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CLONING AND EXPRESSION OF THE *RAS* HOMOLOGUES OF *MUCOR RACEMOSUS*

By

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ABSTRACT

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The ras proteins (GTP-binding regulatory proteins) are thought to play a role in signal transduction pathways in eukaryotic cells by regulating the production of "second messengers" via cyclic adenosine 3'5'monophosphate (cAMP) or phosphatidylinositol (PI) metabolism. PI and cAMP metabolism are correlated to the morphogenetic response in Mucor racemosus, a dimorphic filamentous fungus. The focus of this research was to characterize the Mucor ras genes to initiate our investigation of the molecular mechanisms which regulate morphogenesis. Three DNA restriction fragments were cloned from Mucor gene libraries by hybridization to Saccharomyces cerevisiae YRAS gene probes. Southern hybridization data and restriction endonuclease analysis suggest that these 3 clones represent 3 Mucor ras genes C4, L1B, and E10. Northern blot analysis of Mucor RNA suggests that the three Mucor ras genes are expressed and that one of these genes (L1B) is expressed in a morphology-specific manner.

I would like to dedicate this work to my parents, Shing-Mole and Swae-Siang Chou Lee, my sisters, and brothers.

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INTRODUCTION

Ras genes and ras-related genes

During the past 15 years, a series of oncogenes (cancer associated genes) has been identified through analyses of transforming genes in tumorigenic viruses and in tumor tissue. The transforming genes of Harvey and Kirsten strains of murine sarcoma viruses for example (Harvey, 1964; Kirsten and Mayer, 1967) have been named ras oncogenes. This acronym is derived from the words rat sarcoma. Cellular versions of these transforming genes, called c-ras, have been identified through their nucleotide sequence homology to the retroviral oncogenes. At least three genes are found in the mammalian ras oncogene family: Harvey ras (H-ras) and Kirsten ras (K- ras) which were isolated from their respective murine sarcoma viruses (Ellis et al., 1981), and N-ras which was found as an activated oncogene in a human neuroblastoma (Taparowsky, et al., 1983). Genes exhibiting limited sequence homology to the ras gene family are called ras-related genes that share 36% - 55% sequence homology. ras homologues are at least 55% homologous to the ras gene family. In view of the difficulty of assigning a cellular role for the ras protein, p21, great excitement attended the discovery that genes with remarkable homology to human ras are

widely distributed throughout a variety of different organisms.

Ras homologues have been identified and characterized in simple eukaryotic organisms such as the yeast Saccharomyces cerevisiae (DeFeo-Jones et al., 1983; Power et al.,1984), Drosophila (Neuman-Silberberg et al., 1984) and Dictyostelium (Reymond et al., 1984). However, only in yeast has the cellular function of a ras gene been determined (see below).

Properties of ras genes

The most important goal in ras research is to identify the biological function of ras proteins and to determine the connection between mutations in ras and tumor development in tissue. The presence of ras genes in normal cells and in evolutionarily diverse organisms suggests that these genes have a fundamental role in cellular functions such as proliferation (Noda et al., 1985; Bar-Sagi and Feramisco, 1985; Guerrero, 1986) and terminal differentiation.

All forms of mammalian ras proteins, independent of their phylogenetic origin, effectively bind GTP and GDP (Shih et al., 1980; Papageorge et al., 1982), have GTPase activity (McGrath et al., 1984; Gibbs, Sigal, and Scolnick, 1984), and are associated with the inner surface of the plasma membrane (Willingham et al., 1980; Willumsen et al., 1984). In addition to these properties, the significant sequence homology of ras and G proteins (Hurley et al., 1984; Itoh et al., 1986) has led to speculation that ras proteins, like G proteins, may

participate in the transduction of signals across the cytoplasmic membrane. Little else is known about the biochemical function of the mammalian ras proteins.

The basic biological functions of ras proteins, although unknown, appear to be conserved in evolution. For example: 1) the human HRAS protein can complement yeast strains lacking ras genes; 2) the yeast RAS2 gene product can, in a derivative form, transform mammalian cells; and 3) the GTPase activity of the yeast proteins is impaired by mutations analogous to those present in the oncogenic mammalian ras variants (DeFeo-Jones et al., 1985; Kataoka et al., 1985; Gibbs et al., 1985). Other experimental data support the concept that ras genes of mammals play a dual role in proliferation (Mulcahy and Smith, 1985) and in certain differentiation processes (Noda et al., 1985; Bar-Sagi and Feramisco, 1985).

One experimental approach to understanding ras protein function involves the study of ras-related genes in simple organisms such as yeast (Saccharomyces cerevisiae), Drosophila, and Dictyostelium which are more easily manipulated experimentally than mammalian cells. By a combined genetic and molecular approach, efforts to identify the role of these gene products were initiated in yeast. The results of these studies revealed much about the two encoded yeast RAS proteins, YRAS1 and YRAS2 proteins. The N-terminal 164 amino acids of each protein are 70% homologous to mammalian ras proteins. YRAS proteins bind and hydrolyze GTP, and they are essential for

yeast proliferation and mating (DeFeo-Jones et al.,1983; Tamanoi et al.,1984; Telemeles et al., 1985; Kataobu et al., 1984; Tatchell et al., 1984). In yeast, functional ras genes are required for the maintenance of growth and cell viability (Kataobu et al., 1984; Tatchell et al., 1984). Taken together these observations predict that the biochemical function of ras in yeast may be similar to the function of ras in mammalian cells. These results also suggest that the yeast RAS proteins could be a good model for understanding the function of mammalian ras proteins. Recent experimental evidence demonstrates that one of the two ras homologous genes of S. cerevisiae (YRAS2) activates yeast adenylate cyclase in the presence of guanine nucleotides (Broek et al., 1985; Toda et al., 1985). In spite of the conserved structural and functional properties of YRAS2 and mammalian ras genes, mammalian ras proteins do not appear to activate adenylate cyclase (Beckner et al., 1985; Birchmeier et al., 1985).

Recent studies have suggested that yeast RAS1 protein and vertebrate ras proteins may play an indirect role in the regulation of phosphatidylinositol bisphosphate (PIP_2) hydrolysis (Kaibuchi et al., 1986; Fleischman et al., 1986). These observations have led to the proposal that each of the ras genes in a gene family play different cellular roles in spite of their sequence homology and genetic complementarity. Therefore, model systems other than yeast may be useful in understanding ras protein function and ras interactions with

other proteins.

Mucor racemosus; A model system for study of cellular morphogenesis, fungal food spoilage, and extracellular enzyme biosynthesis

Mucor is a filamentous fungus, belonging to the Class Zygomycetes. The genus Mucor has been and continues to be explored by a great number of microbiologists and mycologists in both the basic and applied areas of biological research. Most of the Mucor spp have properties similar to other Zygomycetes. There is one characteristic, however, which makes several Mucor spp quite distinct from other Zygomycetes and the subject of biochemical study. This is the property of dimorphism. Early in the nineteenth century, the morphological variability of Mucor was first noted. Environmental conditions such as growth medium composition, sparging gas, or temperature are factors which are most often controlled to study Mucor morphogenesis. Dimorphic Mucor spp are those which can grow in a variety of differentiated hyphal morphologies and also in the form of multipolar budding yeasts. Examples of dimorphic Mucor spp include M. racemosus, M. rouxii, M. genevensis, M. bacilliformis and M. subtilissimus (some strains). These organisms have the ability to undergo a morphogenetic change from hyphal to budding yeast-like growth in response to various environmental stimuli (Bartnicki-Garcia, 1963).

Members of the genus Mucor are saprophytes and generally produce high levels of extracellular enzymes resulting in their

wide distribution as food spoilage agents. Foods associated with Mucor spoilage include meat, poultry, bakery goods, dairy products and citrus fruits. Mucor spp are also used to produce ethanol or wines, and several fermented food products in the orient such as sufu and tempeh (Beuchat, 1978). The production of a variety of extracellular enzymes by Mucor also results in industrial interest. Two species of Mucor, M. pusillus and M. miehei are used to produce many useful extracellular enzymes such as lipases, amylases, cellulases, and acid proteases (microbial rennin) which are commonly used in the food industry (Van Heeswijck, 1984). Mucor is usually not a pathogen, but some species are opportunistic pathogens which cause a rare disease , mucormycosis or zygomycosis, which ultimately attack the central nervous system and is often fatal.

Correlates of morphogenesis

Mucor is an especially attractive model system for study of morphogenesis because of the ease of manipulations to alter cell morphology, either yeast or mycelial growth, as a function of thegrowth environment. For example, the organism can be induced to undergo a morphogenetic change from hyphae to budding yeast-like growth by shifting the growth environment from an aerobic to an anaerobic atmosphere. The reverse is also true (Cihlar, 1985). This ability provides an advantage for the researcher to investigate the biochemical and molecular mechanisms that regulate morphogenesis. Morphogenesis in Mucor

represents a useful model for study of cell differentiation in higher eukaryotes. Mucor rouxii and Mucor racemosus are well suited for such studies. They have been more closely studied and perhaps better understood than the other species of Mucor in terms of the biochemical and molecular events that accompany morphogenesis.

Several physiological parameters have been found which are correlated to cellular morphogenesis in M. racemosus. For example, changes in intracellular cyclic AMP levels and specific rates of protein synthesis were correlated to yeast-to-hypha conversion in this fungus (Orlowski and Rose, 1981). The specific activity of ornithine decarboxylase (ODC), the first enzyme leading to polyamine biosynthesis in M. racemosus, was also found to increase when the incubation atmosphere was shifted from CO_2 to air (Inderlied et al., 1980) resulting in a yeast-to-hypha transition. Finally a change in the rate of synthesis of lipids and a change in the turnover of phospholipids (including PIP_2, Ito et al., 1982) accompany morphogenesis in this fungus. Based on these observations, it is possible that ras gene products play an important role in cell morphogenesis in Mucor by interacting with cellular components (membranes) and physiological effectors as part of a signal transducing system.

Research hypotheses

The goal of this research was to test 2 hypotheses. These

are: 1) The M. racemosus genome contains ras homologues. To determine the validity of this hypothesis the ras genes will be cloned in Mucor racemosus. Initial work in this area will involve using known ras genes such as HRAS (human cellular ras gene), YRAS1 and YRAS2 (yeast RAS genes) to screen a Mucor genomic DNA library. The cloned Mucor genes will be further studied by nucleotide sequence analysis. Once ras-related genes are cloned in Mucor, then it is possible to continue the study on their functions by overexpressing normal ras genes in Mucor or by expressing a ras gene with missense mutations and to observe the effects of these in vitro manipulations on cAMP, PI metabolism and morphogenesis in vivo.

2) There are different expression levels of the ras gene products in different cell morphologies in M. racemosus. This hypothesis will be tested by studying the level of ras gene transcripts which accumulate during normal growth and cellular morphogenesis in Mucor by dot blot or Northern analysis (see Methods).

These 2 testable hypotheses were based on the following data: 1. ras proteins, in comparison with G proteins, are similar in nucleotide sequence and putative functional domains; 2. In yeast, data suggest that ras may function in regulation of the production of second messengers cAMP and PIP_2 in signal transduction pathways; 3. In S. cerevisiae, ras activity is correlated with cellular proliferation, differentiation and intracellular cAMP levels ; 4. In Mucor,

morphogenesis, in response to an external signal (O_2) is correlated to cAMP and PIP_2 metabolism ; 5. *ras* genes are ubiquitous in eukaryotes, implying a fundamental role for *ras* in growth and development.

Genetic analysis of *Mucor*

The development of recombinant DNA technology provides us with the tools for engineering of organisms used for production of biochemicals by direct manipulation of their genomes. However, before we can successfully undertake genetic programming of industrial microorganisms such as *Mucor*, we must learn more about the basic genetics and biology of these organisms. A basic understanding of the biochemical and molecular events which regulate growth of *Mucor* will help to increase production of industrial enzymes and control food spoilage by this genus of filamentous fungi.

The methodologies of molecular genetics can help us to generate a greater understanding of the factors that regulate morphogenesis. For example the contribution of a particular gene product (such as *ras*) to the morphogenetic process in *Mucor* can be studied by altering the cloned gene by in vitro mutagenesis and reintroducing the gene back into cell. This protocol requires a cloned gene and a transformation vector. We have available DNA probes (*YRAS1*, *YRAS2*, *HRAS*) which will be useful in isolation of *ras* genes from the *Mucor*. In addition we are developing vectors will be designed and constructed to

contain selectable markers for use in transformation of M. racemosus. Of course, an effective transformation protocol must also be developed in order to introduce recombinant DNA into the cell. Preliminary work in this area was done by Van Heeswijck in Denmark (Van Heeswijck, 1984, 1986; Van Heeswijck and Roncero, 1984) who demonstrated that protoplasts can be efficiently generated from M. racemosus by using chitosanase and chitinase. These protoplasts were transformed at a high frequency (600 leu^+ transformants/ug DNA) using the plasmid vector pLeu4 (see Materials and Methods).

Recently, a leucine requiring strain, M. racemosus R7B, was transformed with the aspartic protease gene (rennin) from M. miehei. The resulting heterologous protease was secreted in active form and had the same apparent molecular weight as the aspartic protease produced in M. miehei. However, the level of enzyme produced in M. racemosus was low (Dickinson et al., 1987). Possible reason for this low level of expression will be examined thoroughly in our laboratory. Mucor gene products from one species can be expressed and secreted in a different Mucor species. These data suggest that we can develop an expression/secretion vector of general use in different species of Mucor. One additional factor which makes M. racemosus potentially a very useful organism is its ability to undergo morphogenesis. We propose to combine experimental tools (i.e. transformation systems, and expression vectors) with an understanding of Mucor growth and development to increase the

ability of Mucor cells grow in high density continuous culture. We also hope to use knowledge gained in our studies to develop an efficient heterologous genes expression system in Mucor miehei and Mucor pusillus.

Preliminary data

The following preliminary data were supplied by Dr. J. Linz as a background to this research. The primary evidence for the existence of ras genes in Mucor was provided by Southern blot analysis of restriction endonuclease digested M. racemosus genomic DNA using yeast RAS genes as probes to detect ras related sequences (as described in detail in Materials and Methods). These analyses revealed several DNA fragments with strong similarity to the heterologous RAS probes and encouraged us to do further isolation of ras related genes. The YRAS2 DNA probe was used by Dr. Linz to screen two lambda/Mucor racemosus libraries (E and L) for ras- related genes. Several phage clones were isolated from the E and L libraries. Two of the stronger hybridizing clones (L1B, E10) were subcloned and analyzed. The results are reported in this thesis. In addition, C bank was screened with the YRAS1 DNA probe to isolate ras homologues in M. racemosus with more similarity to the YRAS1 gene, which shares some sequence similarity with YRAS2 but differs significantly at 3′ end of the gene. This experiment resulted in the isolation of 4 lambda clones, C1-C4, one of which (C4) was also analyzed and reported here.

MATERIALS AND METHODS

Organism, growth media, and culture conditions

Mucor racemosus (M. lusitanicus) ATCC 1216B was the source of DNA and RNA used in cloning and analysis of the ras homologues in this research. Sporangiospore stock cultures were obtained from Paul Sypherd (University of California, Irvine). Mucor cultures were maintained on YPG agar [2% (wt./vol.) glucose, 1% (wt./vol.) Bacto-Peptone, 0.3% (wt./vol.) Bacto-Yeast Extract, and 3% (wt./vol.) Bacto-Agar (Difco Laboratories, Detroit, Michigan)]. The medium was adjusted to PH 4.5 with H_2SO_4. Glucose was always autoclaved separately from the other components of the medium to prevent carmalization. A small amount of a stock spore culture (see below) was inoculated on the center of YPG agar plates (100 mm) which were incubated at room temperature (22^{o}C) for 7 days to produce a confluent growth of mycelium. YPG liquid growth medium was the same as described above except that it did not contain agar.

Spore production

Sporangiospores for germination experiments were produced in 150 mm petri dishes containing 45ml of YPG agar. A small

quantity of pure spore suspension (50 ul of a frozen stored spore culture) was inoculated in the center of YPG agar plates which were incubated at 28°C for a period of 7 to 10 days. At this time the agar surface was completely covered with aerial hyphae bearing the sporangia containing the grey- black sporangiospores. Spores were harvested with ice cold sterile water by scraping the mycelium with a sterile glass rod. Spores were collected by centrifugation at 6,000 xg for 10 min at 4°C and used in RNA preparations (see below).

Germling production

Germlings were prepared by germinating sporangiospores (2 x 10^5 spores/ml) in YPG liquid medium with shaking (180 RPM) at 28°C in a rotatory shaker water bath. The culture was sparged with 2 volumes of sterile air per volume of growth medium per minute. When germ tubes reached 10-12 spore diameters, the germlings were cooled down in a salt ice bath and collected by filtration (Whatman no.1 filter). The cells were frozen immediately in liquid nitrogen and stored at - 70°C, or nucleic acids were extracted immediately, as described below.

Yeast cell production

Yeast cells of Mucor racemosus were prepared by inoculating spore suspensions (2 x 10^5 spores/ml) into YPG broth and incubating with shaking (100 RPM) at 28°C while bubbling CO_2 gas through the culture at a flow rate of 0.5

volume of gas per volume of culture fluid per min. These anaerobic conditions resulted in the germination of spores to yeast cells which continued growth by budding. After a 21 hr incubation period, the culture reached mid-log growth phase, (A_{600} = 0.8; yeast cells doubling time, approximately 4 hr). Yeast cells were harvested by filtration, frozen in liquid nitrogen and stored at -80°C until RNA extraction was carried out.

Yeast-to-hypha cell production

Yeast-to-hypha transition stage cells were obtained from growing yeast cells. Yeasts were grown in YPG with shaking under CO_2 until early log-phase (A_{600} = 0.22). The culture was shifted to an atmosphere of air until 10% of the cells had germ tubes (approximately 3 hr.). The cells were used directly at this time for cellular RNA preparations.

Purification of RNA and DNA from *Mucor racemosus* cells

Total RNA was extracted from *M. racemosus* cells by the procedure of Maramatsu (Maramatsu, 1973) as modified by Horst Domdey (personal communication). The collected cells were broken with grinding for 5 to 10 min in a sterile mortar containing liquid nitrogen. The ground material was transferred to cold 30 ml corex tubes and suspended in 5 volumes of warm (65°C) SDS-RNA extraction buffer (50mM NaOAc, 1.0 mM EDTA, 1% SDS; adjusted to PH 5.0 with acetic acid) treated with

diethylpyrocarbonate (DEPC). The cell suspension was extracted 2 times with 5 volumes of warm RNA extraction buffer-saturated redistilled phenol (65°C) containing 0.1% (wt./vol.) 8-hydroxyquinoline. The aqueous phase was reextracted with 1 volume phenol (RNA extraction buffer saturated) : chloroform : isoamyl alcohol (25:24:1) and then with 1 volume diethyl ether (water-saturated). Finally, the upper phase (ether) was removed and 1/6 volume of 3 M NaOAc (pH 5.2) was added to the aqueous phase followed by 2.5 volumes ethanol to precipitate the RNA (-20°C overnight).

High-molecular-weight M. racemosus genomic DNA was extracted from germlings by the procedure of Cihlar and Sypherd (Cihlar and Sypherd, 1980). M. racemosus germlings were grown, harvested as described earlier, and ground in a mortar under liquid nitrogen for 10 to 15 min to break the cells. The ground material was suspended in 8 volumes of TES buffer (100 mM PH 8.3 Tris, 150 mM NaCl, 100 mM EDTA). SDS (sodium dodecyl sulfate) was added to a final concentration of 0.1% to solubilize the cell membranes. The broken cell suspension was extracted gently (to avoid shearing) with 8 volumes of phenol (TES buffer saturated) and reextracted with 8 volumes phenol (Tris saturated) / $CHCl_3$: isoamyl alcohol (24:1). The DNA was precipitated by the addition of 2 volumes of absolute ethanol (-20°C overnight). The pellet was dissolved in 1X TE (10 mM Tris·Cl, 1 mM EDTA PH 8.0) and treated with RNase (50 ug/ml) and proteinase K (100 ug/ml) for 2 hr at 37°C. The DNA was

extracted with an equal volume of phenol / $CHCl_3$: isoamyl alcohol and ethanol precipitated (2 volumes) at -20°C overnight.

RNA and DNA were quantitated by measuring the absorbance at wavelengths of 260 nm and 280 nm. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} of 1.8 and 2.0, respectively.

Bacterial strains and plasmids

E. coli LE392 (F^- hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 lambda$^-$) (Murray et al., 1977), *E. coli* CES200 (Rec BC^-) and *E. coli* P2392 (P22 lysogen) were used to propagate the *Mucor racemosus* gene library in bacteriophage lambda. *E. coli* JM83 [ara (proA,B-lac) rpsL thi80 dlacZ M15](Messing, 1979) was the host strain for plasmids pUC8, pUC9 (Viera and Messing, 1982), and pBR322 (Bolivar, 1977), which were used in subcloning the ras genes. These plasmids were grown and amplified in LB medium (tryptone [Difco], 10 g/liter; yeast extract [Difco] 5 g/liter; NaCl, 10 g/liter [PH 7.5]) and purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (Birnboim and Doly, 1979; Maniatis et al., 1982). *E. coli* JM83 was made competent for transformation with recombinant plasmids by a $CaCl_2$ procedure reported previously (Mandel and Higa, 1970). The transformed cells were selected and maintained on LB medium supplemented with the chromogenic

substrate 5-bromo, 4-chrolo, 3- indolyl, B-D-galactoside (X-gal, 20 ug/ml) and ampicillin (50 ug/ml, final concentration) for pUC recombinant plasmids. The alkaline lysis method was used in rapid, small-scale or large scale plasmid DNA preparations (Maniatis et al., 1982).

Construction of an *M. racemosus* gene bank in bacteriophage lambda

Mucor racemosus gene banks, were prepared by Dr. Linz. Three *Mucor racemosus* gene banks, C bank, L bank, and E bank, were propagated in *E. coli* CES200, *E. coli* LE392, and *E. coli* LE392 respectively. C bank and L bank were established from *Mbo*I-digested *Mucor racemosus* genomic DNA[10 to 15 Kilobases(kb) fragments] which were purified on sucrose gradients (Maniatis et al., 1978) and ligated to the *Bam*HI arms of the lambda vector EMBL4. *Mucor* DNA, cut with *Ava*I, was ligated to the *Sal*I arms of lambda EMBL3A to construct the E bank. The procedure for library construction is described in more detail by Maniatis et al (1978).

Preparation of bacteriophage DNA

Lambda DNA was purified from the phage clones (plaques) for further analyses by a small-scale, plate lysate method (Maniatis et al., 1982) or large-scale CsCl density gradient method of Carlock (1986).

Radiolabeling of DNA restriction fragments

DNA probes for hybridization were purified by resolving DNA restriction fragments on agarose gels and electroeluting the DNA fragments from gel slices using an electroelution unit from International Biotechnologies Inc (IBI). DNA fragments were then denatured and labeled as described below.

We used [alpha-^{32}P] dGTP in a random primer labeling procedure (Feinberg and Vogelstein, 1984) which allowed DNA probes to be labeled to high specific activity (> 1 x 10^8 CPM/ug DNA). The high specific activity could be achieved with quantities of DNA as low as 10 ng per reaction. A 25-ul mixture was prepared as follows: 10 ul oligo-labeling buffer (OLB buffer, see below), 2 ul of (1 mg/ml) bovine serum albumin, DNA 10-30 ng, 1 ul of 1 mM dATP, 1 ul of 1 mM dCTP, 1 ul of 1 mM dTTP (each triphosphate previously disolved in TE with $MgCl_2$ PH 7.0), 5 ul of [^{32}P]dGTP (DUPONT/NEN, 3000 Ci/mmole, 10 uCi/ul), 2 units of large fragment of Escherichia coli DNA polymerase I (BMB klenow fragment of DNA polymerase). The complete reaction was incubated at room temperature for at least 2 hrs. The reaction was stopped by addition of 2 ul EDTA (0.5 M) and the labeled DNA was purified by a gel filtration column (sephadex G-50-80, 5 ml packed volume) eluted with TE buffer (pH 8.0). The labeled DNA was eluted from the column and collected in the first peak response detected with a Geiger counter. The specific activity of the labeled DNA was measured by liquid scintillation spectroscopy of a 5 ul sample spotted onto a

glass fiber filter.

YRAS1, YRAS2, and HRAS DNA probes

Three different heterologous ras probes, including DNA fragments from two yeast ras genes (YRAS1, YRAS2) and a human cellular ras homologue (HRAS), were used in this study. The YRAS1 gene is carried on a 2.2 kb EcoRI/BamHI fragment cloned into pBR322 also cut with EcoRI/BamHI (total 6.6 kb). The YRAS1 DNA probe is a 1.6 kb DNA fragment generated in a HindIII digest of the 6.6 kb YRAS1-pBR322 DNA. THe YRAS2 gene is carried on a 3.0 kb EcoRI/HindIII fragment cloned into a pUC plasmid cut with EcoRI/HindIII (total 5.7 kb). The YRAS2 probe is a 1.2 kb fragment generated from a HpaI digest of the 5.7 kb YRAS2-pUC DNA. The HRAS gene is carried on a 6.6 kb BamHI fragment from the human genome cloned into BamHI site of pSV2NEO. The HRAS probe is a 2.9 kb SacI fragment which contains all of the exons of the HRAS gene.

Selection and screening of recombinant plasmid clones

The task of isolating a desired recombinant from a population of bacteria or phage was carried out by genetic selection and nucleic acid hybridization.

1.) Genetic selection:

The pUC9 vector carries an ampicillin resistance gene and a multiple cloning site in a B-galactosidase gene. These properties made it possible to screen transformed E. coli cells

for recombinant plasmids by resistance to the antibiotic ampicillin and insertional inactivation of the B- galactosidase gene. Transformed cells containing the recombinant plasmids grew on LB agar plates supplemented with ampicillin as white colonies (hydrolysis of the chromogenic substrate, X-Gal, generates a blue color).

2.) DNA hybridization methods:

Two DNA hybridization methods were used to screen recombinant clones in this study. For in situ plaque hybridization (Benton and Davis, 1977), recombinant DNA from phage in plaques was transferred to a nitrocellulose (N.C.) filter. The N.C. filter was placed face-up (DNA side up) onto a sheet of filter paper saturated with Southern base (0.5M NaOH, 1.5M NaCl) for 5 minutes to disrupt the phage coat and denature the DNA, and then placed on a second filter saturated with neutralization solution (1M Tris-HCl pH 8.0, 1.5M NaCl). Nitrocellulose filters were baked for 2 hr at 80^{o}C in a vacuum oven.

The second method was in situ colony hybridization (Gruntan and Hogness, 1975). Bacteria containing plasmids were spotted onto nitrocellulose filters and grown overnight at 37^{o}C on LB ampicillin medium. The N.C. filters were placed onto a sheet of 3 mm paper saturated with Southern base (5 minute), and then placed on by a second filter saturated with neutralization solution. The filters were air dried and baked at 80^{o}C for 2 hr under vacuum. The DNA on the filters was

hybridized to radioactive probes to detect recombinant clones (see below).

Restriction endonuclease analysis and agarose gel electrophoresis

The optimal reaction conditions for restriction endonuclease digests supplied by the manufacturer were followed. DNA restriction fragments were resolved by electrophoresis through 0.8 to 2.0 % agarose gels (Maniatis et al., 1982, P.150) with a Tris acetate buffer (TAE) system. Fragments of DNA in the gel were visualized by staining with the fluorescent dye ethidium bromide (0.5 ug/ml). Stained gels were photographed by transillumination with UV light (260 nm).

RNA samples were resolved by electrophoresis through denaturing formaldehyde-agarose gels with a diethyl pyrocarbonate (DEPC)-treated autoclaved MOPS/EDTA buffer: 0.2 M MOPS [3-(N-morpholino) propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA adjusted to PH 7.0. Ethidium bromide was added to the sample prior to electrophoresis in order to visualize RNA in agarose gels by transillumination. This method for efficient RNA staining was described by Fourney et al (1988).

Southern and Northern blot analyses of nucleic acids

DNA fragments, separated by electrophoresis through agarose gels, were denatured, transferred and immobilized.

Nitrocellulose filters (Schleicher and Schuell) were used to immobilize DNA fragment sizes greater than 300 bp and nylon membranes (S&S Nytran) were used to immobilize smaller DNA fragments (Southern, 1975). The DNA transfer buffer was 20X SSC (1X SSC is 0.15M NaCl plus 0.015M sodium citrate, PH 7.0). Transfer of DNA was allowed to proceed for 12-24 hr, after which the filter was air dried and baked for 2 hr at 80^{o}C under vacuum prior to hybridization. Transfer of RNA from formaldehyde-agarose gels was carried out by the procedure of Maniatis (1982).

The typical hybridization conditions for nitrocellulose filters were described by Maniatis (1982). Nitrocellulose filters were soaked for 2 hr in prehybridization solution (40% deionized formamide for low stringency or 50% formamide for high stringency, 5X Denhardt solution [1X Denhardt solution is 1% Ficoll (Pharmacia Fine Chemicals), 1% bovine serum albumin, 1% polyvinylpyrrolidone], 6X SSC, 0.1% sodium dodecyl sulfate, denatured salmon testes DNA [100 ug/ml], 5mM EDTA) at room temperature for 2-4 hr. Then a radiolabelled DNA probe was added to the prehybridization solution (5×10^5 to 1×10^6 CPM per ml). Hybridizations were allowed to proceed for 16-24 hr at 37^{o}C for low stringency or 42^{o}C for high stringency in a shaking water bath. Following hybridization, the nitrocellulose filters were washed twice for 15 min in 2X SSC 0.1% sodium dodecyl sulfate at room temperature and then once for 60 min in 2X SSC at 37^{o}C for a low stringency wash or 0.1X SSC at 65^{o}C

for a high stringency wash. Filters were dried in air and exposed to Kodak X-ray film at -70°C with an intensifier screen for 4 hr to several days.

RESULTS

Identification of *ras*-related sequences in *Mucor racemosus* genomic DNA

In order to detect the presence of *ras* homologues in the *Mucor racemosus* genome, Southern blot analysis of *M. racemosus* genomic DNA was conducted using gel purified, random primer-labelled *YRAS1*, *YRAS2* (Figure 1) and *HRAS* DNA probes (data not shown). These three DNA probes were previously described in the Materials and Methods section. *M. racemosus* genomic DNA was digested to completion with *Hin*dIII, *Eco*RI or *Bam*HI to identify genomic DNA fragments which might contain the *ras*-related sequence. Two strongly hybridizing restriction fragments of approximately 6.5 and 3.5 kb and three lightly hybridizing restriction fragments of approximately 24, 9.6 and 7.0 kb were detected in the *Eco*RI- digested DNA using the *YRAS2* probe. *Bam*HI-digested genomic DNA showed three strongly hybridizing restriction fragments of approximately 3.9, 3.8 and 3.6 kb and seven
weakly hybridizing restriction fragments. The *YRAS1* probe hybridized strongly with a 3.8 kb restriction fragment and weakly with a 24 kb (lighter band) fragment in *Bam*HI-digested genomic DNA, and hybridized also with 23, 9.6 and 7.0 kb

restriction fragments in EcoRI-digested DNA. Similarly, HindIII-digested genomic DNA had two restriction fragments (4.7, 3.5 kb) which hybridized with the YRAS1 DNA probe and three restriction fragments (4.7, 4.2, 3.5 kb) which hybridized with the YRAS2 DNA probe (data not shown). The HRAS DNA probe also hybridized strongly with one restriction fragment (7.0 kb) and weakly with one restriction fragment (4.1 kb) in EcoRI-digested M. racemosus genomic DNA.This probe also hybridized strongly with a 3.9 kb restriction fragment and weakly with a 14.5 kb restriction fragment in BamHI-digested genomic DNA (data not shown). The results in this experiment provided the initial evidence for the existence of ras-related genes in Mucor and encouraged us to screen an M. racemosus DNA library in order to clone the putative ras homologues.

Screening lambda/M. racemosus DNA libraries for ras homologous sequences

The C bank of M. racemosus DNA in lambda phage contained 1 x 10^6 plaque forming units per ml (pfu/ml). 1-5 x 10^3 pfu of the lambda library were plated on 3 separate agar plates and the resulting plaques transferred to nitrocellulose as described in Materials and Methods. The radiolabelled YRAS1 DNA probe was used to hybridize to the phage DNA which was fixed nitrocellulose circles (82 mm). Four positive recombinant phage clones (plaques) C1-C4 (Figure 2A) were isolated and subjected to a second in situ hybridization screening. Only 2

recombinants, C3 and C4, showed strong hybridization with YRAS1 (Figure 2B).

In similar fashion, Dr. J. Linz also screened the L and E libraries with the use YRAS2 DNA probe and isolated nineteen phage clones - called L1-L9, and E1-E10. At this time, I selected two strongly hybridizing phage clones (L1B and E10) from Dr. Linz analysis along with C4 for further cloning and nucleotide sequence studies. These clones were selected because they strongly hybridized with YRAS DNA probes and showed different restriction patterns (see below) in restriction endonuclease mapping analysis.

Grouping lambda phage clones by restriction patterns

DNA purified from the phage clones C4, L1B, and E10 were digested to completion with EcoRI, BamHI, HindIII, or EcoRI/HindIII. Restriction fragments were resolved by 1% agarose gel electrophoresis and subjected to Southern blot analysis. The YRAS1 probe hybridized with 10.5 kb EcoRI, 9.4 kb BamHI, and 4.7 kb HindIII restriction fragments in the C4 phage clone. In the L1B phage clone, 7.4 kb EcoRI, 3.8 kb BamHI, and 3.2 kb HindIII restriction fragments hybridized with YRAS1 DNA probe. The E10 phage clone which was screened from the E bank by the YRAS2 DNA probe showed no detectable hybridization with YRAS1 DNA probe (Figure 3). RsaI and Sua3A digests of the phage clones L1B and E10 were also analyzed by Southern hybridization with YRAS2 DNA probe and showed

different hybridization patterns (data not shown). Based on these data, the phage clones which contained ras homologues were grouped into several unique restriction patterns by Southern analysis. These results suggested that more than one ras gene was present in the M. racemosus genome.

Subcloning of M. racemosus ras genes (HC4, BL1B, SE10, and PE10) into the E. coli plasmid vector, pUC9

The 4.7 kb HindIII (HC4), 3.8 kb BamHI (BL1B), 1.25 kb SalI (SE10), and 1.65 kb PvuII (PE10) restriction fragments containing putative ras genes were cloned individually into pUC9 cut with HindIII, BamHI, SalI, and SmaI, respectively. These recombinant plasmids were transformed into JM83. The transformation efficiency of E. coli JM83 by recombinant plasmids containing ras-related gene was low (400 transformants/ug DNA) while the transformation efficiency of control plasmid, pUC9 was 1×10^4 transformants/ug DNA. The reason for the low efficiency was not clear. We screened transformants by in situ colony hybridization with YRAS1 (for HC4) or YRAS2 probes (for BL1B, SE10 and PE10). Only one of two putative transformants included a 4.7 kb C4 HindIII restriction fragment HC4 which was able to hybridize with YRAS1 (Figure 4A, plate a). Restriction mapping and Southern hybridization analysis confirmed that this recombinant plasmid contained the 4.7 kb HindIII restriction fragment (HC4) (Figure 4B). The recombinant plasmids containing SE10 and PE10 and BL1B were identified in a similar manner through restriction analysis and

Southern blot analysis with the YRAS2 DNA probe (data not shown). Dr. J. Linz subcloned the BL1B restriction fragment (3.8 kb BamHI) into pUC9 BamHI site and isolated the recombinant plasmid in the same fashion

Restriction endonuclease maps of HC4, BL1B, and PE10 sequences

HC4, BL1B and PE10 were digested with several restriction endonucleases singly and in combination to produce the restriction maps shown in Figure 5. These data show that the pattern of restriction enzyme recognition sites is unique to each of three clones. The SalI site of HC4 appears to be located at critical sequence for hybridization with the YRAS1 DNA because the probe did not hybridize to any restriction fragments whenever SalI was used to cut HC4.

A comparison of the hybridization pattern of original phage clones (C4, L1B and E10) with recombinant plasmids (pHC4, pBL1B, pPE10 and pSE10)

Southern blot analysis of restriction endonuclease digested phage clones with YRAS1, YRAS2 and HRAS DNA probes (Fig. 6.) showed that each recombinant phage contained a restriction fragment also found in the recombinant plasmid (Figure 5). The C4 phage clone and pHC4 contain a 0.7 kb HindIII/KpnI segment which hybridizes to the YRAS1 DNA probe. The L1B phage clone and pBL1B contain a 2.0 kb HindIII/PvuII segment which hybridizes to YRAS2. The E10 phage clone and pSE10 (or pPE10) contain a 1.25 kb SalI (or 1.65 kb PvuII)

fragment which hybridizes to YRAS2, but did not hybridize detectably with YRAS1. Therefore, each recombinant plasmid contained a subcloned ras gene in the same form as originally isolated from the lambda/Mucor racemosus library.

Determining the location of three ras homologues in the Mucor racemosus genome

In order to specifically measure the expression of individual ras genes in Mucor, we sought to isolate gene-specific DNA probes. Small DNA restriction fragments from each subclone, which hybridize to heterologous ras probes, were identified and gel purified (see Figure 6). These restriction fragments were resolved on an agarose gel and analyzed by Southern hybridization to look for cross hybridization (Figure 8). This experiment shows that the three DNA segments (0.7 kb HindIII/KpnI digest pHC4, 0.69 kb EcoRI/SalI digest M13Sau3A,and 1.25 kb SalI digest pSE10) did not hybridize to each other. These three DNA restriction fragments were then used as probes in Southern blot analysis of M. racemosus genomic DNA digested to completion with HindIII, BamHI, or EcoRI. The restriction fragments were resolved by agarose gel electrophoresis (10 ug of DNA per lane) and transferred to a nitrocellulose filter. Replica filters were then incubated individually with one of the DNA probes shown in Figure 5. Each of the specific probes hybridized to a unique M. racemosus genomic DNA restriction fragment (Figure 7) corresponding to one of the hybridizing restriction fragments which were

detected by Southern analysis of M. racemosus using the heterologous yeast RAS probes (Figure 1). These data demonstrated that the three ras homologues reside at unique locations in the M. racemosus genome.

Northern analysis of M. racemosus RNA with gene-specific probes

The three gene-specific probes (A, B, and C from Figure 5) were labelled to high specific activity with [alpha-^{32}P] dGTP and used to analyze the accumulated levels of ras- related mRNA from several cell morphologies of M. racemosus to determine whether there was a morphology-specific pattern of gene expression for Mucor ras genes. RNA was purified from sporangiospores, germlings, yeasts, and yeast cells which had been induced to undergo morphogenesis to hyphae. Total RNA (20 ug/lane) was resolved on formaldehyde-agarose gels and analyzed by Northern analysis. The pattern of transcript accumulation detected by each probe was quantitated visually. In this analysis only the E10 probe hybridized at detectable levels to an mRNA (Figure 9). In the control experiment (data not shown) an identical nitrocellulose filter was probed with TEF-1, a gene encoding elongation factor 1 alpha, in M. racemosus. This gene is expressed at constant levels in each of morphologies tested (Linz and Sypherd, 1987). In this experiment the TEF-1 probe hybridized at significantly higher levels with germlings mRNA than with any of other three morphologies. These data suggest that the higher signal intensity observed in germling

mRNA with the E10 probe was an artifact in the procedure. This artifact may have arisen as a result of an measuring error The other two probes (C4 and L1B) did not hybridize to mRNA which indicates that the genes were either not transcribed or transcribed at levels too low to be detected (data not shown). These data suggested that there was differential expression of the three genes encoding Mucor ras proteins.

In a recent experiment, the polyadenylated mRNA fraction from M. racemosus cells was purified by oligo(dT) cellulose column chromatography. The mRNA was resolved on a formaldehyde-agarose gel, transferred to nitrocellulose and analyzed by Northern analysis with the gene-specific probes (data not shown). There were different gene transcript levels with each probe. For example, the levels of putative E10 mRNA was several fold higher than the level of putative C4 mRNA. The level of putative C4 and E10 mRNAs did not vary significantly when observing the different cell morphologies suggesting that they are expressed constitutively (i.e., they are always expressed). However, the putative L1B mRNA was detected only in sporangiospores. These preliminary data suggested strongly that there are three genes encoding ras protein in M. racemosus and that at least one gene, L1B, showed a morphology-specific pattern of transcript accumulation.

DISCUSSION

During the past few years, the biological function of *ras* proteins has been studied by comparing their amino acid sequence to other proteins whose function is known or by analysis of their biochemical activities in eukaryotic organisms. In the course of these studies it has become apparent that regardless of their phylogenetic origin, the *ras* proteins have the ability to bind guanine nucleotides. They also have GTPase activity, and are bound to the cytoplasmic membrane. Their significant sequence homology with G proteins suggests that *ras* proteins may participate in signal transduction pathways which allow cells to communicate with their surroundings. Mutations which alter the function of GTP exchange rate, GTP hydrolytic activity, or *ras* transcription level were found to alter cell growth (proliferation) and development (cell differentiation). In *S. cerevisiae*, the experimental data suggest that at least one functional *ras* gene is required to maintain cell growth. Other experimental data indicate that the *ras* gene products of *S. cerevisiae* may be regulatory proteins that control cAMP and PI metabolism. In *S. cerevisiae* cells, the *YRAS2* gene
is involved in regulation of the adenylate cyclase pathway.

However, the role of genes coding for *ras* proteins in higher eukaryotic organisms is not clear. There are several things that we want to discover about *ras* protein function using *Mucor* as a model system including the biological function of *ras* and the identity of effector proteins, receptor proteins, and other components of a putative signal transduction pathway. These data will hopefully allow us to clarify the connection between expression of mutant *ras* and tumor formation. *Mucor* is a good model for the study of a possible correlation between signal transduction and cell differentiation because it can grow in 4 different morphologies with different growth rates in response to an external signal. These observations directly led to our hypothesis that one or more *ras* proteins are involved in signal transduction pathways which allow *Mucor* to respond to an external signal to change its morphology and growth rate.

The identification and isolation of the *ras* genes from *M. racemosus* was accomplished by the use of three heterologous DNA probes, *YRAS1*, *YRAS2*,and *HRAS*. The *Hin*dIII, *Bam*HI, *Sal*I and *Pvu*II restriction fragments of phage clones C4, L1B, and E10 which hybridized to these probes in Southern blot analysis, were subcloned into a pUC9 plasmid vector and used to generate restriction endonuclease maps (Figure 5). The restriction sites located in each of the three subclones did not overlap. Southern blot analysis of *M. racemosus* genomic DNA with ^{32}P-labelled gene-specific DNA probes from each of the

subcloned *Mucor ras* genes (Figure 7), provided additional evidence for the presence of three genes in the *M. racemosus* genome. These gene-specific probes hybridized to unique restriction fragments in the genomic DNA but did not hybridize to each other suggesting that the *Mucor ras* genes are located at unique locations in the genome.

The three *Mucor ras* gene-specific DNA probes were also used in standard Northern analysis of RNA purified for several cell morphologies of *M. racemosus*. These preliminary data suggest that all three genes are expressed in the cell morphologies studied. However, the accumulated level of transcript derived from each gene varied considerably, with E10 mRNA present in several fold greater levels than either the C4 transcript or L1B transcript. The level of putative E10 and C4 mRNAs varied little among the cell morphologies studied suggesting that they are expressed constitutively. The putative L1B mRNA was only present in detectable quantity in sporangiospores. These data suggest that there is differential expression of the genes encoding *ras* proteins in *M. racemosus*. At least one gene, L1B, shows a morphology- specific pattern of transcript accumulation. These data are preliminary and must be confirmed by further experiments.

Expression of each of the *Mucor ras* genes may be regulated during cellular proliferation such that changes in the requirements for individual *ras* proteins results in changes the level of each these genes' activity. This may provide a crude

mechanism to regulate the *ras* protein level and activity in the cell. The *ras* genes in *M. racemosus* may represent a gene family in which the individual genes are developmentally regulated. Because we do not know the exact location for each of the *ras* gene in the three gene-specific probes, the data must be regarded as preliminary. We do know that each of these *Mucor* probes hybridizes with more than one heterologous *ras* gene. However, only the L1B gene containing the 690 bp (base pair) gene-specific probe has been subjected to nucleotide sequence analysis. The L1B gene fragment has been completely sequenced in our laboratory. These data showed that the 690 bp L1B probe contains an open reading frame of 390 bp which shares 70-80% homology at the amino acid level with the 5' end of the yeast *ras* genes. We speculate that the remaining 300 bp in the L1B gene fragment are flanking sequences, which represent the *ras* promoter sequence and an intergenic sequence which is rich in adenine and thymine residues. We currently are unable to determine if the *ras* sequences of the L1B probe hybridize with mRNA or if it is the flanking sequence. To clarify the data from Northern analysis, we must identify internal restriction fragments from C4, L1B, and E10 to use as probes. After more nucleotide sequence data from the three *Mucor* *ras* genes is available, we will repeat these experiments with new DNA probes.

In future work, we plan to analyze the regulation of transcription of the *ras* genes using molecular genetic

technology. We also will conduct S1 nuclease mapping to locate the ras promoter and terminator sequences and confirm the presence of intervening sequences which have been tentatively located in the nucleotide sequence of L1B. These experiments are designed to carefully explore if this gene family is being regulated differentially during cellular morphogenesis.

Now that we have cloned several ras genes in Mucor, we can use these genes as tools to initiate studies which are designed to reveal the biological function of ras genes in Mucor. We will change the activity of these genes through in vitro mutagenesis or to introduce multiple copies of ras gene into Mucor cells to overexpress ras gene and observe any differences in the phenotype of the cells and any effects of altered ras expression/activity on cAMP or PIP_2 metabolism. Finally we plan to generate a series of monoclonal antibodies (MAb) which will bind specifically to each of the Mucor ras proteins. These MAb can be used to 1) confirm changes in the quantity of ras proteins; 2) determine the location of ras proteins in Mucor cells; and 3) inhibit or alter ras protein activity by binding to the protein in cell extracts of membrane preparations. We can then measure any effect of MAb binding on cAMP or PIP_2 metabolism.

The dimorphic fungus Mucor racemosus is a useful model for studying the possible relationship between ras genes expression and cell differentiation. A basic understanding of growth and development in Mucor racemosus should help us to more

effectively use the industrial Mucor spp to improve yield of food products such as organic acids, fermented food, enzymes, or to prevent food spoilage.

SUMMARY

Two hypotheses were established at the beginning of the research: 1) *M. racemosus* contains *ras* homologues. 2) There are different expression levels of the *ras* gene products. The experimental data suggest that these two hypotheses are correct. Southern hybridization data and restriction endonuclease analysis suggest that the 3 clones represent 3 *Mucor ras* genes. These genes are C4, L1B, and E10. Northern blot analysis of *Mucor* RNA suggests that the three *Mucor ras* genes are expressed at different levels and that one of these genes (L1B) is expressed in a morphology- specific manner.

APPENDIX

Figure 1. Southern blot analysis of M. racemosus genomic DNA with ^{32}P-labelled YRAS DNA probes. 20 ug of M. racemosus genomic DNA were fragmented with the indicated restriction endonucleases and resolved by agarose gel electrophoresis. Lanes: YRAS1,B; we used YRAS1 as a probe to detect putative ras homologues in a BamHI digest of M. racemosus genomic DNA: YARS1,E; YRAS1 probe, EcoRI digest: YRAS2,B; YRAS2 probe, BamHI digest: YRAS2,E; YRAS2 probe, EcoRI digest: kb; molecular size markers (HindIII digest of lambda DNA). (Autoradiogram provided by Dr. J. Linz).

Figure 1.

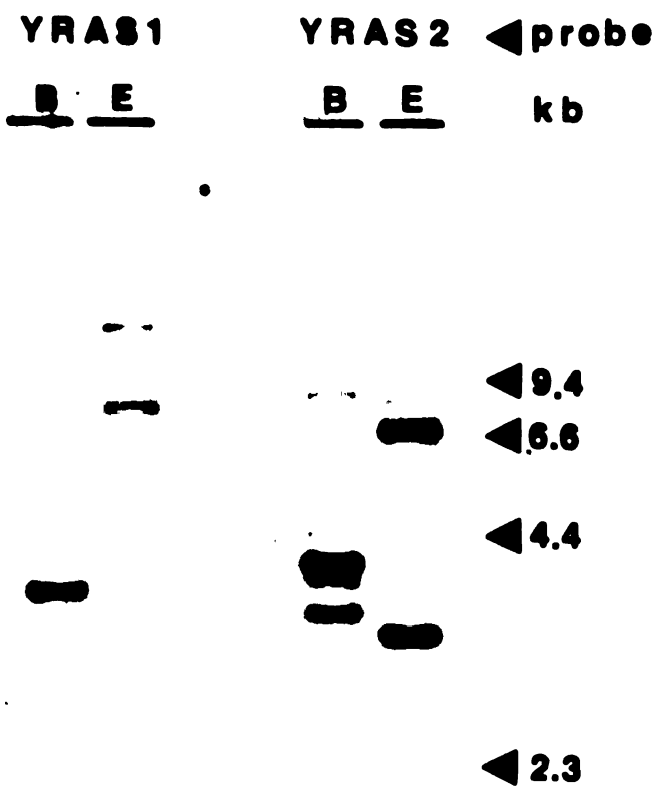


Figure 2: Autoradiograms of recombinant DNA library screening by in situ plaque hybridization. A radiolabelled YRAS1 DNA probe was used to hybridize duplicate nitrocellulose filters using the low stringency conditions which are outlined in Materials and Methods.

A; 1. Control, E. coli only. 2. First round screening of approximately 3,000 recombinant lambda plaques (C bank).

B; Duplicate filters of second round screening of one positive recombinant phage (C3) isolated from first round screening.

C; Duplicate filters of second round screening of a second positive recombinant phage (C4) isolated from first round screening.

Figure 2.

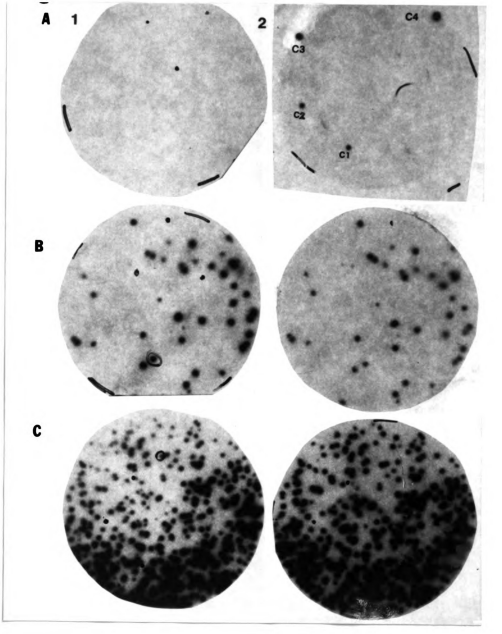


Figure 3: Southern blot analysis of purified recombinant phage DNA with a ^{32}P-labelled YRAS1 DNA probe. DNA purified from the phage clones C4, L1B, and E10 was digested with the indicated restriction endonucleases and analyzed by Southern hybridization under low stringency conditions (see Materials and Methods). Hybridization was performed at 37^{o} for 12-18 hours. Lanes: 1-3; EcoRI digest of C4, E10, L1B phage clones DNA. 4-6; BamHI digest. 7-9; HindIII digest. 10-12; EcoRI/HindIII digest. kb; molecular size markers (HindIII digest of lambda DNA). Approximate DNA fragment sizes are indicated in kbp. (E = EcoRI, B = BamHI, H = HindIII, E/H = EcoRI/HindIII)

Figure 3.

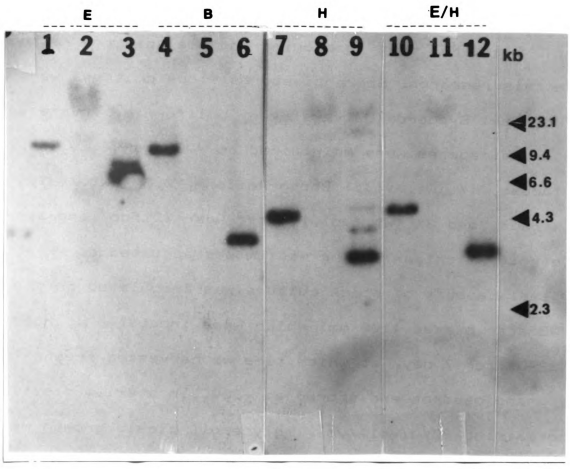


Figure 4: A; Autoradiograms of colony screening by in situ colony hybridization using ^{32}P labelled YRAS1 DNA probe. Two colonies from a primary in situ colony hybridization thought to contain a 4.7 kb HindIII fragment from C4 recombinant phage were streaked onto agar plates containing LB medium, X-Gal, and Ampicillin. 12 single colonies from each plate were then patched onto nitrocellulose filters on new agar plates. In situ hybridization was carried out on these filters with a radiolabelled YRAS1 DNA probe under low stringency conditions (see Materials and Methods). In plat a, the colonies strongly hybridized with the YRAS1 probe; In plate b, the colonies did not hybridize, including the control colony which represents a pUC9 plasmid with no insert. B; Autoradiogram of Southern blot analyses of the subcloned C4 gene. Plasmid DNA was prepared from five colonies from plate a using the alkaline lysis minipreparation procedure (see Materials and Methods) and cut with HindIII to generate a 4.7 kb HindIII insert fragment from the C4 lambda clone and a 2.7 kb pUC9 vector fragment. The DNA was resolved on a 1% agarose gel transferred to nitrocellulose filter. Southern blot analysis was conducted with the YRAS1 DNA probe under low stringency conditions.

Figure 4.

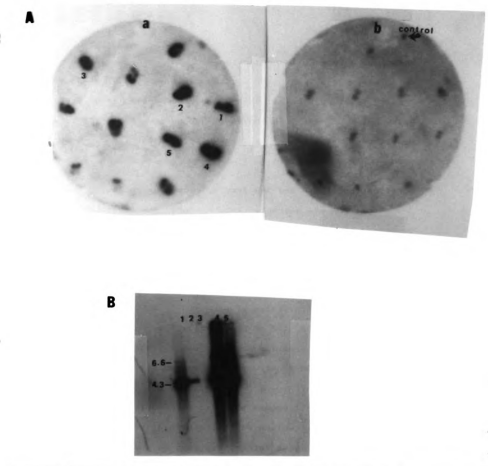
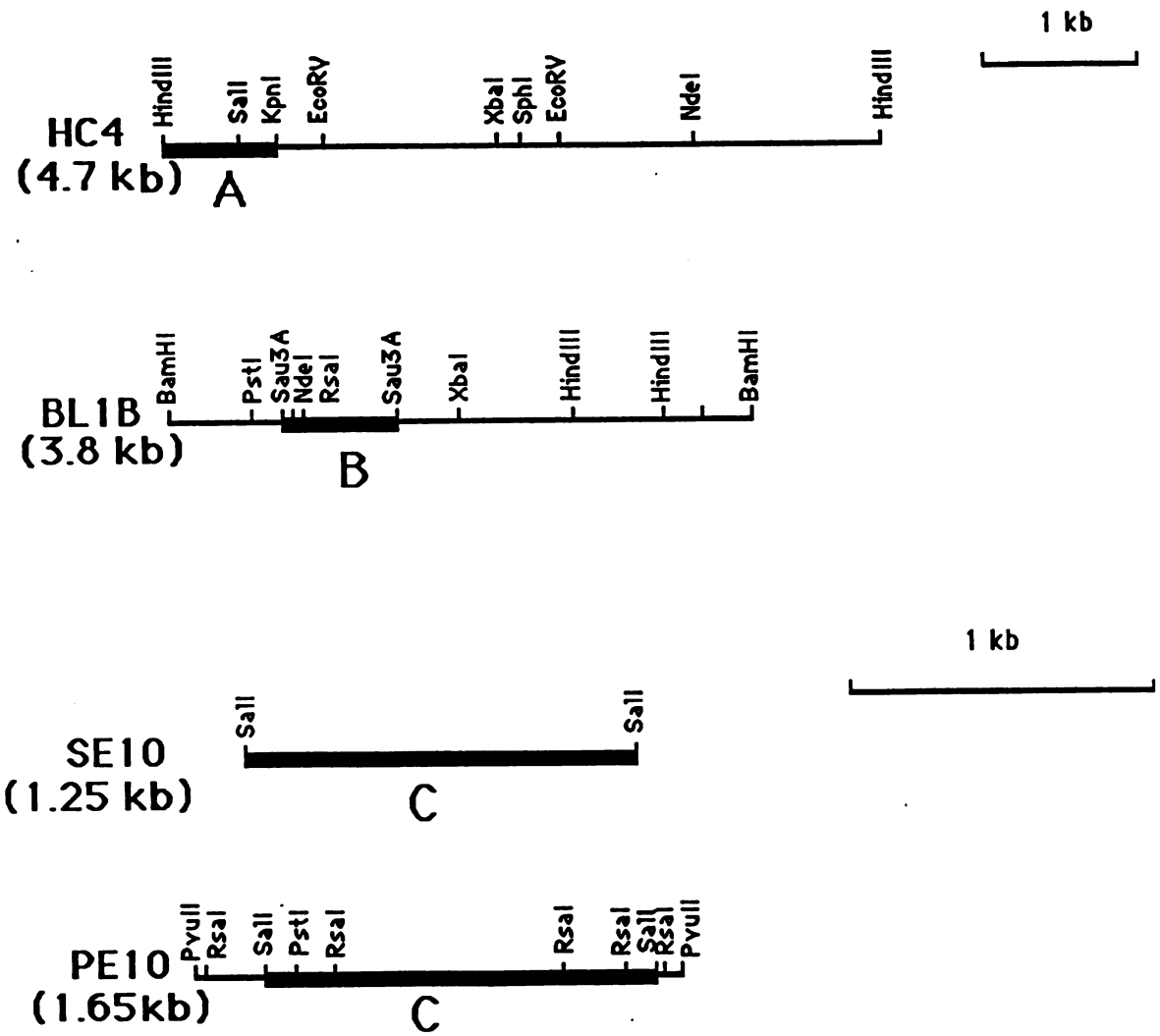


Figure 5: Restriction endonuclease maps of M. racemosus ras homologues subcloned into pUC9. the ras containing recombinant plasmids pHC4, pBL1B, pSE10, and pPE10 were cut with several restriction endonucleases. The DNA restriction fragments were resolved by electrophoresis through a 1% agarose gel and their sizes were analyzed to generate restriction maps. Only the M. racemosus DNA inserts are shown in the maps. The ras homologous region in each clone as determined by Southern blot analysis of this gel with YRAS1 and YRAS2 probes (data not shown) is indicated by the highlighted line segment.

Figure 5.


1 kb
HindIII
SalI
KpnI
EcoRV
XbaI
SphI
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NdeI
HindIII
HC4
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BamHI
PstI
Sau3A
NdeI
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XbaI
HindIII
HindIII
BamHI
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B
1 kb
SalI
SalI
SE10
(1.25 kb)
C
PvuII
RsaI
SalI
PstI
RsaI
RsaI
RsaI
SalI
RsaI
PvuII
PE10
(1.65kb)
C

Figure 6: Southern blot analysis of M. racemosus ras genes in recombinant lambda clones using ^{32}P-labelled DNA probes. Three different ^{32}P-labelled probes were used to hybridize to replicate sets of DNA restriction fragments to generate the autoradiograms shown in panel a, b and c. These restriction fragments were resolved by agarose gel electrophoresie and transferred to nitrocellulose filters. Low stringency hybridization conditions were used. a; A ^{32}P-labelled probe prepared from YRAS1 DNA was used in hybridizations against; lane2, C4 digested with HindIII/KpnI; lane3, L1B digested with HindIII/PvuII; lane4, E10 digested with SalI; lane5, E10 digested with PvuII. b; A YRAS2 ^{32}P-labelled probe was used in hybridizations against C4 lambda, L1B lambda, E10 lambda restriction enzyme digested segment. c; A HRAS ^{32}P-labelled probe was used. The DNA in lanes 2 through 5 in b and c are replica sets of the DNA in panel a. The hybridizations were performed under low stringency conditions.

Figure 6.

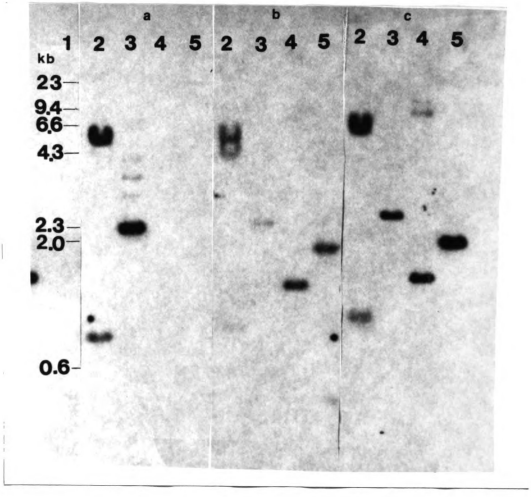


Figure 7: Southern blot analysis of M. racemosus genomic DNA with ^{32}P-labelled DNA probes from the ras homologous regions of pHC4, pBL1B, pSE10. In panels a, b and c, three different ^{32}P-labelled probes were used to probe 3 replica sets of M. racemosus genomic DNA digested with restriction endonucleases. High stringency hybridization conditions (42^{o}C, 50% formamide, 5X SSC) and high stringency wash conditions (65^{o}C, 0.1X SSC for 1 hr)were used. Panel a, A ^{32}P-labelled probe was prepared from a gel purified HindIII/KpnI restriction fragment from pHC4 (0.7 kb); panel b, Gel purified Sau3A restriction fragment from pBL1B (0.69 kb); Panel c, Gel purified SalI restriction fragment from pSE10 (1.25 kb) (See Figure 5. the regions of highlighted line segment). Lanes: S, molecular size standard (HindIII digest of lambda DNA). lane1; HindIII digest of M. racemosus DNA. lane2; BamHI digest of M. racemosus DNA. lane3; EcoRI digest of M. racemosus DNA. (H = HindIII, B = BamHI, E = EcoRI)

Figure 7.

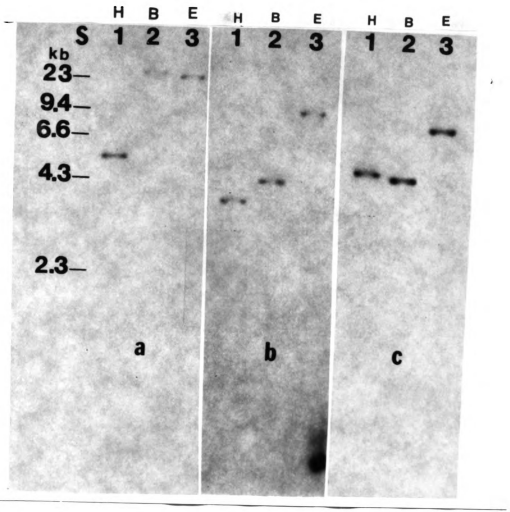


Figure 8: Cross hybridization of three Mucor racemosus ras homologous genes by Southern blot analysis. DNA restriction fragments were purified by agarose gel electrophoresis and used as DNA probes. Lanes: kb, molecular size makers. In panel a, b, and c; three different DNA probes indicated by DNA fragment A, B, and C see in Figure 5 were used to probe nitrocellulose filters. lane1; SalI digest pSE10. lane2; SalI/EcoRI digest M13Sau3A. lane3; HindIII/KpnI digest pHC4. High stringency hybridization and wash conditions were used (65^{o}C, 0.1 X SSC for 8 hr).

Figure 8.

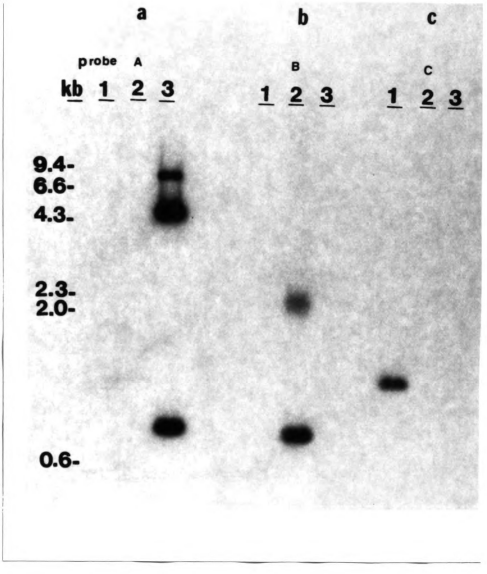
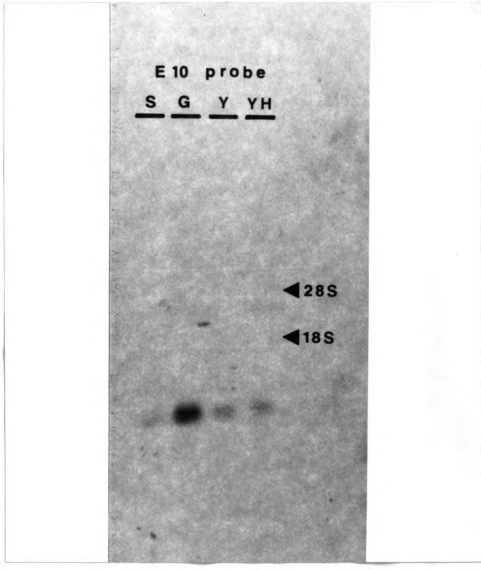


Figure 9: Northern analysis of Mucor racemosus RNA with three M. racemosus gene-specific probes A, B, C (shown in Figure 5). Total RNA was purified from sporangiospores (S), yeast cells (Y), germilings (G), and yeast cells which were induced to undergo morphogenesis (YH) as described in Materials and Methods. The purified total RNA (20 ug/lane) was resolved on formaldehyde- agarose gels and analyzed by Northern hybridization analysis with ras gene specific probes A, B, C under high stringency conditions. Only the E10 probe hybridized detectablly with an mRNA. Two other probes did not show any detectable hybridization with the RNA (data not shown).

Figure 9.



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