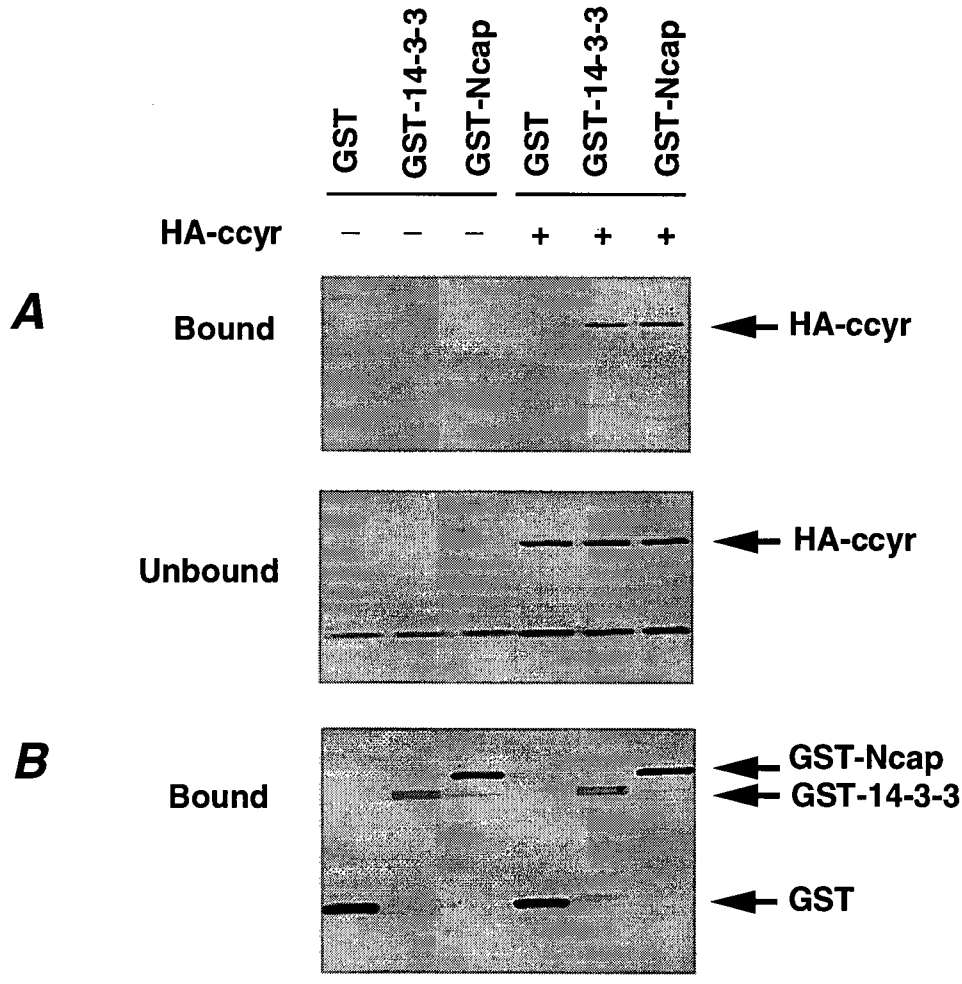

Figure 14. Interaction of 14-3-3 with the C-terminal domain of adenylyl cyclase *in vitro*

Purified GST, GST-14-3-3 or GST-Ncap protein immobilized on glutathione agarose beads was incubated with cell lysates prepared from yeast cells expressing (+) or not expressing (-) hemagglutinin epitope-tagged C-cylase protein (HA-ccyr). After 3 hours of incubation at 4 °C, the unbound fraction was sampled, mixed with SDS sample buffer, and boiled for 5 minutes. The beads containing bound fractions were washed, resuspended in 40 µl of SDS sample buffer, and boiled for 5 minutes. 15 µl of bound samples and 1/30 of unbound samples were analyzed by SDS-PAGE electrophoresis and immunoblotted with monoclonal antibody 12CA5 to detect HA-ccyr protein (panel A) or with monoclonal antibody GST-12 to detect GST and GST-fusion proteins (panel B).

interact with the regulator of cyclase. Two hybrid experiments with VP16-gpa2 and LexA-ccyr were also conducted and the results were negative (data not shown) indicating that Gpa2 does not interact with the C-terminal domain of cyclase. Whether it interacts with full length cyclase is unknown. Previous studies proposed that GTP-binding proteins are unlikely to be the regulator of adenylyl cyclase in *S. pombe*, based on the observation that adenylyl cyclase activity is not stimulated *in vitro* in a GTP-dependent manner (Engelberg et al., 1990; D. Young, personal communication). So although genetic evidence suggests that Gpa2 modulates adenylyl cyclase activity, Gpa2 may not be the direct regulator of *S. pombe* adenylyl cyclase.

We failed to identify clones encoding proteins that interact with the leucine-rich repeat region of adenylyl cyclase. There is no clear explanation to account for this result. The possibilities are that there is an inhibitory domain within the protein region used for the two hybrid screen; the LexA-mcyr fusion protein is not in the right conformation; the leucine-rich repeat region alone is not sufficient for associating with other proteins; or the interaction is too weak and unstable to be detected by the yeast two hybrid system. Although the leucine-rich repeat region of *S. cerevisiae* adenylyl cyclase associates with RAS proteins, the interaction is weak when tested by the yeast two hybrid system (data of our lab). Previous experiments showed that expression of the N-terminal 1281-amino acid region results in a similar phenotype to that of cells containing the *cyr1* disruption (Kawamukai et al., 1991), suggesting that expression of this domain interferes with adenylyl cyclase function, possibly by binding to its

positive regulator. If this is the case, the middle region of adenylyl cyclase used in the two hybrid screen may not be sufficient for interaction with the regulator of cyclase.

The *S. pombe* 14-3-3 protein is capable of associating with the C-terminal domain of adenylyl cyclase, both in the yeast two hybrid system and *in vitro*. The interaction between 14-3-3 and C-cyclase is strong in the yeast two hybrid system, while only approximately 3% of HA-epitope-tagged C-cyclase in yeast extract binds specifically to purified GST-14-3-3 *in vitro*. We did not identify the *S. pombe* Cap by the yeast two hybrid screen, presumably because the cDNA library was made by cloning the *Eco*RI-*Xho*I cDNA fragments into pGADGH vector and *Eco*RI cuts within the *S. pombe cap* coding region.

The 14-3-3 clone identified in the two hybrid screen lacks the N-terminal 70 amino acids of the full length protein. It would be more convincing if we can show that full length 14-3-3 protein can be coimmunoprecipitated with C-cyclase protein from yeast lyates. To do that, the full length coding sequence of 14-3-3 can be amplified from a *S. pombe* cDNA library and cloned into the myc-epitope expression vector. This myc-epitope-tagged 14-3-3 protein will be coexpressed in yeast with HA-epitope-tagged C-cyclase protein, followed by immunoprecipitation with antibody against HA-epitope and immunoblot analysis with antibody against myc-epitope.

The 14-3-3 proteins have been found in most eucaryotic cells and are highly conserved (for review, see Morrison, 1994). Recent studies suggest that mammalian 14-3-3 proteins bind to the Raf protein kinase and play a role in Raf activation. Two genes, *rad24* and *rad25*, encode *S.*

pombe homologs of 14-3-3 proteins (Ford et al., 1994). The *rad24* gene product is 71% identical to that of *rad25* and both are homologous to *S. cerevisiae* and mammalian 14-3-3 proteins. The *rad24-rad25* double null mutant is inviable indicating an essential role for the 14-3-3 homologs in *S. pombe*. Studies also showed that *rad24* and *rad25* are required for DNA damage checkpoint in *S. pombe*. *S. pombe rad24* null mutants, and to a lesser extent *rad25* null mutants, are sensitive to UV and to ionizing radiation; and such cells enter mitosis prematurely.

The functional significance of the interaction of 14-3-3 with the C-terminal domain of adenylyl cyclase remains to be determined. It is not known whether 14-3-3 protein is involved in the regulation of adenylyl cyclase activity in *S. pombe*. Determination of adenylyl cyclase activity of *S. pombe* cells overexpressing *rad24* or with a *rad24* deletion should provide us some information to address this question.

CHAPTER 4. SIGNIFICANCE AND PROPECTIVE STUDIES

Cyclic nucleotides play an essential role in controlling many of the activities of eucaryotic and procaryotic cells. In mammals and yeast, cyclic AMP acts as an important second messenger in signal transduction pathways. The cAMP signaling pathways appear to control different responses in different organisms. In the budding yeast *S. cerevisiae*, cAMP plays a role in growth control (Kataoka et al., 1984). In the fission yeast *S. pombe*, cAMP is dispensable for cell growth, but it appears to be involved in regulating sexual development (Kawamukai et al., 1991). Stimulation of the cAMP pathway in *S. pombe* inhibits mating and sporulation, while attenuation of this pathway derepresses *S. pombe* cells for initiating sexual development. Growth of mammalian cells is in general inhibited by a high concentration of cAMP, although conflicting results have been reported (for review, see Pastan et al., 1975; Dumont et al., 1989).

The cAMP signaling pathway and the components involved in the cascade have shown conserved and diverged aspects during the course of evolution. The *S. cerevisiae* and *S. pombe* express structurally related adenylyl cyclases. These cyclases show striking homology within their respective C-terminal catalytic domains, and both enzymes contain tandemly repeated leucine-rich consensus sequences. The mammalian adenylyl cyclase contains two large multi-membrane-spanning domains and two 40-kD domains that are proposed to be catalytic (Krupinski et al., 1989). This adenylyl cyclase shows very little homology to the *S. cerevisiae* and *S. pombe* adenylyl cyclases. Since the *cyr1*$^-$ *S. cerevisiae*

strains (Kataoka et al., 1985) and *S. pombe* strains (Kawamukai et al., 1991) contain no measurable cAMP or adenylyl cyclase activity, it is unlikely that either yeast contains a homolog to the adenylyl cyclase gene that has been identified in mammals. It is not yet clear if mammals contain a homolog of the adenylyl cyclase gene found in yeast.

The regulator of adenylyl cyclase appears to have switched during evolution. RAS proteins activates adenylyl cyclase in *S. cerevisiae* (Broek et al., 1985; Toda et al., 1985). It was shown that mammalian Ras could activate the *S. cerevisiae* adenylyl cyclase (Broek et al., 1985) but neither mammlian Ras nor *S. cerevisiae* RAS could regulate mammlian adenylyl cyclase (Beckner et al., 1985). Instead, a guanine nucleotide-binding protein (G_S) mediates hormone-dependent activation of mammalian adenylyl cyclase (for review, see Gilman et al., 1987). Ras does not regulate adenylyl cyclase in *S. pombe* and the regulator of adenylyl cyclase in this yeast is still unknown. Genetic evidence suggests that *S. pombe* Gpa2, a G-protein α-subunit, is involved in the determination of the cAMP level according to nutritional conditions, and is likely to be a positive regulator of adenylyl cyclase. However, Gpa2 does not interact with the leucine-rich repeat region of adenylyl cyclase, as tested by our yeast two hybrid experiments (Figure 12). Although there could be many explanations, it is possible that Gpa2 is not the direct activator of adenylyl cyclase. This hypothesis is also supported by the observation that *S. pombe* adenylyl cyclase activity is not stimulated *in vitro* in a GTP-dependent manner (Engelberg et al., 1990; D. Young, personal communication).

By using the yeast two hybrid system to screen an *S. pombe* cDNA library, *S. pombe* Rad24, a homolog of the mammalian 14-3-3 proteins, was identified and shown to be capable of interacting with the C-terminal domain of adenylyl cyclase (Figure 13). Seven mammalian isoforms of 14-3-3 have been identified and these proteins are highly conserved. It has been shown that mammalian 14-3-3 family members associate with the products of proto-oncogenes and oncogenes, including Raf, Bcr-Abl and the polyomavirus middle tumor antigen, suggesting that 14-3-3 proteins participate in cell transformation and mitogenic signaling pathways. Recent studies showed that in yeast cells expressing Raf and Mek, mammalian 14-3-3 activates Raf (Freed et al., 1994), and bacterially synthesized mammalian 14-3-3 stimulates the activity of Raf prepared from yeast cells expressing Raf (Irie et al., 1994). Thus, the mammalian 14-3-3 may participate in or be required for activation of Raf. In addition, members of the 14-3-3 family appear to play a role in the Ca^{2+}-dependent activation of enzymes involved in neurotransmitter systhesis (Ichimura et al., 1988) and the regulation of protein kinase C (Aitken et al., 1990). *S. cerevisiae* has a single known 14-3-3 homolog (BMH1) (van Heusden et al., 1992). BMH1 is 60% identical with members of the 14-3-3 family and its function is nonessential. *S. pombe* has two homologs of 14-3-3, Rad24 and Rad25, which together provide a function that is essential for cell proliferation (Ford et al., 1994). Both Rad24 and Rad25 are required for the DNA damage checkpoint in *S. pombe*. The mechanism of this checkpoint control is yet unclear, nor is the functional significance of the interaction between Rad24 and adenylyl cyclase. Perhaps Rad24 is

involved in the regulation of adenylyl cyclase, playing a similar role to that of mammalian 14-3-3 in the regulation of Raf.

Much more remains to be learned about Rad24 and its association with adenylyl cyclase, nevertheless, our results provide a clue for future research in identifying the regulator of adenylyl cyclase in *S pombe*. First we have to determine whether the interaction of adenylyl cyclase with Rad24 is direct, or whether other proteins are involved. Since the N-terminal domain of *S. pombe* Cap associates with the C-terminal domain of adenylyl cyclase, and human N-cap has been shown to be able to interact with mammalian 14-3-3 in the yeast two hybrid system and *in vitro* (Gang Yu, personal communication), we can not rule out the possibility that in *S. pombe* Rad24 binds to the N-terminal domain of Cap, which in turn associates with the C-terminal domain of adenylyl cyclase, or that these three proteins form a trimer-complex. To make this point clear, we can test whether Rad24 interacts with *S. pombe* Cap in the yeast two hybrid system. We can also determine whether Rad24 and the C-terminal domain of adenylyl cyclase coimmunoprecipitate from yeast extracts prepared from a *cap*$^-$ strain. Alternatively, we can study the interaction of GST-Rad24 with MBP-adenylyl cyclase fusion protein purified from bacterial lysates *in vitro*.

If the interaction between Rad24 and adenylyl cyclase is shown to be direct, then we can explore further to determine whether it could be a direct activator of adenylyl cyclase in *S. pombe*. Previous experiments have shown that deletion of adenylyl cyclase leads to conjugation and sporulation under conditions that normally inhibit wild-type strains, and overexpression of adenylyl cyclase leads to a partially sterile

phenotype (Kawamukai et al., 1991). We will determine if deletion or overexpression of *rad24* leads to similar phenotypes, and we will test the ability of *rad24*, when overexpressed, to suppress *cyr1*$^-$, and the ability of *cyr1*, when overexpressed, to suppress deletion of *rad24*. We can also express Rad24 in bacteria and study whether the purified Rad24 could stimulate adenylyl cyclase activity in *S. pombe* membrane extracts.

This thesis also presents the cloning and characterization of *S. pombe pka1* encoding the catalytic subunit of cAMP-dependent protein kinase. Our results also showed that, while *S. pombe* cells overexpressing adenylyl cyclase have a partially sterile phenotype, cells overexpressing Pka1 is completely sterile (Figure 7). Interpretation of this observation could be that the overexpressed adenylyl cyclase is not fully activated, or its activity is regulated by a feedback mechanism. Previous studies on the *S. cerevisiae* and *S. pombe* adenylyl cyclase and cAMP phosphodiesterase support the later choice. It was shown that cAMP levels were only modestly elevated in cells overexpressing adenylyl cyclase (Kawamukai et al., 1991), and were only modestly reduced in cells overexpressing cAMP phosphodiesterase (Mochizuki and Yamamoto, 1992). Thus, a strict feedback mechanism at the enzyme level is likely to regulate the activity of adenylyl cyclase and cAMP phosphodiesterase. *S. pombe* cells exhibit a completely sterile phenotype when overexpressing Pka1, presumably because such cells express an excess of the cAMP-dependent protein kinase catalytic subunit.

The cAMP cascade in *S. pombe* is also involved in the regulation of expression of the glucose-repressible gene *fbp1*, which encodes fructose-1, 6-bisphosphatase (Hoffman et al., 1991). This gene is expressed in the absence of glucose, where the level of intracellular cAMP is decreased. Mutants in which transcription of *fbp1* is constitutive have been isolated and named *git* (glucose-insensitive transcription). These mutations identified 10 genes. The *S. pombe cyr1*, *pka1* and *gpa2* are found among the *git* genes. It is an interesting question how the two pathways, one for sexual development and the other for *fbp1* expression, are differentially regulated by cAMP-dependent protein kinase in *S. pombe*. Identification of the downstream effectors and substrates of Pka1 will help to answer this question, and will enrich and expand our understanding of the cAMP signaling pathway in *S. pombe*.

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