This is to certify that the

thesis entitled

Differential Expression of ras Genes

During Growth and Development in Mucor racemosus

presented by

Sung-Yuan Wang

has been accepted towards fulfillment
of the requirements for

M.S. degree in Food Science

John E. Linz

Dr. John E. Linz

Major professor

Date 11-8-89

DIFFERENTIAL EXPRESSION OF RAS GENES DURING GROWTH AND DEVELOPMENT IN *Mucor racemosus*

By

Sung Yuan Wang

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements

of

MASTER OF SCIENCE

Food Science and Human Nutrition

1989

ABSTRACT

DIFFERENTIAL EXPRESSION OF RAS GENES
DURING GROWTH AND DEVELOPMENT IN Mucor racemosus

By

Sung Yuan Wang

ras genes are an ubiquitous eukaryotic gene family implicated in cell proliferation and differentiation. The ras proteins bind guanine nucleotide have GTPase activity and are thought to play an important role in human tumor formation. Evidence suggests the involvement of ras in cellular morphogenesis in Mucor racemosus in response to environmental signals. This dimorphism fungus is a useful model to study ras function and its potential role in regulation of morphogenesis. Three MRAS gene homologues have been previously cloned from this organism. We subcloned DNA fragments from the 3' end hypervariable region of each MRAS gene and utilized them as DNA probes for Northern analysis of poly(A)$^+$-RAN from 8 unique morphological stages to determine transcript levels of MRAS genes accumulated in a morphology-specific manner. The results of this study suggest that at least two MRAS genes (MRAS1 and MRAS2) are expressed differentially and these MRAS transcripts are accumulated in a morphology-related pattern.

To my dear parents

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation and deepest gratitude to his major advisor, Dr. John E. Linz for his continuous encouragement, assistance and understanding in the course of his graduate study. The author also wishes to extend his thank to the members of his Master's degree committee, Dr. James J. Pestka, Dr. William G. Helferich, Dr. C.N. Shih for their involvement in his research. Special acknowledge and appreciation are given to Dr. David G. McConnell for his compassionate assistance and encouragement to the author's academic course.

Sincere thanks are extended to the author's laboratory members, especially to Dr. William L. Casale and Jason Horng for their technical advice, Roscoe Warner for his help in photographs production, Chris Skory for his valuable advice in image analysis. Many thanks go to Mary Streng and Yi-Yu Yen for their help in laboratory works and friendship.

Last but not least, the author wishes to express deepest appreciation to his parents, Mr. P. H. Wang and Mrs. P. Wang and his brother and sister for their love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

ras genes are an ubiquitous eukaryotic gene family. Nucleotide sequence analyses of ras genes and their products in mammals, birds, insects, plants, and fungi revealed a high degree of conservation (Shilo and Weinberg 1981). The functional similarity of ras genes has also been studied. The mammalian H-ras gene can complement nonviable ras1$^-$ and ras2$^-$ S. cerevisiae mutants and enable diploid spores of this organism to germinate and produce viable haploid cells (Kataoka et al. 1985, DeFeo-Jones et al. 1985). In a gene transfer assay, chimeric yeast-mammalian ras genes in which the amino terminus of the mammalian H-ras gene was replaced with the amino terminus of the yeast ras gene were able to efficiently transform mouse NIH3T3 cells in culture (Defeo-Jones et al. 1985). A similar result was observed when mammalian cells were transformed with chimeric ras genes formed by fusing the amino terminus of human H-ras oncogenes with the carboxy terminus of Drosophila ras genes (Schejter and Shilo 1985). These data suggest a high degree of functional conservation of ras genes during evolution. The products of ras proto-oncogenes have also been implicated in regulation of cell proliferation (Mulcahy et al. 1985) and cell differentiation (Guerrero et al 1986,

Bar-Sagi and Feramisco 1985).

Despite the phylogenetic origin of ras genes, they encode proteins that bind guanine nucleotides (Temeles et al. 1985); have GTPase activity (Temeles et al. 1985, Manne et al. 1985) and associate with the plasma membrane (Fujiyama and Tamanoi 1986). These biochemical properties of ras proteins, along with their significant sequence similarity with G proteins (Lochrie et al. 1985) which are involved in membrane signal transduction systems, suggest that ras proteins may play a crucial role, like G proteins, in transmembrane signaling systems and that altered ras function may lead to abnormalities in cell differentiation and proliferation.

Three ras gene homologues (MRAS) have been identified in the filamentous fungus Mucor recemosus by screening a genomic DNA library using H-ras, yeast RAS1 and RAS2 genes as probes. This filamentous fungus contains a small genome, is easy to grow, can differentiate rapidly under simple laboratory conditions and is relatively easy to manipulate genetically. Therefore, this dimorphic fungus provides a useful model system to study ras genes and their potential function in cellular morphogenesis.

Morphogenesis of Mucor cells is regulated by a variety of environmental conditions (Cihlar 1985). Cyclic adenosine 3', 5'-monophosphate (cAMP) and phosphatidylinositol (PI) have been implicated in regulation of cellular morphogenesis of Mucor (Larsen and Sypherd 1974, Orlowski 1979, Orlowski 1980, Forte and Orlowski 1980, Ito et al. 1982). These molecules are thought to play important roles in signal transduction pathways in eukaryotic cells, and may therefore play a role in mediation of the morphogenetic response in Mucor. A signal transduction pathway involving adenylate cyclase, phosphodiesterase, protein kinases and/or phospholipid metabolism may be linked to morphogenesis in Mucor.

Based on the above data, we proposed the following hypothesis: If one or more Mucor ras genes are involved in regulation of morphogenesis, the expression levels of the genes may correlate with changes in morphology of Mucor cells. As an initial step in testing this hypothesis, this project focused on determining the expression levels of three Mucor ras transcripts in several different morphologies of Mucor cells.

LITERATURE REVIEW

Structural Aspects of ras

The acronym ras is derived from rat sarcoma because the ras genes were initially discovered as oncogenes of Harvey and Kirsten strains of rat sarcoma viruses (Harvey 1964, Kirsten and Mayer 1967). Three ras genes, H-ras-1, K-ras-2 and N-ras, have been identified in the mammalian genome (DeFeo et al. 1981, Hall et al. 1983) and two pseudogenes, H-ras-2 and K-ras-1, are present in genomes of most mammals (Eills et al. 1981, McGrath 1983). Two ras genes, RAS1 and RAS2, which are highly homologous to the mammalian ras genes, were found in the yeast Saccharomyces cerevisiae (Powers et al. 1984). Other ras homologues, including H-ras in chickens (Shih et al. 1981), Dras1, Dras2/64B and Dras3 in the fruit fly Drosophila melanogaster (Neuman-Silberberg et al. 1984, Schejter and Shilo 1985), Apl-ras in the mollusk Aplysia (Swanson et al. 1986) and Ddras in the slime mold Dictyostelium discoidium (Reymond et al. 1984) have also been identified.

Comparative analysis of the deduced amino acid sequences of the products of these ras genes with the

mammalian ras p21 protein showed a high degree of sequence similarity except in the hypervariable carboxy terminus region. The pattern of predicted structural domains is also conserved (Barbacid 1987).

The X-ray crystallographic structure of the c-H-ras p21 protein has been resolved (De Vos et al. 1988). This structural analysis reveals four α helices and six-strands of β sheet which are connected by nine loops, L1 through L9. The biosynthetic pathway of RAS1 and RAS2 gene products of S. cerevisiae has been elucidated (Fujiyama et al 1986). The RAS1 and RAS2 proteins appear to be initially synthesized as 37 Kd and 41 Kd precursor molecules, respectively, and are rapidly converted to 36 Kd and 40 Kd intermediate forms. These two processed proteins are further modified by fatty acid acylation. After the attachment of palmitic acid, the RAS1 and RAS2 proteins are localized in the plasma membrane. The site for palmitic acid acylation is presumably located at the carboxy terminus of the RAS proteins. The processing of precursor molecules appears to be necessary for the fatty acid acylation (Deschenes and Broach 1987). It has been suggested that the sequence Cys-A-A-X-COOH (A is aliphatic amino acid and X is any amino acid) located at the carboxy terminus of ras proteins is involved in the fatty acid acylation of p21 ras proteins (Willumsen et al. 1984, Weeks et al. 1985). The

exact site of the fatty acid acylation may be located at the carboxy terminus cystein residue. A modification of H-ras precursor proteins distinct from fatty acid acylation in NIH3T3 cells has also been detected (Buss and Sefton 1986), however, the nature of this modification is not clear. Phosphorylated yeast RAS proteins have also been found (Temeles et al. 1984, Fujiyama and Tamanoi 1986).

Biochemical Properties of ras Gene Products

The biochemical properties of ras proteins have been studied intensively after they were first identified in human tumors. The ras proteins have been shown to bind guanine nucleotides (GDP and GTP) effectively (Tamanoi et al. 1984, Temeles et al. 1985) and hydrolyze GTP to GDP (Manne et al. 1985, Sweet et al. 1984, Temeles et al. 1985). Activated ras proteins often show reduced GTPase activity (Manne et al. 1985, Temeles et al. 1985). In addition to GDP/GTP binding and GTPase activity, activated p21 ras proteins possess an autokinase activity (Shih et al. 1982). A recent study of the GTPase catalytic domain of human c-H-ras p21 protein demonstrated that the hydrolysis of GTP may result in the simultaneous release of the gamma phosphate group of GTP causing autophosphorylation of Thr^{59} which is located adjacent to phosphate binding loop 1 of the

activated ras protein (De Vos et al. 1988). The GTP binding ability of purified ras p21 protein was shown to be inhibited by antibodies against epitopes located at the amino terminus region of the protein. The binding of antibodies to ras proteins was inhibited by GTP or GDP already bound to the protein (Clark et al. 1985).

Biological Function of ras Genes

The ras genes appear to play an important role in human tumor formation (Cooper 1982, Bos et al. 1987, Forrester et al. 1987). Some tumor cells contain mutant ras genes which are capable of transforming NIH3T3 cells. By a random mutagenesis analysis, mutants carrying amino acid substitutions at amino acid residues including Gly^{12}, Gly^{13}, Ala^{59} and Glu^{63} of the mammalian p21 ras protein activate the transforming potential of the H-ras gene. (Fasano et al. 1984). These residues are conserved in the yeast RAS protein (Tamanoi 1988) and correspond to amino acid residues 19, 20, 66 and 70 in yeast. Mutants having valine substituted for glycine at position 19 of the yeast RAS protein resulted in physiological changes of yeast cells, such as failure to accumulate carbohydrate, inefficient sporulation, sensitivity to nutrient starvation, and sensitivity to heat-shock treatment (Kataoka et al. 1984,

Marshall et al. 1987, Toda et al, 1985). In contrast, ras2⁻ mutants exhibited a different phenotype, such as accumulation of carbohydrate and premature sporulation. These mutations in yeast and mammalian ras proteins appear to alter ras function by altering the GTPase activity or affinity of GTP binding to the ras protein. X-ray crystallography studies (De Vos et al. 1988) showed that the GDP molecule bound to ras p21 is embedded in a pocket formed by loops L1, L2, L7 and L9 of the protein structure. Loop 1, which contains amino acid residues 10 to 16 is located near the phosphate groups of GTP and appears to be the active site for GTPase function. Mutations in amino acids 12 and 13 may therefore activate the ras transforming potential by altering GTPase function. Some studies have indicated that inhibition of GTPase activity may result in activation of oncogenic ras protein (Temeles et al. 1985, McGrath et al. 1984, Der et al. 1986). Mutants which revealed an increase in dissociation rate of guanine nucleotide of ras proteins may also exert the transforming properties (Sigal et al. 1986, Walter et al. 1986). This may be due to a higher availability of GTP molecules in the cells for ras proteins, or to an induced conformational change of ras proteins resulting in a stable active state regardless of guanine nucleotide binding. Residues 59, 61 and 63 belong to loop 4 which indirectly contacts loop 1. Mutation at these positions in loop 4 may possibly activate

ras p21 through an indirect conformational change of loop 1 and may in addition affect GTP binding or GTPase activity. Amino acid residues 116,117, 119 and 120 of loop 7 and residues 145 to 147 of loop 9 form a site for guanine base binding. Mutation at these residues may directly affect the binding of the guanine base. This structural model agrees with previous studies (Jurnak 1985, McCormick et al. 1985. Sigal et al. 1986) which predict that substitution of amino acid residues at these positions may affect the GDP or GTP binding and reduce GTPase activity of ras proteins.

The effector activities of ras proteins have been studied. By substitution of amino acid residues at position 35 to 40, the biological effect of ras proteins appeared to be reduced, but the biochemical activities (GTPase activity or guanine nucleotide binding) were not disrupted (Sigal et al. 1986, Willumsen et al. 1986). A mutated yeast RAS2 protein (Ser^{42} which is located in the putative effector region) expressed in yeast cells also resulted in reduced biological function (Marshall et al. 1988). X-ray crystallography of the H-ras protein showed that amino acids 35 to 40 lie near the GDP molecule when embedded in the protein but do not directly contact GDP (De Vos et al. 1988). These data suggested that amino acid residues 35 to 40 of the mammalian ras protein may be involved in the effector activity of ras proteins, since downstream

activities of the effector protein are inhibited by mutations in this region

A model explaining the mechanism of ras protein biological function has been proposed (Barbacid 1987). The ras proteins appear to exist in equilibrium between an active and inactive state. Most of the ras proteins which bind GDP exist in an inactive form until they receive upstream stimulus in the putative signal transduction pathway. This stimulus activates ras proteins by replacing GDP with GTP. The activated ras proteins convey this signal to the putative effector molecules. Under normal conditions, the stimulated ras proteins may be inactivated by the intrinsic GTPase activity. Adenylate cyclase in yeast has been identified as a RAS2 effector molecule.

Mutants capable of inducing transformation of cells in vivo or in vitro suggest that ras genes may play a role in cell proliferation. The protein product of ras proto-oncogene is required for initiation of S-phase in NIH3T3 cells (Mulcahy et al. 1985). Transformation of an activated N-ras gene or microinjection of ras oncogene proteins into PC12 cells induce these cells to differentiate into neuron-like cells (Bar-Sagi and Feramisco 1985, Guerrero et al. 1986) which suggests ras may play a role in cell differentiation. It has been reported that the addition of

epidermal growth factor (EGF) stimulates the GDP-binding activity of ras oncogene proteins in transformed rodent cells (Kamata and Feramisco 1984). This suggests a certain degree of interaction occurs between ras protein and EGF receptor pathway. The exact biological function of mammalian ras proteins is still poorly understood.

Perhaps the best understanding of ras biological function has been achieved in S. cerevisiae. Yeast RAS proteins are essential for yeast cell proliferation and mating (DeFeo-Jones et al. 1983, Kataoka et al. 1984). At least one functional yeast RAS gene is required for the maintenance of growth and cell viability. Spores lacking both the RAS1 and RAS2 gene are nonviable. Disruption of both genes appears to block the spores in the G1 phase of the cell division cycle. (Tatchell et al. 1985, Fraenkel 1985). Yeast RAS gene products have been shown to be involved in the regulation of the formation of secondary messengers. Yeast RAS1, RAS2 or human H-ras genes activate yeast adenylate cyclase in the presence of guanine nucleotides (Broek et al. 1985). The yeast RAS2 gene has been demonstrated to be directly involved in regulation of adenylate cyclase (Toda et al. 1985). For example, the cAMP levels in $RAS2^{val19}$ mutants (analogous to activated H-ras p21) were about 4 fold higher than cAMP levels in wild-type strains. ras1$^-$RAS2 mutants have only slightly decreased

cAMP levels while RAS1ras2$^-$ mutants showed at least 4 fold lower levels of cAMP than that of RAS1RAS2 strains. ras1$^-$ ras2$^-$ strains revealed 20 fold lower cAMP than wild-type cells. These data suggested that RAS1 and RAS2 proteins modulate cAMP levels and that the RAS2 protein is the major controlling element in yeast cells while RAS1 may only be a minor determinant. In vitro studies showed that the yeast ras1$^-$ras2$^-$ mutants appeared to have a low level of adenylate cyclase activity which regulates cAMP formation. RAS2^{val19} mutants revealed a high levels of adenylate cyclase activity. Moreover, the wild type adenylate cyclase activity was induced in the presence of a nonhydrolyzable GTP analog (Casperson et al. 1985, Toda et al. 1985). The high availability of the GTP analog may allow it to be incorporated into ras proteins and overstimulate adenylate cyclase because the proteins are unable to hydrolyze GTP resulting in a permanently active GTP-bound conformation. In summary, yeast cells carrying mutant ras genes have altered regulatory function of adenylate cyclase, altered cAMP levels, and altered morphology. GTPase activity of yeast RAS proteins also correlate to ras biological function. Unlike yeast, there is no current evidence which demonstrates that mammalian ras genes are related to the stimulation of adenylate cyclase activity in mammalian cells (Beckner et al. 1985).

However, recent studies have suggested that certain ras genes may play a regulatory role in the phosphatidylinositol pathway in mammalian cells. Fibroblast cells transformed with ras genes showed significantly reduced phosphatidylinositol-4,5-bisphosphate (PIP_2) levels and increased levels of the breakdown product 1,2-diacylglycerol (DAG) compared with normal cells (Fleischman et al. 1986). Microinjection of transforming ras p21 into Xenopus oocytes resulted in increased levels of PIP_2 and DAG (Lacal et al. 1987). Phospholipase C is responsible for the breakdown of PIP_2 into DAG and IP_3 (Kikkawa and Nishizula 1986). This led to the proposal that ras proteins may mediate the activation of phospholipase C which acts as an effector molecule in the signal transduction pathway (Wakelam et al. 1986). However, microinjection of ras proteins into rat fibroblasts also resulted in stimulation of phospholipase A_2 (Bar-Sagi and Feramisco 1986). In addition, it has been reported that cells transformed with H-ras oncogenes resulted in elevation of DAG in the absence of a detectable increase in inositol triphosphate (IP_3). This led to the suggestion that a source other than phosphatidyinositol bisphosphate may exist for generation of DAG and that activated ras gene may participate in this novel pathway for DAG production (Lacal et al 1987).

The yeast RAS1 gene has also been implicated in the regulation of inositol phospholipid (PI) turnover (Kaibuchi et al 1986). ras1$^-$ mutants revealed a higher level of phosphatidylinositol bisphosphate and the breakdown products inositol monophosphate, inositol diphosphate and inositol triphosphate. A slightly elevated turnover was observed with ras2$^-$ mutant cells. Thus, the RAS1 gene appeared to play the major role in regulation of phosphatidylinositol phosphate turnover. However, another study reported that ras genes are unlikely to function in control of phospholipase activity in yeast (Yu et al 1988). The connection between ras proteins and phospholipid metabolism is not clear at this time.

Other Molecules in ras Mediated Signalling Pathway(s)

The amino acid sequence and biochemical properties of ras proteins are similar to G proteins which are involved in signal transduction pathways (Gilman 1987). G proteins interact with cell surface receptors, are stimulated into activity, and in turn stimulate (G_s) or inhibit (G_i) effector proteins such as adenylate cyclase. The similarity between G proteins and ras proteins has lead to a search for analogous receptor and effector proteins. A function upstream of ras (CDC25 gene) in a signalling pathway has

been identified in S. cerevisiae (Robinson et al. 1987, Broek et al. 1987). Disruption of CDC25 arrests yeast cell sporulation in G1 phase of the cell cycle. An activated $RAS2^{val19}$ gene will suppress mutant CDC25 function, but normal RAS2 will not replace this function. This suggests that the CDC25 product regulates RAS2 function. This regulatory function also may be related to GTP binding of RAS2 proteins because $RAS2^{val19}$ proteins bind GTP constitutively. Moreover, the CDC25 gene product activates both RAS1 and RAS2 but apparently results in interaction of RAS1 and RAS2 with different downstream effectors. This suggests that RAS1 and RAS2 genes play different cellular roles.

A GTPase activating protein (GAP) that stimulates the GTPase activity of normal ras proteins but not oncogenic ras proteins in mammalian cells has been discovered (Trahey and McCormick 1987). This cytoplasmic protein appears to interact with ras protein at the putative effector domain (Adari et al. 1988, Cales et al. 1988). The interaction of GTP-bound of ras protein with GAP may lead to cellular proliferation. The activated GTP-bound ras may be converted to inactivated GDP-bound of ras protein (Sigal 1988). Oncogenic ras proteins which binds GTP in a stable complex may prolong the activation by GAP. This suggests that GAP may be the biological target for regulation by ras proteins.

Mucor racemosus as a Model System to Study ras Genes

Mucor racemosus is a filamentous fungus in the class Zygomycetes. This dimorphic fungus can be induced to undergo cellular morphogenesis in response to a change in the gaseous environment and was selected here as an experimental system for study of ras genes and their potential function in cellular morphogenesis. Although it is a relatively simple organism with a small genome, M. racemosus exhibits a variety of morphogenetic processes in response to stimuli including chemicals, nutrients and gases (Cihlar 1985). Mucor racemosus alternates between growth as single-cell yeast and filamentous hyphae. The fungus sporulates in several ways resulting in either of two asexual spore-types, sporangiospores and athrocanidia or sexually-produced zygospores. Each of these spore-types can undergo germination with the appropriate stimuli. Mucor grows from spore to a yeast under anaerobic conditions in the presence of fermentable hexose. By a switch of gaseous environment from CO_2 or N_2 to air, the yeast cell of Mucor responds to this single environmental stimulus and changes its morphology to hyphae. A change of gaseous condition from air to CO_2 or N_2 causes a hyphae-to-yeast shift. Morphogenesis in a population is synchronous, complete, and easily induced or inhibited by a variety of conditions. The ability to mediate the morphogenetic transition provides

an advantageous system in which to investigate the biochemical and molecular mechanisms that operate during differentiation.

Biochemical changes in Mucor cells which accompany morphogenesis have been the subject of intensive study. Two key observations in Mucor cellular morphogenesis involve cAMP and phosphatidylinositol. These molecules are thought to play important roles in signal transduction pathways in eukaryotic cells and were of interest because of their potential regulatory roles in mediation of the morphogenetic response. The yeast cell of Mucor contains about four times the level of intracellular cAMP as hyphal cells. During yeast-to-hyphae morphogenesis, there is a large burst in cAMP synthesis (Larsen and Sypherd 1974). The cAMP levels increase during spherical growth of sporangiospores but drop prior to the emergence of germ tubes and remain low throughout further hyphal growth. This observation suggested a possible role for cAMP in sporangiospore germination. A change in the activity of adenylate cyclase, the enzyme responsible for cAMP synthesis, appears to be responsible for large variations in cAMP observed during morphogenesis (Orlowski 1980). During sporangiospore germination, adenylate cyclase activities increased but the cAMP phosphodiesterase activities remained constant (Orlowski 1980). The effect of cAMP on morphogenesis may be

mediated by cAMP binding proteins (Orlowski 1979, Orlowski 1980). In Mucor racemosus, cAMP binding protein activity was shown to decrease during yeast-to-hyphae morphogenesis (Forte and Orlowski 1980) and during spore germination (Orlowski 1980). A protein kinase activity may be involved in a cAMP sensing capacity (Pall 1981).

A similar study showed that there is a burst of lipid synthesis during yeast-to-hyphae morphogenesis of Mucor. More importantly, a large increase in phospholipid turnover was observed during morphogenesis, especially turnover of phosphatidylethanolamine and phosphatidylinositol. The rate of lipid turnover was inhibited by the addition of cerulenin which is a fatty acid synthesis inhibitor (Ito et al. 1982). Taken together, these data suggest that a signal transduction pathway involving adenylate cyclase, phosphodiesterase, cAMP binding protein and protein kinases may be linked to morphogenesis in Mucor. Phosphatidylinositol (PI) turnover may also be involved.

Three ras genes have been cloned previously from Mucor racemosus. These genes may encode proteins involved in morphogenesis in Mucor. Based on the above observations, we proposed the following hypothesis: if one or more Mucor ras genes are involved in regulation of cellular morphogenesis, the expression levels of the genes may correlate with

changes in morphology of M. racemosus. The objective of this study was to detect the expression levels of three Mucor ras transcripts, MRAS1, MRAS2 and MRAS3 using gene-specific DNA probes.

The long term goals of this study are to understand the nature of regulation of ras activity, the receptor and effector proteins which interact with ras, and the potential of ras mediated signalling in altering gene expression at the level of the nucleus. The yeast RAS2 gene, the model which is currently understood in the most detail, is unique among eukaryotic ras genes in regulation of cAMP metabolism. Mammalian ras genes appear not to be involved in this process. A new model system may provide new clues for mammalian ras function. The involvement of ras genes in PI turnover is possibly conserved during evolution, but the exact biochemical pathway is still not known. Mucor provides a system to elucidate the role of ras in PI turnover on a biochemical basis. The study of Mucor RAS genes could also increase the understanding of the biosynthetic pathway of the mammalian ras proteins. Other genes which are not yet identified may be involved in the processing of ras proteins. The activity and cellular localization of these processing enzymes may lead to the elucidation of a pathway for the process and transport of ras proteins to the plasma membrane.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions

The filamentous fungus Mucor racemosus ATCC 1216B was the source of DNA and RNA used in this research for cloning of ras genes and analysis of ras transcripts. Mucor cultures were maintained on YPG agar medium (2% wt./vol. glucose, 1% wt./vol. Bacto-peptone, 0.3% wt./vol. Bacto-yeast extract and 3% wt./vol. Bacto-agar). The pH of the medium was adjusted to 4.5 with H_2SO_4. Glucose was autoclaved separately from other components of the medium to prevent carmelization and browning of the medium.

A small quantity of pure spore suspension (5 μl of a frozen stored spore culture) was inoculated in the center of YPG agar medium (150 mm petri dish containing 45 ml YPG agar) and incubated at 28°C to produce sporulating cells and sporangiospores. During the incubation, the agar surface became covered with aerial hyphae. After 2 days of incubation, aerial hyphae (sporangiophores) with spore sacs (sporangia) were formed which contained immature sporangiospores. Sporangiophores, sporangia, and immature spores were harvested from these sporulating cultures by

pouring liquid nitrogen directly into the plate, scraping the frozen material into a sterile tube and storing at -70°C for later used. Other cultures were incubated for 7 to 10 days. At this time, the agar surface was completely covered with aerial hyphae bearing the sporangia containing grey-black mature sporangiospores. Mature sporangiospores were harvested with ice cold sterile distilled water by scraping the mycelium with a sterile glass rod, collected by centrifugation at 6,000xg for 10 min at 4°C, and stored at -70°C until RNA preparation. Alternatively, sterile glycerol (50% stock solution) was added to the spore suspension (15% final concentration) to retain viability for inoculation of growth media.

An aliquot of sporangiospores was inoculated into YPG liquid growth medium (2×10^5 spores/ml) and incubated at 28°C in a rotatory shaker water bath to prepare germinating spores and germlings. The culture was provided with 2 volumes of sterile air per volume of growth medium per minute. Germinating spores were harvested from the culture after 1 hour and 4 hours of incubation. Germling cultures were cooled down in a salt ice bath when germ tubes reached 10-12 spore diameters in length (ca. 12 hours). Other incubations of the 12 hour germlings were shifted to CO_2 and incubated at 20°C with shaking (200rpm) to produce germling-to-yeast transition cells. These were harvested after 1

hour and 3 hours incubation. The different morphologies of Mucor cells were collected by filtration through Whatman No. 1 filter paper. The cells were frozen immediately in liquid nitrogen and stored at -70°C until RNA extraction was carried out.

Yeast cells of Mucor were prepared by inoculating sporangiospores into YPG broth (2×10^5 spores/ml) and incubating with CO_2 gas bubbling through the culture at a flow rate of 0.5 volume of gas per vol of culture fluid per min and shaking at 28°C. The anaerobic conditions resulted in germination of spores to yeast cells and continued growth by budding of yeast cells. The doubling time of Mucor yeast cells is approximately 4 hours. After 21 hours of incubation, the culture reached mid-log growth phase (A_{600}=0.8). Yeast cells were harvested by filtration through Whatman No.1 filter paper and frozen in liquid nitrogen. The cells were stored frozen at -70°C for RNA preparation.

Yeast-to-hyphae transition cells were obtained by growing yeast cells in YPG liquid medium at 28°C with shaking under CO_2 until early log growth phase (A_{600}=0.22) was reached. The gaseous environment of the growing culture was then shifted to air. The culture was incubated at 28°C with shaking. Yeast-to-hyphae transition cells were collected at 3 hours of incubation, respectively. The cell

sample was frozen in liquid nitrogen and used directly for RNA extraction.

Bacterial Strains, Plasmids and Bacteriophage

E. coli K12 DH5α was the host strain for plasmid pUC19 (Yanisch-Perron et al. 1985, Roberts 1987), which was used in subcloning DNA fragments of *Mucor* RAS genes. *E. coli* K12 JM101 (Messing et al. 1981) was used to propagate the MRAS DNA fragments in bacteriophage M13mp18 and mp19 (Norrander et al. 1983).

Isolation and Purification of Plasmid and Bacteriophage DNA

Plasmid DNA was prepared by the procedure of Maniatis et al. (1982). A single colony of bacteria carrying the desired plasmid DNA was inoculated into 5 ml of rich medium (LB) containing 50 μg/ml of ampicillin and incubated at 37°C overnight with vigorous shaking. An aliquot (0.1 ml) of the overnight culture was inoculated to 25 ml of LB/ampicillin medium and incubated with shaking at 37°C until the culture reached late-log phase of growth (OD_{600}=0.6). This culture was transferred to 500 ml of LB/ampicillin medium and incubated for 2.5 hours at 37°C with shaking.

Chloramphenicol solution (34 mg/ml in ethanol) was added to this culture (final concentration 170 μg/ml) and the incubation continued overnight with shaking at 37°C. The bacterial cells were harvested by centrifugation (4,000xg at 4°C, Sorvall GSA rotor). Plasmid DNA was isolated by alkaline lysis of bacterial cells and purified by centrifugation (60,000xg for 12 hours at 20°C, Beckman VTI65-1 rotor)) to equilibrium on Cesium Chloride-Ethidium Bromide density gradients. The purified circular double-strand plasmid DNA was collected. Ethidium bromide was removed by extraction with isoamyl alcohol several times. DNA was desalted by dialysis using a membrane impermeable to proteins of Mr > 3,500 with several changes of TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) for 24 hours. The concentration of the DNA solution was quantitated by spectrophotometric measurement. A_{260}=1 unit corresponds approximately to 50 μg/ml for double-strand DNA. A pure preparation of DNA has an A_{260}/A_{280} ratio of 1.8.

A single plaque of Bacteriophage M13mp18 was inoculated into 10 ml of YT broth containing 5 μl of an overnight culture of JM101. At the same time, 100 μl of overnight culture of JM101 was inoculated into 100 ml of YT growth medium. Both cultures were incubated at 37°C with vigorous shaking for 3 hours. One ml of the phage-infected culture was then mixed with 100 ml of the JM101 culture to obtain a

multiplicity of infection of 1 to 10 and incubated for an additional 5 hours at 37°C with shaking. The cells were collected by centrifugation at 8,000xg (Sorvall SS-34 rotor) for 5 minutes at 4°C. Because the replicative form (RF) of bacteriophage M13 occurs in the bacterial cell as a circular double-strand DNA molecular, phage DNA was isolated, purified and quantitated by the same procedure as that for plasmid DNA.

Subcloning DNA Fragments of *Mucor* RAS Genes in Plasmid pUC19 for use as Gene Specific Probes

DNA restriction fragments containing the 3' hypervariable region of MRAS1 and MRAS3 genes were subjected to restriction endonuclease digestion of plasmids containing the MRAS genes. The restriction fragments were resolved by electrophoresis on a 1% agarose gel and purified by electroelution using an apparatus from International Biotechnology Instrument (IBI). Ligation was carried out as described by Maniatis et al. (1982). Vector DNAs (plasmid pUC19) were linearized by incubating the plasmid with restriction endonucleases which would generate ends complementary to the cloned MRAS DNA fragment. A 3:1 molar ratio of MRAS DNA fragment to 100 ng of linearized plasmid pUC19 DNAs were mixed with 2 μl of 10x ligation buffer (0.5

M Tris pH 7.5, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermidine, 10mM ATP, 1 mg/ml DNA enzyme grade bovine serum albumin) and 10 units of T4 DNA ligase. The mixture was made up to a final reaction volume of 20 µl with water. Two microliters of the preincubation mixture was saved for ligation analysis by 1% of agarose minigel. The reaction mixture was incubated at 16°C for 12-16 hours. After ligation, 2 µl of the reaction was removed to analyze for successful ligation by gel electrophoresis. The remaining ligation mixture was then diluted with TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) to 80 µl and saved for transformations.

Transformation of Plasmid and Bacteriophage into *E. coli*

E. coli DH5α was the host strain for plasmid DNA. Competent cells for plasmid transformation were prepared by the Calcium Chloride procedure (Hanahan 1983, Maniatis et al. 1982). A single colony of *E. coli* DHα grown on LB agar (Luria-Bertani, 1% wt./vol. Bacto-tryptone, 0.5% wt./vol. Bacto-yeast extract, 10 mM NaCl, pH 7.5 with 1.5% wt./vol. Bacto-agar) was inoculated into 3 ml of LB medium and incubated with shaking at 37°C overnight. The overnight culture was diluted 1/200 in LB medium and incubated with shaking at 37°C until mid log phase (OD_{590}=0.4-0.6. ca.2-3

hours). Cells were harvested by centrifugation at 7,000xg for 5 minutes at 4°C and resuspended in 1/5 volume of ice cold 0.1 M $MgCl_2$ and kept on ice for 15 minutes. The cells were then collected by centrifugation (7,000xg, 5 minutes at 4°C) and resuspended in 1/50 original volume of ice cold 0.1 M $CaCl_2$ and chilled in an ice bath for 60 minutes to generate competent cells. Samples of diluted (4-fold dilution) ligation mixture (5 µl and 20 µl) were added to separate tubes containing 100 µl of competent cells, and mixed by gently tapping the tubes. The transformation reaction was kept in an ice bath for 30 minutes allowing the cells to take up DNA. The transformed cells were heat shocked for 2 min at 42°C and then placed on ice for 2 min. Transformed cells were recovered by adding 1 ml of SOC (2% wt./vol. Bacto-tryptone, 0.5% wt./vol. Bacto-yeast extract, 10 mM NaCl, 25 mM KCl, 20 mM $MgSO_4/MgCl_2$ (2 M Mg^{++} filter sterilized stock), 20 mM Glucose (2 M filter sterilized stock); mixed and filter steriled) at rm. temp. and incubated with shaking at 37°C for 60 minutes. Aliquot (100 µl and 1 ml concentrated to 100 µl) of the transformed cell suspension were spread onto LB agar medium containing ampicillin (50 µg/ml) and incubated at 37°C overnight.

E. coli strain K12 JM101 was used as a recipient strain for transformation by M13mp18 and mp19 to propagate this bacteriophage. Cells were first grown on minimal agar

medium (500 ml of steriled 1.2% wt./vol. Na_2HPO_4, 0.6% wt./vol. KH_2PO_4, 0.1% wt./vol.NH_4Cl, pH 7.4 M9 salts combined with 500 ml of 3% autoclaved Bacto-agar; to this solution, 1 ml 1.0 M $MgSO_4$, 1 ml 0.1 m $CaCl_2$, 1 ml 1.0 M Thiamine-HCl and 5 ml 40% glucose were added) to ensure that the F episome of the bacteria was not lost. Competent cells of E. coli K12 JM101 were generated by a procedure similar to that for competent cells of E. coli DH5α. JM101 cells were grown on YT medium (0.8% wt./vol. Bacto-tryptone, 0.5% wt/vol. Bacto-yeast extract, 0.5% wt./vol NaCl and adjusted to pH 7.4, 1.5% wt./vol. Bacto-agar was added for agar plates before autoclaving). An aliquot (3 μl) of bacteriophage DNA was added to 100 μl of competent cells and incubated on ice for 40 minutes with occasional shaking. The cells were then heat shocked at 42°C for 2 minutes. One μl, 10 μl and 90 μl of the transformation reaction were added to 3 tubes containing 0.2 ml of plating culture which was generated by inoculating a single colony of JM101 into 3 ml of YT broth and incubating at 37°C until late-log phase of growth (OD_{550}=1.0). Molten YT medium (3 ml, 45°C) containing 0.1 ml 2% (wt.vol.) X-gal (5-bromo-4-chloro-3-indolylbeta-D-galactoside in dimethylformamide) and 0.2 ml 100 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) was added and quickly mixed in each tube. The mixtures were then poured onto YT agar medium. The plates were kept at room temperature for 15 minutes to allow the top agar to harden

and then incubated at 37°C overnight.

Radiolabeling DNA Restriction Fragments

DNA restriction fragments to be used as gene-specific DNA probes for hybridization were gel purified by electrophoresis on agarose gels and electroeluted from gel slices. DNA fragments were denatured by heating to 95-100°C in a sealed eppendorf tube for 5 minutes and immediately chilled to 0°C in an ice bath for 5 minutes. The random primer labeling procedure (Feinberg and Vogelstein 1983) was used to radiolabel the probes. The reaction was carried out by mixing H_2O (to a total volume of 50 ul) , 13 ul of oligo-labeling buffer (OLB: 10 μl mixture of 1.8% 2-mercaptoethanol :2 M Hepes:1 ug/ml hexadeoxyribonucleotides (2:5:3) and 3 μl of 1 mM dATP, dCTP and dTTP dissolved in TE buffer pH 7.0, 10 mM $MgCl_2$ mixture), 2 μl of 1 mg/ml solution of bovine serum albumin (molecular biology grade, BRL), 10-30 ng of denatured DNA, 5 μl of [α-^{32}P]dGTP (DUPONT/NEN research products, 3000Ci/mmol, 10 mCi/ml) and 2 units of large fragment of E. coli DNA polymerase I (Boehringer Mannheim Biochemicals; labeling grade Klenow fragment). The complete reaction was incubated at rm. temp. at least 2 hours. The reaction was stopped by adding 1 μl of 0.5 M EDTA. The labeled probes were purified by gel

exclusion column chromatography (Sephadex G50-80, 5 ml of packed volume) by elution with TE buffer (pH 8.0). The radiolabelled DNA was collected in the first peak detected with a Geiger counter. Two aliquots (5 ul) of eluted DNA were spotted onto glass-fiber filters. The DNA sample on one filter was precipitated with Trichloroacetic acid (TCA) (Maniatis et al. 1982). The specific activity of the labeled DNA was then quantitated by liquid scintillation spectroscopy (United Technologies, MINAXI TRI-CARB 4000 series scintillation counter). A high specific activity (for example 1 x 108 cpm/μg DNA) was achieved by using as little as 10 ng of DNA in each reaction.

Selection and Screening for the Presence of Recombinant Plasmids in E. coli Cells

The desired recombinant from a population of bacteria was isolated by genetic selection and in situ hybridization of bacterial colonies. The plasmid pUC19 carries an ampicillin resistance gene and a multiple cloning site within the β-galactosidase gene. It is therefore possible to screen E. coli cells for the presence of a recombinant plasmid by selecting for resistance to the antibiotic ampicillin and insertional inactivation of the β-galactosidase gene. Bacteria which carry an active β-

galactosidase gene on plasmid pUC19 can hydrolyze the chromogenic substrate, X-gal, and generate a blue colony. A DNA fragment inserted into the β-galactosidase gene of pUC19 inactivates the gene. Cells containing these recombinant plasmids appear white on medium supplemented with X-gal.

For in situ colony hybridization (Grunstein and Hogness 1975, Maniatis et al. 1982.), randomly selected bacterial colonies suspected to carry recombinant plasmids were spotted by a toothpick onto a nitrocellulose (N.C) filter (Schleicher & Schuell) which was placed on LB/ampicillin agar medium and incubated at 37°C overnight. The filter with bacterial colonies was placed with the colony side up on a sheet of 3MM filter paper saturated with 10% SDS (sodium dodecyl sulfate) solution for 5 minutes to limit the size of hybridization signal and then transferred onto another sheet of 3MM filter paper saturated with base solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes to denature the DNA. The filter was transferred to a third sheet of 3MM filter paper saturated with neutralizing solution (1 M Tris-Cl pH 8.0, 1.5 M NaCl) for 10 minutes and then transferred onto a 3MM filter paper saturated with 2x SSC solution for 10 minutes.. The filter was air dried for 60 minutes and baked at 80°C for 2 hours under vacuum. The DNA on the filter was hybridized with a gene-specific probe to detect recombinant clones.

Purification of mRNA from M. racemosus

RNA from Mucor cells of several morphologies was prepared by the procedure of Maramatsu (1973). The glasswares used for RNA purification were treated with 0.1% of diethylpyrocarbonate (DEPC) and baked at 250°C for 12 hours. Cells were placed into a sterile mortar containing liquid nitrogen and broken by grinding with a pestle for 10 minutes. The frozen cell material was transferred to an ice cold 30 ml Corex tube and resuspended with 1 ml of cold SDS-RNA-extraction buffer (50 mM sodium acetate, 1.0 mM EDTA and 1% SDS; adjusted to pH 5.0 with glacial acetic acid and treated with DEPC). The suspension was then mixed with an additional 4 ml of SDS-RNA-extraction buffer warmed to 65°C followed immediately by 5 ml of hot phenol (65°C saturated with buffer, containing 0.1% wt./vol. 8-hydroxyxyquinoline and 0.2% β-mercaptoethanol). The samples were vortexed for 30 seconds. The mixture was placed at 65°C for 15 minutes. The aqueous phase containing the RNA was recovered and extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then with 1 volume of diethyl ether (water saturated). The aqueous phase was then mixed with 1/6 volume of 3 M sodium acetate followed by an additional 2.5 volumes of ethanol. The RNA was allowed to precipitate by storing the solution at -20°C overnight. It was recovered by centrifugation at 5,000xg for 10 minutes at 4°C. The

pellet was washed with 75% ethanol containing 0.1 M sodium acetate (pH 7.5). The purified total RNA was dissolved in TE buffer (pH 8.0). The concentration and purity of RNA were measured by spectrophotometric determination. An A_{260} of 1 corresponds to approximately 40 ug/ml of RNA. A pure preparation of RNA has an A_{260}/A_{280} ratio of 2.0.

Polyadenylated RNA was separated from nonpolyadenylated RNAs by oligo(dT)-cellulose chromatography (Edmonds et al. 1971, Aviv and Leder 1972, Maniatis et al. 1982). Dry powder oligo(dT)-cellulose (0.25 g) was used to pack a 1 ml column for 10 mg of total RNA. The column was prepared by equilibrating the oligo(dT)-cellulose powder in sterile loading buffer (20 mM Tris-Cl, pH 7.6; 0.5 M NaCl, 1 mM EDTA and 0.1% SDS). The solution was then poured into a sterile silanized pasteur pipette plugged with silanized glass wool. The column was packed by washing with 3 column-volumes each of sterile water, 0.1 M NaOH and 5 mM EDTA solution, and sterile water (until the pH of the column effluent was less than 8) and then with 5 volumes of sterile loading buffer. The RNA sample was dissolved in sterile water and heated to 65°C for 5 minutes following the addition of an equal amount of 2x loading buffer. The sample was cooled to rm. temp. and loaded onto the column. The effluent was collected, heated to 65°C, cooled and reapplied to the column. The column was then washed with 5-10 column-volumes of loading

buffer followed by 4 column-volumes of loading buffer containing 0.1 M NaCl. The poly(A)$^+$RNA was eluted with 2-3 column-volumes of sterile elution buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA and 0.05% SDS). Samples containing poly(A)$^+$RNA were pooled and sodium acetate (3 M, pH 5.2) was added to a final concentration of 0.3 M. The RNA was allowed to precipitate with the addition of 2.2 volumes of ethanol and stored at -20°C overnight. It was recovered by centrifugation (5,000xg,4°C for 10 min) and dissolved in sterile water (DEPC-treated).

Restriction Endonuclease Analysis and Gel Electrophoresis

Restriction endonuclease digestions of DNA were carried out according to the optimal reaction conditions as described by manufacturers. DNA restriction fragments were separated by gel electrophoresis. Gels varied in percentage of agarose (0.8%-1.0%) depending on the size of DNA fragments under analysis. A Tris-acetate buffer system (TAE: 0.04 M Tris-acetate, 0.02 M EDTA) (Maniatis et al. 1982) containing 0.5 μg/ml of the fluorescent dye ethidium bromide (Sharp et al. 1973) was used to make up agarose gels and for the electrophoresis buffer to stain the separated DNA fragments. Alternatively, 0.5 μg/ml ethidium bromide solution was applied upon completion of electrophoresis in a

45 min soak. DNA fragments separated in the gel were visualized with a short wave (254 nm) UV-light transilluminator and photographed through a yellow filter with Polaroid type 667 film.

RNA samples were fully denatured by treating the sample with formaldehyde and formamide and resolved by electrophoretic separation through 1% to 1.5% formaldehyde-agarose gels (Maniatis et al. 1982, Fourney et al. 1988) with a MOPS/EDTA buffer system (10x stock solution: 0.2 M MOPS (3-(N-morpholinolino)-propanesulfonic acid), 50 mM sodium acetate, 10 mM EDTA, pH 7.0; treated with 0.1% DEPC and autoclaved). After electrophoresis, one lane on the agarose gel containing a commercial size marker RNA ladder (Bethesda Research Labs) was separated from the other RNA samples and post-stained with 0.5 μg/ml ethidium bromide in 10 mM $MgSO_4$ solution for 30 minutes. The RNA ladder was photographed on a short wave UV-light (254 nm) transilluminator through a yellow filter with Polaroid type 667 film. The formaldehyde in the sample gel was removed by soaking the gel in 1 mM $MgSO_4$ solution for 60 minutes.

Southern and Northern Analyses of Nucleic Acid

Similarities between particular sequences of DNA were analyzed by Southern transfer and hybridization (Southern 1975, Southern 1980, Maniatis et al. 1982). DNA fragments were separated by electrophoresis through agarose gels and denatured by soaking the gel in several volumes of base solution at room temperature for 60 minutes. The gel was then neutralized with 3 changes of the neutralization solution with constant shaking at room temperature for 60 minutes. Denatured DNA fragments which were greater than 300 base pairs in size were generally transferred and immobilized to a nitrocellulose filter (Schleicher & Schuell). A Nylon membrane filter (Schleicher & Schuell, Nytran) was used to immobilize smaller DNA fragments. A 10x SSC buffer system was used for transfer of DNA by capillary action and allowed to proceed for 12 to 24 hours. The filter with bound DNA was air dried for 60 minutes. The DNA on the filter was then immobilized by baking the filter at 80°C under vacuum. Alternatively, DNA was bound on the nylon filter covalently by exposing the DNA on the filter to a short wave (254 nm) UV transilluminator for 5 minutes.

The RNA was transferred to nitrocellulose filters or nylon membranes with 10x SSC by capillary action for 12 to 24 hours, and fixed to the membrane by baking for 2 hours at

80°C under vacuum or by placing nylon membranes with the RNA side face on a short wave (254 nm) transilluminator for 5 minutes.

Southern and Northern blots for hybridization were soaked in prehybridization solution containing deionized formamide (40% for low stringency hybridization, 50% for high stringency), 5x Denhardt solution (5% ficoll, 5% bovine serum albumin, 5% polyvinyl-pyrrolidone), 6x SSC solution, 100 μg/ml denatured salmon testis carrier DNA and 5 mM EDTA for 2 to 4 hours with shaking at rm. temp. For Southern analysis, 1-5 x 10^5 cpm/ml of a radiolabeled gene-specific DNA probe was then added to the prehybridization solution. For Northern analysis, 5 x 10^5 cpm/ml to 1 x 10^6 cpm/ml of radiolabeled gene-specific probe in hybridization fluid was utilized. The hybridization was carried out at 37°C (low stringency) or 42°C (high stringency) in a shaking water bath for 12 to 36 hours. Following hybridization, the filters were washed twice in 2x SSC/0.1% SDS washing solution at room temperature for 15 minutes followed by 0.1x SSC/0.1% SDS washing solution for 60 minutes at 42°C for a low stringency wash or 65°C for a high stringency wash. The filters were air dried and sealed with plastic wrap and exposed to Kodak XAR5 diagnostic film with or without an intensifier screen for 2 hours to several days depending on the intensity of hybridization signal. The image of the

hybridization signal on X-ray film was developed by soaking the film in Kodak developer for 5 minutes and a Kodak rapid fixer for 5 minutes.

Image Analysis of Autoradiography

The image of Southern and Northern hybridization signals on autoradiographs was analyzed by a video densitometer (Biomed Instruments, Inc.). The intensity of these hybridization signals was integrated as the scanned area of hybridization signal in a unit of pixel square.

Dot Matrix analysis of Nucleotide sequences

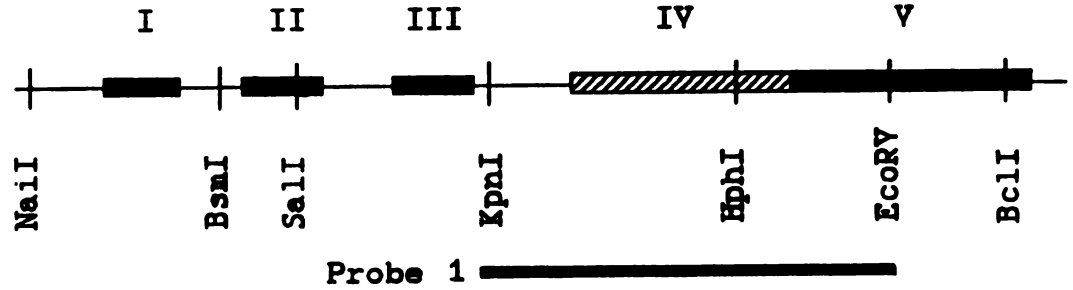
The similarity of nucleotide sequences was compared by a computer assisted dot matrix analysis. This method was developed by Dr. George Gutman and Brian Ward, University of California, Irvine.

RESULTS

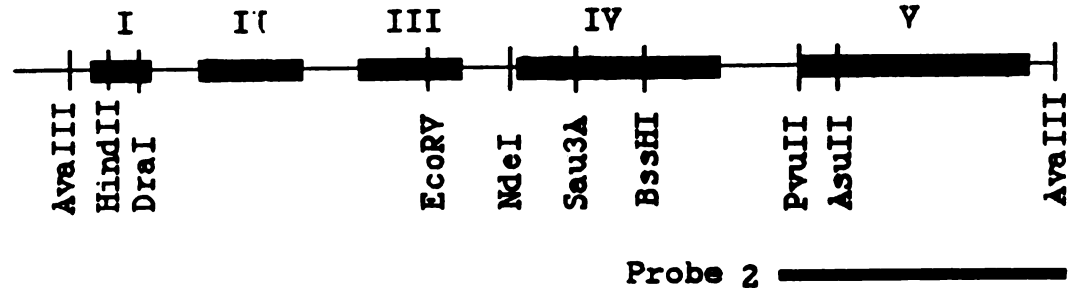
The research hypothesis of this study is that if one or more Mucor racemosus genes (MRAS) are involved in regulation of morphogenesis, the expression levels of the genes may correlate with changes in morphology. Therefore, it was necessary to specifically measure the expression of individual MRAS genes. The nucleotide sequences of 3 MRAS genes have been previously determined. Based on these data we were able to generate restriction maps of MRAS1, MRAS2 and MRAS3 and to predict the intron/exon structures of these genes (Figure 1). The nucleotide sequence data were compared to select regions among the 3 MRAS genes with a low degree of similarity. A sequence comparison was conducted using computer assisted dot matrix analysis and was based on a 8 bp match in a 10 bp window (80% stringency). The hypervariable region of the MRAS genes with less than 20% sequence similarity was identified in exon V located at the 3' end of each gene. Because of difficulty in identifying and purifying DNA restriction fragments containing only the hypervariable region (exon V) of each MRAS gene, longer restriction fragments which included part of exon V of each MRAS gene along with additional DNA sequences were selected to prepare DNA probes for analysis of MRAS transcript levels.

Figure 1. Predicted intron/exon structure and restriction maps of Mucor racemosus MRAS1, MRAS2 and MRAS3 genes. Solid and hatched segments represent the predicted exons.Probe 1, probe 2 and probe 3 were used for detection of MRAS gene transcripts. Probe 1, 310 bp KpnI/EcoRV restriction fragment (MRAS1-1). Probe 2, 580 bp PvuII/BamHI (BamHI site not shown) restriction fragment (MRAS2-0). Probe 3, 450 bp SacI/HindIII restriction fragment (MRAS3-21).

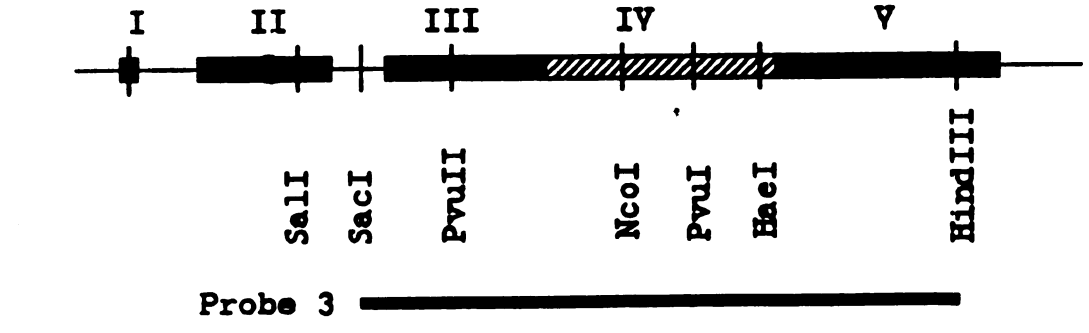
HRAS1



HRAS2



HRAS3



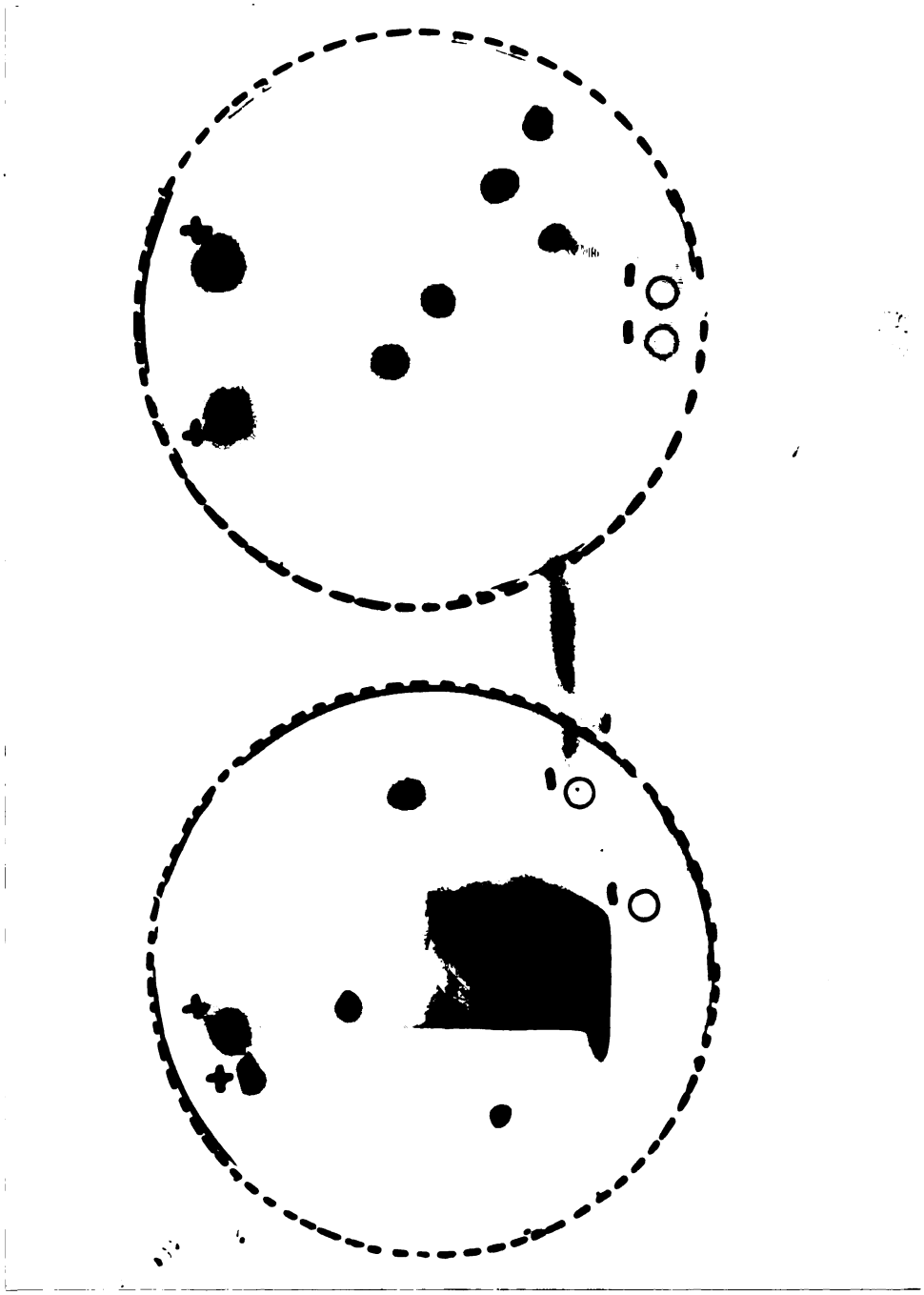
Subcloning DNA Restriction Fragments of MUCOR RAS Genes into Plasmid pUC19

MRAS1. A 310 base pair KpnI/EcoRV restriction fragment containing a portion of the predicted hypervariable region of exon V and all of exon IV of the MRAS1 gene was subcloned into the plasmid vector pUC19 which was linearized with the complementary restriction endonucleases KpnI/SmaI. The resulting recombinant plasmid was transformed into E. coli strain DH5α. The transformants were screened by the in situ colony hybridization technique using the 310 bp KpnI/EcoRV DNA restriction fragment of MRAS1 gene (Figure 1) as a DNA probe. Eight of 72 bacterial colonies screened were found to have a recombinant plasmid containing the 310 KpnI/EcoRV restriction fragment of the MRAS1 gene (Figure 2).

MRAS2. A 580 bp PvuII/BamHI restriction fragment containing the entire exon V of MRAS2 gene and 3' 300 bp of flanking sequence was cloned into bacteriophage M13mp18. This subcloned restriction fragment had been used for analysis of the MRAS2 nucleotide sequence (contributed by Dr. William Casale).

MRAS3. A 450 bp SacI/HindIII restriction fragment, which contains the predicted exon III, IV and most of the exon V hypervariable region of MRAS3 (Figure 1) was also

Figure 2. In situ colony hybridization analysis of colonies transformed with MRAS1-1. Bacterial colonies transformed with recombinant plasmids were screened by using the 310 bp KpnI/EcoRV DNA restriction fragment of MRAS1 gene as gene-specific probe. Nitrocellulose filter was washed under low stringency conditions (0.1XSSC/0.1%SDS solution at 42°C for 60 minutes). The filter was then exposed to X-ray film without an intensifier for 4 hours at rm temp. Colonies marked + represent the positive control bacteria carrying recombinant plasmid containing the MRAS1 gene. Colonies marked - represent negative control bacteria carrying plasmid pUC19 only.



cloned into plasmid pUC19 which had been linearized by SacI/HindIII restriction endonucleases to generate complementary ends. The recombinant plasmid was transformed into E. coli DH5α. Transformants containing the recombinant plasmid were screened by colony hybridization using a 450 bp SacI/HindIII fragment of MRAS3 gene (Figure 1) as a probe. Selected transformants (72 colonies) were screened and 8 clones showed positive hybridization (Figure 3).

Analysis of Subcloned Fragments from MRAS1-1, MRAS2-0 and MRAS3-21

Recombinant plasmids containing the subcloned MRAS1 DNA fragment were prepared from several transformants by the alkaline lysis miniprep procedure and analyzed by restriction endonuclease digestion with KpnI/BamHI and EcoRI/BamHI. In the correct plasmid construct, these pairs of enzymes were predicted to generate 310 bp and 330 bp restriction fragments, respectively. These restriction endonuclease fragments were resolved by agarose gel electrophoresis (Figure 4). Only two transformant clones carried a recombinant plasmid containing a 310 bp KpnI/BamHI fragment (BamHI site located in the polylinker region of the plasmid) or a 330 bp EcoRI/BamHI fragment (both restriction sites located within the polylinker region of plasmid). One

Figure 3. In situ colony hybridization analysis of colonies transformed with MRAS3-21. *E. coli* DH5α cells were transformed with a recombinant plasmid containing a 450 bp SacI/*Hind*III restriction fragment of MRAS3 gene and screened by using a 450 bp *Sac*I/*Hind*III DNA restriction fragment as DNA probe. Nitrocellulose filter was washed under low stringency conditions (0.1XSSC/0.1%SDS solution 42°C for 1 hour). The washed filter was then exposed to X-ray film for 4 hours without an intensifier at rm. temp. Colonies marked + represent transformants containing the MRAS3 gene as a positive control. Colonies marked - represent negative control bacteria carrying plasmid pUC19 only.

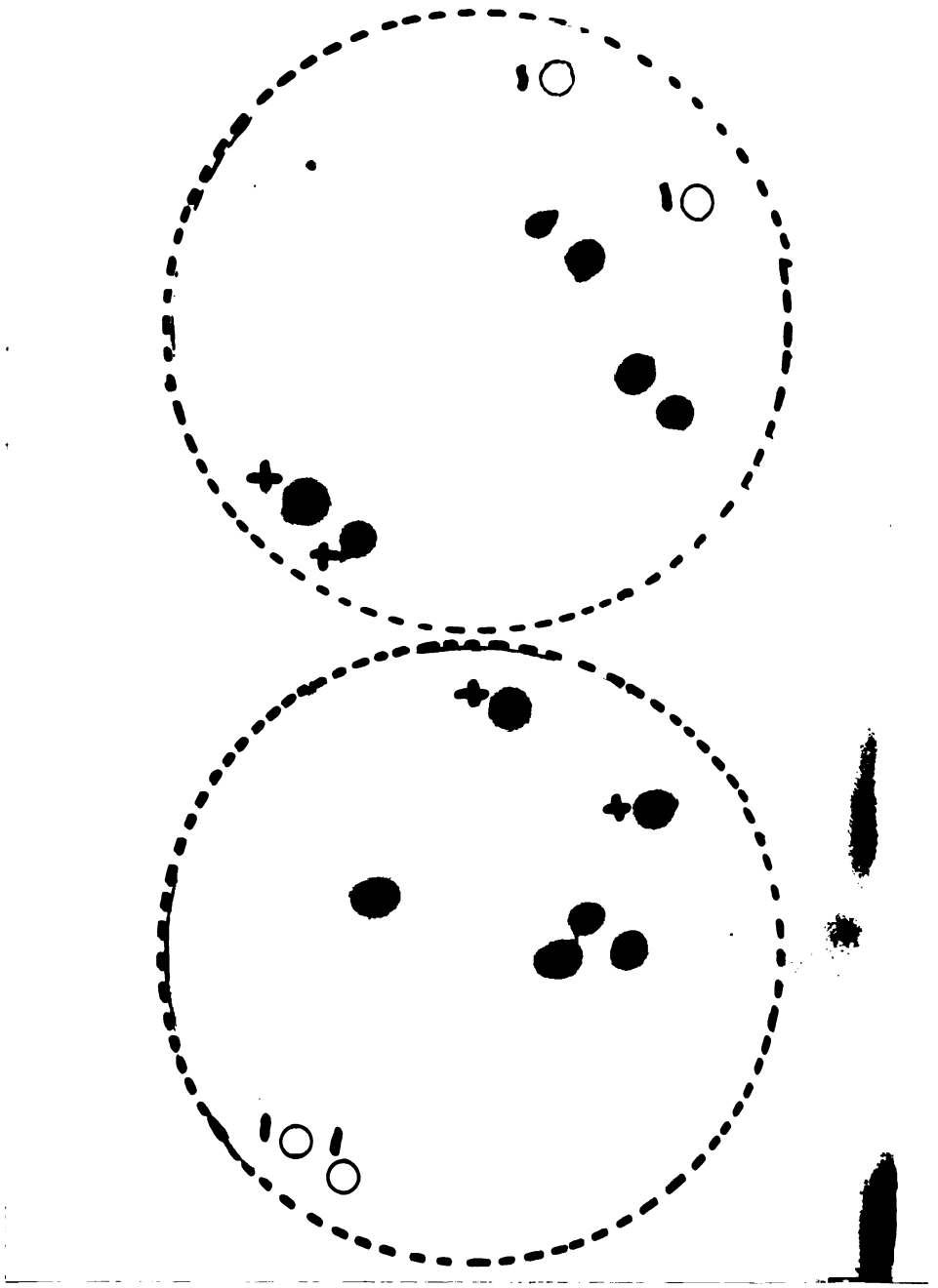
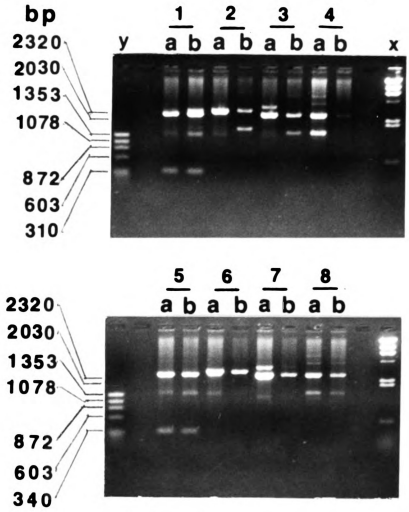


Figure 4. Restriction endonuclease analysis of eight transformants carrying recombinant plasmids containing the subcloned MRAS1 DNA fragment. Plasmid constructs from transformants (1 through 8) were digested with restriction endonucleases KpnI and BamHI (lane a) or EcoRI and BamHI (lane b), respectively, and the DNA fragments resolved on a 1% agarose gel. Lane x: Lambda DNA-HindIII digest size marker; lane y: PhiX174 RF DNA-HaeIII digest size marker (New England BioLabs).


bp
2320
2030
1353
1078
872
603
310
y
1
2
3
4
a b a b a b a b
x
5
6
7
8
a b a b a b a b
2320
2030
1353
1078
872
603
340

transformant was selected and large amounts of recombinant plasmid were prepared by cesium chloride density gradient centrifugation. The resulting recombinant plasmid was digested with KpnI and BamHI restriction endonucleases in combination to produce a 310 bp restriction fragment (Figure 6a). Southern analysis of this blot showed the presence of a 310 bp KpnI/BamHI restriction fragment hybridized with a DNA probe prepared from MRAS1 KpnI/EcoRV restriction fragment (figure 6a). This experiment demonstrated that the 310 bp KpnI/EcoRV restriction fragment was subcloned in plasmid pUC19 and transformed into E. coli DH5α. This recombinant plasmid containing the subcloned DNA fragment of MRAS1 gene was assigned the name MRAS1-1.

A 580 bp PvuII/BamHI DNA restriction fragment containing exon V and about 300 bp of flanking DNA from MRAS2 (Figure 1) was previously subcloned into bacteriophage M13mp18 (W. Casale unpublished data). E. coli JM101 was infected with this recombinant phage to propagate the phage DNA. Restriction endonuclease analyses (EcoRI and BamHI; EcoRI site located in polylinker region of Mp13mp18) were used to confirm the structure of the recombinant phage (Figure 6b). The size of the restriction fragment was predicted to be about 600 bp (20 bp were added from polylinker region), but the resolved restriction fragment appeared slightly larger than 600 bp, possibly due to the

Figure 5. Restriction endonuclease analysis of eight transformants carrying recombinant plasmids containing the subcloned MRAS3 fragment. Three different double restriction endonuclease digestions, EcoRI and HindIII (lane a), PvuII and HindIII (lane b) or PvuI and EcoRI (lane c) were conducted and the DNA fragments resolved on a 1% agarose gel to analyze recombinant plasmids prepared from 8 MRAS3 subclone transformants (1 through 8). Lane x: Lambda DNA-HindIII digest size marker; lane y: PhiX174 RF DNA-HaeIII digest size marker.

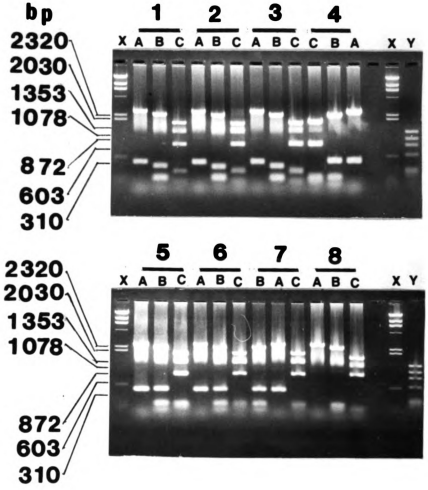
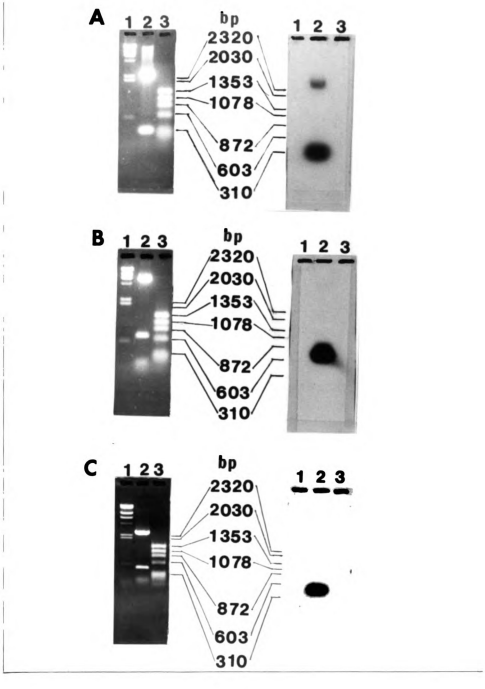

bp
2320
2030
1353
1078
872
603
310
1
2
3
4
X A B C A B C A B C C B A X Y
5
6
7
8
X A B C A B C B A C A B C X Y

Figure 6. Southern analyses of plasmid DNAs containing MRAS1 and MRAS3 DNA fragments, and bacteriophage DNA containing an MRAS2 DNA fragment. Panel A, an MRAS1 recombinant plasmid was digested with SacI and HindIII and a 310 bp fragment was generated. Panel B, an MRAS2 phage clone was digested with EcoRI and BamHI and a DNA fragment slightly larger than 600 bp was generated. Panel C, a 460 bp EcoRI and HindIII restriction fragment was generated by digest MRAS3 recombinant plasmid. These restriction digests were resolved on 1% agarose gel and photographed by transillumination with short wave UV-light. Photographs of the gels are shown on the left in panel A, B, C. The MRAS1 310 bp KpnI/EcoRV fragment, the MRAS2 600 bp EcoRI/BamHI fragment and the MRAS3 450 bp SacI/HindIII fragment were used to prepare DNA probes and hybridized to individual blots in panel A, B, C respectively. The nitrocellulose filters were washed under low stringency condition (0.1XSSC/0.1%SDS at 40°C for 60 minutes). The washed filters were exposed to X-ray film without an intensifier for 2 hours at rm.temp. The autoradiographs are shown on the right side of each panel.


A
1 2 3
bp
2320
2030
1353
1078
872
603
310
1 2 3
B
1 2 3
bp
2320
2030
1353
1078
872
603
310
1 2 3
C
1 2 3
bp
2320
2030
1353
1078
872
603
310
1 2 3

co-migration of restriction endonucleases with the DNA restriction fragment resulting in gel retardation. In order to ensure this restriction fragment was the subclone from MRAS2 gene, Southern analysis was conducted on DNA transferred from this gel to a nitrocellulose filter using the 580 bp PvuII/BamHI fragment as the probe. This restriction fragment of the MRAS2 gene (Figure 1) hybridized to the EcoRI/BamHI fragment released by digestion of the M13 clone (Figure 6b). The recombinant bacteriophage M13mp18 containing the subcloned PvuII/BamHI restriction fragment of MRAS2 was named MRAS2-0.

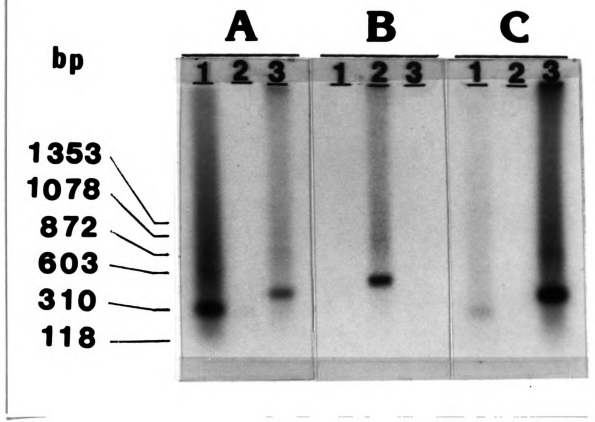
Transformant clones containing the subcloned SacI/HindIII fragment of the MRAS3 gene were analyzed using a procedure similar to that described for the MRAS1-1 subclone. Three different double restriction endonuclease digestions conducted on the recombinant plasmids demonstrated that all clones contained the expected restriction fragment (Figure 5). One transformant was selected to prepare large quantities of the recombinant plasmid. The resulting recombinant plasmid was analyzed by digestion with EcoRI and HindIII endonucleases. A 460 bp EcoRI/HindIII restriction fragment (EcoRI site located in the polylinker region of plasmid) was generated (Figure 6c). In Southern blot analysis, this 460 bp EcoRI/HindIII restriction fragment hybridized with a DNA probe (Figure 6c)

prepared from the SacI/HindIII restriction fragment of MRAS3 gene demonstrating that the 450 bp SacI/HindIII restriction fragment (Figure 1) had been ligated into plasmid pUC19 and transformed into E. coli DH5α. This plasmid containing the subclone of the MRAS3 gene was named MRAS3-21.

Determining the Similarity between the Three Mucor RAS Gene Subclones-MRAS1-1, MRAS2-0 and MRAS3-21

In order to minimize the risk of cross-hybridization in the analysis of MRAS gene expression using MRAS1-1, MRAS2-0 and MRAS3-21 as gene-specific probes, it was necessary to examine the similarity of these three DNA restriction fragments in advance. The subcloned restriction fragments from MRAS1-1, MRAS2-0 and MRAS3-21 were purified by agarose gel electrophoresis followed by electroelution. The purified DNA fragments were resolved in triplicate on a agarose gel and subjected to Southern hybridization analysis using MRAS1-1, MRAS2-0 and MRAS3-21 as DNA probes (Figure 7). The MRAS1-1 and MRAS3-21 subclones cross-hybridized to each other to a limited extent. The hybridization signals were analyzed by scanning the autoradiograph with a densitometer. The strength of cross-hybridization of MRAS1-1 to MRAS3-21 was only about 12% of the specific binding of the MRAS1-1 probe to itself; ie. the strength of MRAS3-21

Figure 7. Nucleotide sequence similarity between the three Mucor ras gene subclones. The subcloned restriction fragments from MRAS1-1 (sample 1), MRAS2-0 (sample 2) and MRAS3-21 (sample 3) were purified by gel electrophoresis and resolved in triplicate on an agarose gel and subjected to Southern blot hybridization using MRAS1-1 (panel A), MRAS2-0 (panel B) and MRAS3-21 (panel C) as DNA probes. Nitrocellulose filters were washed under high stringency conditions (0.1XSSC/0.1%SDS solution 65°C for 60 minutes) and then exposed to X-ray film for 2 hours without intensifier at rm temp. Autoradiographs resulting from these Southern analyses are shown.


A
B
C
bp
1 2 3
1 2 3
1 2 3
1353
1078
872
603
310
118

specific binding was more than 8 fold greater than cross-hybridization to MRAS1-1 (Table 1). According to the nucleotide sequence data, the hypervariable region of the MRAS genes is located in exon V of each gene. The MRAS1-1 restriction fragment contains the entire region of exon IV and approximately half of exon V. The MRAS3-21 restriction fragment includes exon III, exon IV and most of exon V. The cross-hybridization of these two restriction fragments is likely to be due to the higher similarity between MRAS1-1 and MRAS3-21 in exon IV. Based on computer assisted dot matrix analysis (80% stringency) data, a 50 bp fragment of MRAS1-1 and a 90 bp fragment of MRAS3-21 showed the least similarity between each other. Both fragments were located within exon V of each gene. As expected, MRAS2-0 did not cross-hybridize to either MRAS1-1 or MRAS3-21 because this restriction fragment contains only exon V and a 3' segment of flanking sequence. The Southern hybridization data and dot matrix data showed good agreement and suggested that the MRAS probes would be useful for preliminary analyses of MRAS transcript levels. It was believed that these probes would not cause significant artifacts in detecting MRAS gene transcript levels and would differentiate among transcripts of the three MRAS genes.

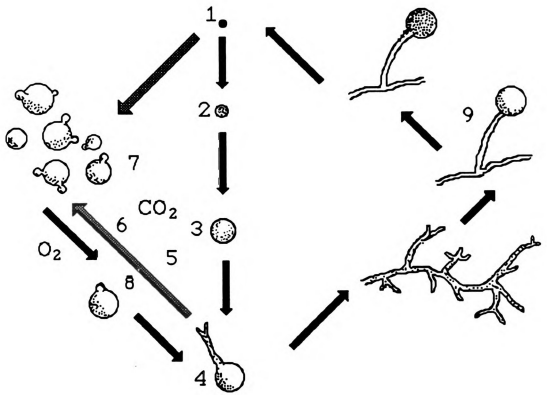
Table 1. Analysis of cross-hybridization of MRAS probes. Columns contain data for DNA probes used for hybridization. Column A, MRAS1-1 DNA fragment; probe 1. Column B, MRAS2-0 DNA fragment; probe 2 and column C, MRAS3-21 DNA fragment; probe 3. Numerical values represent integrated area of absorbance peak measured in square pixels where one pixel represents a unit information resolved by densitometer from the densitometer scan of the autoradiographs shown in figure 7. Rows contain data from the target DNAs on the nitrocellulose filter DNA samples. Row 1, target DNA fragment MRAS1-1; Row 2, MRAS2-0 as target DNA; and Row 3, MRAS3-21 as target DNA.

	A	B	C
1	3842	-	495
2	-	3741	-
3	574	-	4019

Northern Analysis of M. racemosus RAS Transcript levels with DNA Probes

MRAS1-1, MRAS2-0 and MRAS3-21 were used to prepare hybridization probes to analyze the Mucor ras gene transcripts in this study. The DNA probes MRAS1-1, MRAS2-0 and MRAS3-21 were labeled with [α-^{32}P]dGTP to a high specific activity (1-5 x 10^8 cpm/μg DNA). These three DNA probes were used to analyze the accumulated levels of ras mRNA from several cell morphologies of M. racemosus (Figure 8) to determine whether there was a morphology-related change in expression of Mucor ras genes. mRNAs were purified from immature spores and sporangiophores (sporulating cells), sporangiospores, germinating spores (1 hour and 4 hours incubation), germlings (12 hours germination), germling-to-yeast transition cells (1 hour and 3 hours), yeast cells, and yeast cells which has been induced to undergo morphogenesis to hyphae (see materials and methods). Polyadenylated RNA was isolated from these different morphologies of M. recemosus, resolved on formaldehyde-agarose gels and transferred to nitrocellulose filters. The probes described above were used to hybridize to the RNA samples on filters under high stringency conditions.

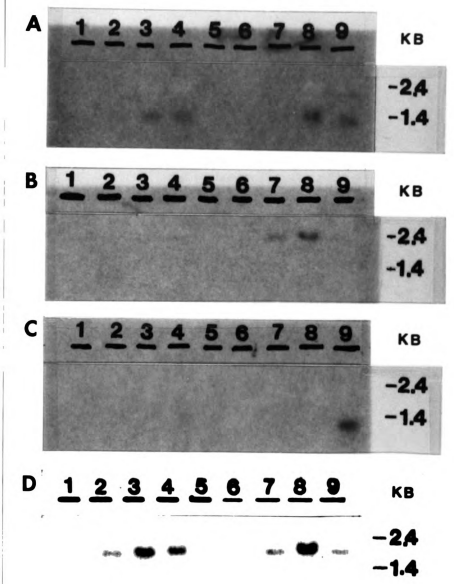
Figure 8. Morphologies of Mucor racemosus selected for isolation of poly(A)$^+$-RNA to be used in Northern analysis. Solid segments represent aerobic growth conditions and hatched segments represent anaerobic processes. 1, sporangiospores; 2, one hour-old germinating spores; 3, four hour-old germinating spores; 4, twelve hour-old germlings; 5, germling-to-yeast one hour transition cells; 6, germling-to-yeast three hours transition cells; 7, yeast cells; 8, yeast-to-hyphae transition cells, and 9, sporangiophores, sporangia and immature spores (sporulatintg cells).


1
2
3
4
5
6
7
8
9
CO_2
O_2

The pattern of transcript accumulation detected by each probe was first quantitated visually and differed significantly between the different probes. The MRAS1-1 probe hybridized at detectable levels to transcripts from several different morphologies of Mucor mRNA (Figure 9). The MRAS1 gene appeared to be expressed specifically in germinating spores (4 hours of incubation), germlings, yeast-to-hyphae transition cells and sporulating cells. The size of the MRAS1 transcript detected in these morphologies was 1.3 Kb. The transcript which hybridized to MRAS2-0 probe was found in yeast and yeast-to-hyphae cells and was about 2.3 Kb in size (Figure 9). The MRAS3-21 probe hybridized only to a 1.3 Kb transcript in sporulating cells (Figure 9). The MRAS3 transcript detected in sporulating cells is not likely to be due to cross-hybridization of MRAS3-21 probe to the MRAS1 transcript because the pattern of transcript accumulation of MRAS1 and MRAS3 is different. Moreover, the accumulated level of MRAS3 transcript in sporulating cells is similar to that of the MRAS1 transcript (Table 2), which would most likely not be the case if the signal was due to cross hybridization.

In a control experiment, the identical blots were hybridized with a TEF-1 probe (Figure 9). This gene encodes elongation factor-1α in M. racemosus and is expressed at constant levels in 4 of the morphologies tested here (ie.

Figure 9. Northern analysis of M. racemosus poly(A)$^+$-RNA with MRAS DNA probes. Poly(A)$^+$-RNA was isolated from several different morphologies of Mucor cells, resolved on formaldehyde-agarose gels, and blotted to nitrocellulose filters. Lane 1, polyadenylated RNA from sporangiospore; Lane 2, germinating spores (1 hour); Lane 3, germinating spores (4 hours); Lane 4, germlings; Lane 5, germling-to-yeast cells (1 hour); Lane 6, germling-to-yeast cells (3 hours); Lane 7, yeast cells; Lane 8, yeast-to-hyphae cells, and Lane 9, sporulating cells. MRAS DNA probes were radiolabelled and used to probe Northern blots: Panel A, MRAS1-1 probe; Panel B, MRAS2-0 probe; Panel C, MRAS3-21 probe. Panel D was probed with a TEF-1 probe. Shown are autoradiographs from each hybridization reaction. Nitrocellulose filters were washed 0.1XSSC/0.1%SDS solution at 65°C for 60 minutes (high stringency condition). Blots A, B and C were exposed to X-ray film with an intensifier at -70°C for 72 hours. Blot D was exposed to X-ray film with an intensifier at -70°C for 16 hours.


A
1 2 3 4 5 6 7 8 9
KB
-2.4
-1.4
B
1 2 3 4 5 6 7 8 9
KB
-2.4
-1.4
C
1 2 3 4 5 6 7 8 9
KB
-2.4
-1.4
D
1 2 3 4 5 6 7 8 9
KB
-2.4
-1.4

sporangiospores, germlings, yeast, yeast-to-hyphae 3 hours transition cells, Linz and Sypherd 1987). In the control experiment, the TEF-1 probe hybridized at significant levels to a 1.5 Kb transcript in germinating cells (1 and 4 hours), germlings, yeast cells, yeast-to-hyphae transition cells and sporulating cell mRNAs and at reduced levels with the same size transcript germling-to-yeast (3 hours of incubation) transition cells. No transcript was detected in sporangiospores and germling-to-yeast (1 hour) transition cells with the TEF-1 probe. This suggests that either no TEF-1 transcript is present at this stage of growth or that the mRNA in this sample was degraded. Since no transcript was detected in RNA from sporangiospores or germling-to-yeast (1 hour) transition cells using the MRAS probes, this may be due to artifacts arising from absence of detectable levels of mRNA in these samples. The control experiment suggests that the quantities of mRNAs from each morphological stage used in the Northern analysis of MRAS genes transcript were not constant (Table 2), especially in the sample from germling-to-hyphae (1 hour) cells. Further work is needed to normalize mRNA quantities for each sample using the accumulated levels of the TEF-1 transcript as the control level.

Table 2. Analysis of Northern hybridization of Mucor RNA with DNA probes. Columns contain data from Mucor poly(A)$^+$-RNA samples isolated from different morphologies of Mucor cells used in Northern analysis of figure 9. Column 1, sporangiospore; 2, germination spores (1 hour); 3, germination spores (4 hours); 4, germlings; 5, germling-to-yeast (1 hour); 6, germling-to-yeast (3 hour); 7, yeast cells; 8., yeast-to-hyphae and 9, sporulating cells. Numerical values represent integrated area of absorbance peak measured in square pixels where one pixel represents a unit of information resolved by densitometer peak from the densitometer scan of autoradiographs shown in figure 9. Rows contain data from the DNA probes used for Northern hybridization analysis of M. racemosus RAS transcript levels.

	1	2	3	4	5	6	7	8	9
TEF-1	-	5177	7268	5766	-	4304	3487	16437	7355
MRAS1-1	-	-	1487	2897	-	-	-	7920	3704
MRAS2-0	-	-	-	-	-	-	295	2737	-
MRAS3-21	-	-	-	-	-	-	-	-	3271

DISCUSSION

It has been proposed that ras genes may play a fundamental role in basic cellular functions in eukaryotic cells. Two cellular functions which have been experimentally linked to ras expression are cell differentiation and cell proliferation. We are studying M. racemosus as a simple model system for cell proliferation and differentiation in eukaryotes. Three ras genes have been cloned previously from Mucor racemosus. We hypothesized that if one or more ras genes are involved in regulation of morphogenesis (cell differentiation) in this organism, the expression levels of the genes may correlate with changes in morphology of M. racemosus. This project focused on the preliminary detection of the expression levels of three Mucor ras transcripts, MRAS1, MRAS2 and MRAS3 using DNA probes.

DNA restriction fragments containing a portion of the 3' end hypervariable region (exon V) and additional sequences were subcloned from each MRAS gene. The similarity among these three MRAS gene subclones was tested by Southern hybridization and computer assisted dot matrix analyses. Both types of analysis demonstrated that MRAS1-1

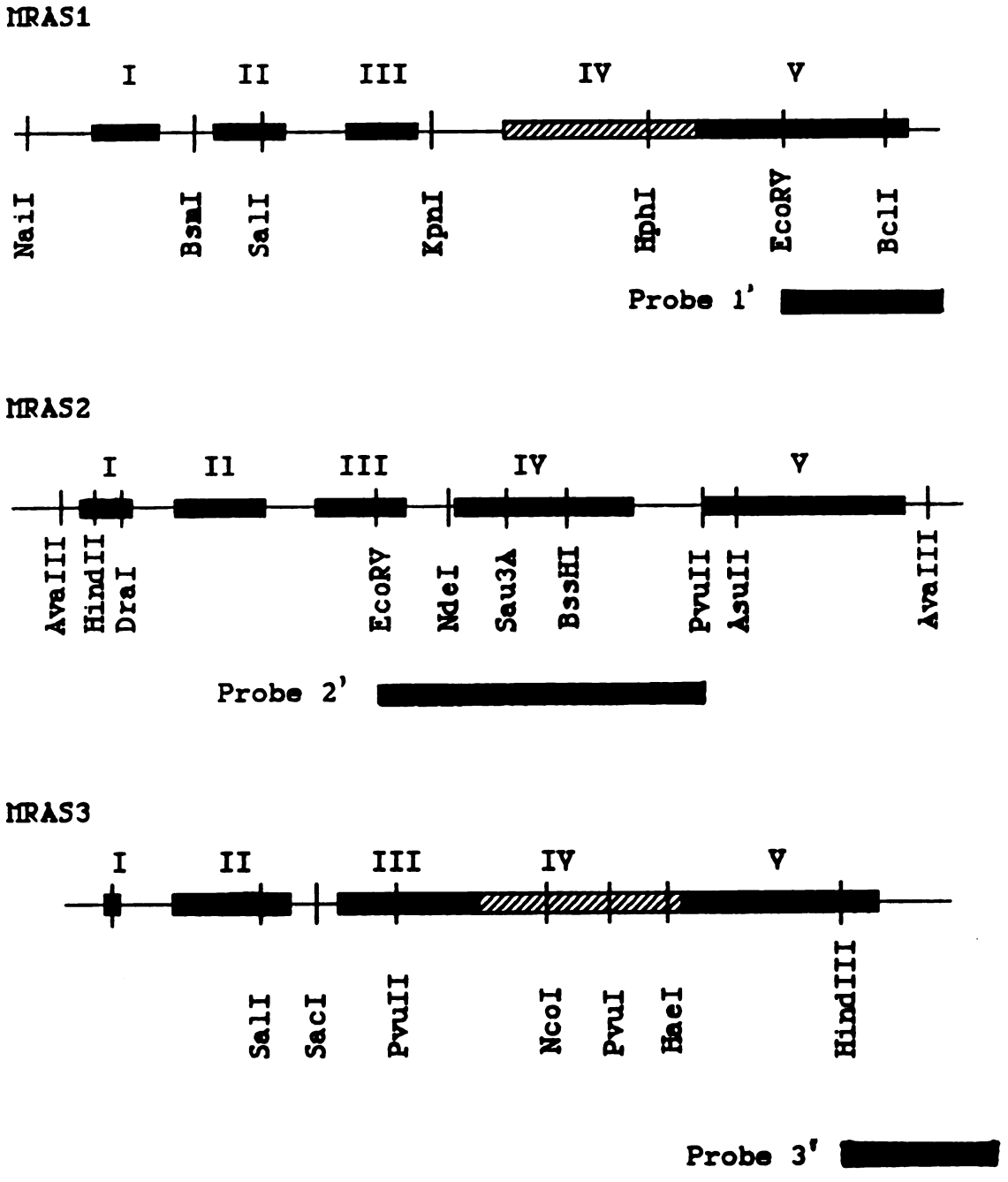
and MRAS3-21 did share some sequence similarity. However, the cross-hybridization between MRAS1-1 and MRAS3-21 probes was only about 12% to 15% of the specific hybridization level. Significant similarity between MRAS2-0 and MRAS1-1 or MRAS3-21 was not detected. Based on these data, we concluded that these three subclones were useful in preparing DNA probes for preliminary detection and quantitation of MRAS transcripts.

Polyadenylated mRNA from eight morphologies of Mucor cells were analyzed. The preliminary data from this study suggested that MRAS1 and MRAS3 genes were expressed in a morphology-specific pattern because the accumulated transcript levels of these two MRAS genes correlated with the morphological changes of Mucor racemosus. However, the MRAS2 transcript, which was detected in yeast and yeast-to-hyphae transition cells was still in doubt because this probe contained DNA sequences downstream from the MRAS2 gene (flanking sequence). At least two ras genes, MRAS1 and MRAS3, therefore may be involved in regulation of Mucor morphogenesis.

In a recent study, the quantities of polyadenylated mRNA purified from several cell morphologies of M. racemosus have been normalized (by TEF-1 transcript levels as control) and used to repeat this Northern blot analysis (J. Linz,

unpublished data). DNA restriction fragments containing a portion of exon V and flanking sequences of MRAS1 and MRAS3 genes were used to prepare DNA probes. In addition, a third fragment located totally within the predicted open reading frame (protein coding sequence) of MRAS2 was used (Figure 10). The results revealed that the MRAS2 gene was not expressed at detectable levels in any of the morphologies of Mucor cells. The previous experiment which showed a putative 2.3 Kb (figure 9) MRAS2 transcript in yeast and yeast-to-hyphae cells was due to the hybridization of flanking sequence of MRAS2-0 probe to Mucor mRNA. The expression data and nucleotide sequence data suggested that the MRAS2 gene may be a pseudogene (ie. totally inactive), is expressed at extremely low levels, or is expressed at times not sampled in this study. DNA probes prepared from the MRAS1 gene hybridized to a 1.3 Kb mRNA in all morphologies of Mucor cells and the accumulated transcript level varied considerably. MRAS3 DNA probes which hybridized strongly to the mRNA of sporulating cells, hybridized to mRNA of germlings moderately, and slightly to yeasts in the more refined analysis. These new data confirm the previous result that two MRAS genes are differentially expressed. These MRAS transcripts are accumulated in a morphology-related pattern.

Figure 10. DNA probes used for the refined Northern analysis of MRAS transcripts. Probe 1', the EcorV/EcoRV restriction fragment (3' EcoRI site not shown)containing part of the exon V of MRAS1 gene and 3' flanking sequence; Probe 2', the EcoRV/PvuII internal restriction fragment of MRAS2 gene, and Probe 3', the HindIII/HinDIII restriction fragment (3' HindIII site not shown) containing a portion of exon V of MRAS3 gene and 3' flanking sequence.


MRAS1
I
II
III
IV
V
NaiI
BsmI
SalI
KpnI
HphI
EcoRV
BclI
Probe 1'
MRAS2
I
II
III
IV
V
AvaIII
HindII
DraI
EcoRV
NdeI
Sau3A
BssHI
PvuII
AsuII
AvaIII
Probe 2'
MRAS3
I
II
III
IV
V
SalI
SacI
PvuII
NcoI
PvuI
HaeI
HindIII
Probe 3'

The results of this study indicated that expression of Mucor ras genes may be regulated during cellular morphogenesis. Changes in the requirement for individual ras protein may result in the alteration of expression of each Mucor ras gene. However, investigation of the biological function of Mucor ras genes is necessary in future studies. One approach to discovery of the normal biological function of the MRAS genes is to qualitatively and quantitatively alter MRAS gene products and observe changes in cell growth and development. Expression of antisense mRNA or overexpression of Mucor ras genes may be useful tools to explore the correlation of MRAS gene activity and the physiological response of Mucor cells. These tools may provide a crude mechanism to artificially regulate the ras protein level and activity in the cell.

Due to the correlation of signal transduction and morphogenesis in Mucor, the effects of ras gene expression on cAMP and phosphatidylinositol phosphate (PI) metabolism, two important secondary messengers, may also be observed. The identity of effector proteins, receptor proteins and other components of a putative signal transduction pathway with which MRAS proteins may interact will be helpful in clarifying the function of individual Mucor ras proteins. Monoclonal antibodies, specific to individual Mucor ras proteins, may also be useful to determine the location of

ras proteins in Mucor cells and to confirm the biological function of Mucor ras proteins by blocking these activities in vitro.

Normal mechanisms for control of Mucor ras gene transcription are also important to study. The alteration in level of transcript may be due to either the rate of transcription or rate of mRNA degradation. To investigate this problem, the identification of promoter regulatory regions, and regulatory proteins involved in MRAS gene expression would be helpful to clarify the regulatory mechanism at the transcriptional level. The exploration of transport and processing mechanisms of ras hnRNA may be useful in understanding the alterations in accumulated transcript level and RNA degradation.

CONCLUSION

The finding of three ras gene homologues in the filamentous fungus Mucor racemosus led to these studies designed to explore the correlation of MRAS gene expression and cellular morphogenesis in this organism. This study showed that at least two MRAS genes were expressed differentially and that the accumulated transcript levels of the MRAS1 and MRAS3 genes varied considerably in a morphology-related pattern. These experimental data confirm one part of the original research hypothesis that the expression levels of the MRAS genes may correlate with changes of Mucor cell morphology if one or more MRAS genes are involved in regulation of morphogenesis. Further exploration into the expression of the MRAS genes and the diversity of Mucor ras protein function will be helpful to understand the possible relationship between ras gene expression and cell differentiation in higher eukaryotes.

Literature Cited

Adari, H., D. R. Lowy, B. M. Eillumsen, C. J. Der, and F. McCormic. Guanosin Triphosphatase Activating Protein (GAP) Interact with the p21 ras Effector Binding Domain. 1988. Science 240:518-521.

Aviv, H and P. Leder. 1972. Purification of Biologically Active Globin Messensger RNA by Chromatography on Oligothymidylic Acid-Cellulose. Proc. Natl. Acad. Sci. USA 69:1408-1412.

Bar-Sagi, D and J. R. Feramisco. Microinjection of the ras Oncogene Protein into PC12 Cells Induces Morphological Differentiation. 1985. Cell 42:841-848.

Barbacid, M. ras genes. 1987. Ann. Rev. Biochem. 56:779-827.

Beckner, S. K., S. Hottori, and T. Y. Shih. The ras Oncogene product p21 is not a Regulatory Component of Adenylate Cyclase. 1985. Nature 317:71-72.

Bos, J. L., E. R. Fearson, S. R. Hamilton, M. Verlaan-de Vries, J. Van Boom, A. J. van der Eb, and B. Vogelstein. Prevalence of ras Gene Mutations in Human Colorectal Cancers. 1987. Nature 327:293-297.

Broek, D., N. Samiy, O. Fasano, A. Fujiyama, F. Tamanoi, J. Northup, and M. Wigler. Differential Activation of Yeast Adenylate Cyclase by Wild-Type and Mutant RAS Proteins. 1985. Cell 41:763-769.

Broek, D., T. Toda, T. Michaeli, L. Levin, C. Birchmeier, M. Zoller, L. Powers, and M. Wigler. The S. cerevisiae CDC25 Gene Product Regulates the RAS/Adenylate Cyclase Pathway. 1987. Cell 48:789-799.

Buss, J.,and B. M. Sefton. Direct Identification of Palmitic Acid as the Lipid Attached to $p21^{ras}$. 1986. Mol. Cell. Biol. 6:116-122.

Cales, C., J. F. Hancock, C. J. Marshall, and A. Hall. The Cytoplasmic protein GAP is Implicated as the Target for Regulation by ras Gene Product. 1988. Nature 332:548-550.

Casperson, G., N. Walder, A. Brasier, and H. Bourne. A Guanine Nucleotide-Sensitive Adenylate Cyclase in the Yeast Saccharomyces cerevisiae. 1983. J. Biol. Chem. 258:7911-7914.

Cihlar, R. 1985. Morphogenesis and Dimorphism of Mucor. In "Gene Manipulation in Fungi" pp.449-467. Bennett, J. and L. Lasure (ED).

Clark, R., G. Wong, N. Amheim. D. Nitechi, and F. McCormick. Antibodies Specific for Amino Acid 12 of the ras Oncogene Product Inhibit GPT binding. 1985. Proc. Natl. Acad. Sci. USA 82:5280-5284.

Cooper, G. M. Cellular Transforming Genes. 1982. Science 217:801-806.

Dechenes, R. J. and J. R. Broach. Fatty Acylation is Important but not Essential for Saccharomyces cerevisiae RAS Function. 1987. Mol. Cell Biol. 7:2344-2351.

Defeo, D., M.A. Gonda, H. A. Young, E. H. Chang and D.R. Lowy, E. M. Scolnick, and T. W. Ellis. Analysis of Two Divergent Rat Genomic Clones Homologous to the Transforming Gene of Harvey Murine Sarcoma Virus. 1981. Proc. Natl. Acad. USA 78:3328-3332.

Defeo-Jones, D., K. Tatchell, L. C. Robinson, I. S. Sigal and W. C. Vass, D. R. Lowy, and E. M. Scolnick. Mammalian and Yeast ras Gene Products: Biological Function in their Heterologous System. 1985. Science 228:179-184.

De Vos, A. M., L. Tong, M. V. Milburn, P. M. Matas, J. Jancarik, S. Noguchi, S. Nishimura, K. Miura, E. Ohtsuka and S. H. Kim. Three-Dimensional Structure of an Oncogene Protein:Catalytic Domain of Human c-H-ras p21. 1988. Science 239:888-893.

Der, C. J., T. Finkel, and G. M. Cooper. Biological and Bichemical Properties of Human ras^H Genes Mutated at Codon 61. 1986. Cell 44:167-176.

Edmonds, M., M. H. Vaughn, Jr., and H. Nakazato. Polyadenylic Acid Sequences in Heterogeneous Nuclear RNA and Rapidly-Labeled Polyribosomal RNA of HeLa Cells:Possible Evidence for Precursor Relationship. 1971. Proc. Natl. Acad. Sci 68:1336-1340.

Eills, R. W., D. Defeo, T. Y. Shih, M. A. Gonda and H. A. Young. The p21 src Genes of Haryey and Kirsten Sarcoma Viruses Originate from Devergent Members of a Family of Normal Vetebrate Genes. 1981. Nature 292:506-511.

Fasano, O., T. Aldrich, F. Tamanoi, E. Taparowsky, M. Fruth, and M. Wigler. Analysis of the Transforming

Potential of the Human H-ras Gene by Random Mutagenesis. 1984. Proc. Natl. Acad. Sci. USA 81:4008-4012.

Feinberg, A. P. and B. Vogelstein. A Techinque for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity. 1983. Analytical Biochem. 132:6-13.

Fleischman, L. F., S. B. Chahwala, and L. Cantley. RAS-Transformed Cells:Altered Levels of Phosphatidylinositol-4,5-bisphostphate and Catabolites. 1986. Science 231:407-410.

Forte, J. M., and M. Orlowski. Profile of Cyclic Adenosine 3',5'-Monophosphate-Binding Proteins During the Conversion of Yeast to Hyphae in Fungus Mucor. 1980. Exptl. Mycol. 4:78-86.

Forrester, K., C. Almoguera, K. Han, W. E. Grizzle, and M. Perucho. Detection of High Incidence of K-ras Oncogene During Human Colon Tumorigenesis. 1987. Nature 327:298-303.

Fourney, R. M., J. Miyakushi, T. S. Day III, and M. C. Paterson. Northern Blotting : Efficient RNA Staining and Transfer. 1988. Focus. 10:5-7.

Fraenkel, D. G. On ras Gene Function in Yeast. 1985. Proc. Natl. Acad. Sci. USA 82:4740-4744.

Fujiyama, A., and F. Tamanoi. Processing and Fatty Acid Acylation of RAS1 and RAS2 proteins in Saccharomyces cerevisiae. 1986. Proc. Natl. Acad. Sci. USA 83:1266-1270.

Gilman, A. G. G Proteins:Transducers of Receptor-Generated Signals. 1987. Ann. Rev. Biochem. 56:615-649.

Guerrero, I., H. Wong, A. Pellicer, and D. E. Burstein. Activated N-ras Gene Induces Neuronal Differentiation of PC12 Rat Pheochromocytoma Cells. 1986. J. Cell. Physiol. 129:71-76.

Grunstein, M. and D. Hogness. Colony Hybridization:A Method for the Isolation of Cloned DNAs that Contains a Specific Gene. 1975. Proc. Natl. Acad. Sci. USA 72:3961-3965.

Hall, A., C. J. Marshall, N. K. Spurr and R. A. Weiss. Identification of Transforming Gene in Two Human Sarcoma Cell Lines as a New Member of the ras Gene

Family Located on Chromosome 1. 1983. Nature 303:396-400.

Hanahan, D. Studies on Transformation of Escherichia coli with Plasmids. 1983. J. Mol. Biol. 166:557-580.

Harvey, J. J. An Unidentified Virus which Causes the Rapid Production of Tumours in Mice. 1964. Nature 204:1104-1105.

Ito, E., T. Cihlar, and C. Inderlied. Lipids Synthesis During Morphogenesis of Mucor racemosus. 1982. J. Bacteriol. 152:880-887.

Jurnak, F. 1985. Structure of the GDP Domain of EF-Tu and Location of the Amino Acids Homologous to ras Oncogene Proteins. Science 230:32-36.

Kaibuchi, K., A. Miyajima, K. I. Arai, and K. Matsumoto. Possible Involvement of RAS-Encoded Proteins in Glucose-Induced Inositolphospholipid Turnover in Saccharomyces cerevisiae. 1986. Proc. Natl. Acad. Sci. USA 83:8172-8176.

Kamata, T., and J. R. Feramisco. Epdermal Growth Factor Stimulates Guanine Nucleotide Binding Activity and Phosphorylation of ras Oncogene Proteins. 1984. Nature 310:147-150.

Kataoka, T., S. Power, C. McGill, O. Fasano, J. Strathern, J. Broach and M. Wigler. Genetic Analysis of Yeast RAS1 and RAS2 Genes. 1984. Cell 37:437-445.

Kataoka, T., S. Powers, S. Cameron, O. Fasano, M. Goldfarb, J. Broach and M. Wigler. Functional Homology of Mammalian and Yeast RAS Genes. 1985. Cell 40:19-26.

Kikkawa, U., and Y. Nishizuka. The Role of Protein Kinase C in Transmembrane Signalling. 1986. Ann. Rev. Cell Biol. 2:147-178.

Kirsten, W. H. and L. A. Mayer. Morphologic Responses to a Murine Erythroblastosis Virus. 1967. J. Natl. Cancer Inst. 39:311-335.

Lacal, J, C., P. De La Pena, J. Moscat, P. Garcia-Barreno, P. S. Anderson, and S. A. Aaronson. Rapid Stimulation of Diacylglycerol Production in Xenopus Oocytes by Microinjection of H-ras p21. 1987. Science 238:533-536.

Lacal, J. C., J. Moscat, and S. A. Aaronson. Novel Source of 1,2-Diacylglycerol Elevated in Cells Transformed by Ha-

ras Oncogene. 1987, Nature 310:279-272.

Larsen, A., and L Sypherd. Cyclic Adenosine 3',5'-Monophosphate and Morphogenesis in Mucor racemosus. 1974. J. Bacteriol. 117:432:438.

Linz, J. E., and P. S. Sypherd. Expression of Three Genes for Elongation Factor 1α during Morphogenesis of Mucor racemosus. 1987. Mol. Cell. Biol. 7:1925-1932.

Lochrie, M., J. Hurley, and M. I. Simon. Sequence of the Alpha Subunit of Photoreceptor G Protein: Homologies Between Transducin, ras and Elongation Factors. 1985. Science 228:96-99.

Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Molecular Cloning, A Lab Manual. Cold Spring Harbor Laboratory, N. Y.

Manne,V., E. Bekes, H. F. Kung. Ha-ras Proteins Exhibit GTPase Activity:Point Mutations that Activate Ha-ras Gene Products Result in Decreseased GTPase activity. 1985. Proc. Natl. Acad. Sci. USA 82:376-380.

Maramatsu, M. 1973. Preparation of RNA from Animal Cells. Methods Cell Biol. 7:23-51.

Marshall, M. S., J. B. Gibbs, E. M. Scolnick, and I. S. Sigal. Regulatory Function of the Saccharomyces cerevisiae RAS C-Terminus. 1987. Mol. Cell Biol. 7:2309-2315.

Marshall, M. S., J. B. Gibbs, E. M. Scolnick, and I. S. Sigal. An Adenylate Cyclase from Saccharomyces cerevisiae that is Stimulated by RAS proteins with Effector Mutations. 1988. Mol. Cell. Biol. 8:52-61.

McCormick, F., B. F. Clark, T. F. la Cour, M. Kjeldgaard, L. Norskov-Lauritsen, and J. Nyborg. A Model for the Tertiary Structure of p21, the Product of the ras Oncogene. 1985. Science 230:70-82.

McGrath, J. P., D.J. Capon, D. H. Smith, E.Y.Chen and P. H. Seeburg. Structure and Organization of the Human Ki-ras Proto-oncogene and related Processed Pseudogenes. 1983. Nature 304:501-506.

Messing, J., R. Crea, and P. H. Seeburg. A System for Shotgun DNA Sequencing. 1981. Nucl. Acid Res. 9:309-321.

Mulcahy, L. S., M. R. Smith and D. W. Stacey. Requirement

for ras Proto-oncogene Function during Serum-Stimulated Growth of NIH3T3 Cells. 1985. Nature 313:241-243.

Neuman-Silberberg, F. S., E. Schejter, F. M. Hoffmann, and B. Z. Shilo. The Drosophila ras Oncogenes:Structure and Nucleotide Sequence. 1984. Cell 37:1027-1033.

Norrander, J., T. Kempe, and J.Messing. Construction of Improved M13 Vector Using Oligodeoxynucleotide-Directed Mutagenesis. 1983. GENE 26:101-106.

Orlowski, M. Changing Pattern of Cyclic AMP-Binding Proteins during Germination of Mucor racemosus Sporangiospores. 1979. Biochem. J.182:547-554.

Orlowski, M. Cyclic Adenosine 3',5'-Monophosphate and Germination of Sporangiospores from the Fungus Mucor. 1980. Arch. Microbiol. 126:133-140.

Pall, M. Adenosine 3',5'-Phosphate in Fungi. 1981. Microb. Rev. 45:462-480.

Power, S., T. Kataoka, O. Fasano, M. Goldfarb, J. Stratherm, J. Broach, and M. Wigler. Genes in Sarccharomyces cerevisiae Encoding Proteins with Domains Homologous to the Mammalian ras Proteins. 1984. Cell 36:607-612.

Reymond, C. D., R. H. Gomer, M. C. Mehdy, and R. A. Firtel. Developmental Regulation of a Dictyostelium Gene Encoding A Protein Homologous to Mammalian ras Protein. 1984. Cell 39:141-148.

Robinson, L. C., J. B. Gibbs, M. S. Marshall, I. S. Sigal, and K. Tatechell. CDC25: A Component of the RAS Adenylate Cyclase Pathway in Saccharomyces cerevisiae. 1987. Science 235:1218-1221.

Roberts, R. J. Restriction Enzyme and their Isoschizomers. 1987. Nucl. Acid Res. (Sequences Supplement) 15:r189-r217.

Seeburg, P. H., W. W. Colby, D. J. Capon, D. V. Foeddel, and A. D. Levinson. Biological Properties of Human c-Ha-ras Genes Mutated at Codon 12. 1984. Nature 312:71-75.

Sharp, P. A., B. Sugden, and J. Sambrook. Detection of Two Restriction Endonuclease Activities in Hemophilus parainfluenzae Using Analytical Agarose-Ethidium Bromide Electrophoresis. 1973. Biochem. 12:3055-3063.

Schejter, E. D., and B. Z. Shilo. Characterization of Functional Domains of p21 ras by Use of Chimeric Genes.

1985. EMBO J. 4:407-412

Shih, C., L. C. Padhy, M. Murray, and R.A. Weinberg. Transforming Genes of Carcinomas and Neuroblastomas Introduced into Mouse Fibroblasts. 1981. Nature 290:261-264.

Shih, T. Y., P. E. Stokes, G. W. Smythers, R. Dhar, and S. Oroszlan. Characterization of the Phosphorylation Sites and the Surrounding Amino Acid Sequences of the p21 Transforming Proteins Coded for By the Harvey and Kirsten Strains of Murine Sarcome Viruses. 1982. J. Bio. Chem. 257:11767-11773.

Sigal, I. S. The ras Oncogene: A Structure and Some Function. 1988. Nature 332:485-486.

Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, and E. M. Scolnick. Identification of Effector Residues and a Neutralizing Epitope of Ha-ras-encoded p21. 1986. Proc. Natl. Acad. Sci. USA 83:4725-4729.

Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, G. L. Temeles, and B. S. Wolanski. Mutant ras-encoded Proteins with Altered Nucleotide Binding Exert Dominant Biological Effects. 1986. Proc. Natl. Acad. Sci. USA 83:952-956.

Southern, E. Detection of Specific Sequence Among DNA Fragments Separated by Gel Electrophoresis. 1975. J. Mol. Biol. 98:503-517.

Southern, E. Gel Electrophoresis of Restriction Fragments. 1980. Method Enzymol. 68:152-176.

Swanson, M. E., A. M. Elste, S. M. Greenberg, J. H. Swartz, T. H. Aldrich, and M. E. Furth. Abundant Expression of ras Proteins in Aplysia Neurons. 1986. J. Cell Biol. 103:485-492.

Sweet, R. W., S. Yokoyam, T. Kamata, J. R. Feramisco, M. Rosenberg, and M. Gross. The Product of ras is a GTPase and the T24 Oncogenic Mutant is Deficient in this Activity. 1984. Nature 311:273-275.

Tatchell, L., L. C. Robinson, and M. Breitenbach. RAS2 of Sacchromyces cerevisiae is required for Glucogenic Growth and Proper Response to Nutrient Limitation. 1985. Proc. Natl. Acad. Sci. USA 82:3785-3789.

Tamanoi, F. Yeast RAS Genes. 1988. Bioch. Biophy. Acta. 948:1-15.

Tamanoi, F., M. Walsh, T. Kataoka, and M. Wigler. A Product of Yeast RAS2 gene is a Guanine Nucleotide Binding Protein. 1984. Proc. Natl. Acad. Sci. USA 81:6924-6928.

Temeles, G. L., D. DeFeo-Jones, K. Tatchell, M. S. Ellinger, and E. M. Scolnick. Expression and Characterization of ras mRNA from Sacchromyces cerevisiae. 1984. Mol. Cell Biol. 4:2298-2305.

Temeles,G. C., J. B. Gibbs., J. S. D'Alonzo, I. S. Sigal and E. M. Scolnick. Yeast and Mammalian ras Proteins have Conserved Biological Properties. 1985. Nature 313:700-703.

Toda,T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Carmeron, J. Broach, K. Matsumoto and M. Wigler. In Yeast, RAS Proteins are Controlling Element of Adenylate Cyclase. 1985. Cell 40:27-36.

Trahey, M., and F. McCormick. A Cytoplasmic Protein Stimulates Normal N-ras p21 GTPase, but does not Affect Oncogenic Mutants. 1987. Science 238:542-545.

Wakelam, M. J. O., S. A. Davies, M. D. Houslay, I. McKay, C. J. Marshall. and A. Hall. Normal $p21^{ras}$ Couples Bombesin and other Growth Factor Receptor to Inositol Phosphate Production. 1986. Nature 323:173-176.

Walter, M., S. G. Clark, and A. D. Levinson. The Oncogenic Activation of Human $p21^{ras}$ by a Novel Mechanism. 1986. Science 233:649-652.

Weeks, M. O., G. L. Hager, R. Lowe, and E. M. Scolnick. Development and Analysis of a Transformation-Defective Mutant of Harvey Murine Sarcoma tk Virus its Gene Product. 1985. J. Virol. 54:586-597.

Willumsen, B., A. Christensen, N. L. Hubbert, A. G. Papageorge, and K. Lowy. The p21 ras C-Terminus is required for Transformation and Membrane Association. 1984. Nature 310:583-586.

Yanisch-Perron, C., J. Vieira, and J. Messing. Improved M13 Phage Cloning Vectors and Host Strains: Nucleotide Sequenes of the M13mp18 and pUC19 Vector. 1985. GENE 33:103-119.

Yu, C. L., M. H. Tsai, and D. W. Stacey. Cellular ras Activity and Phospholipid Metabolism. 1988. Cell 52:63-71.