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Expression of a Gene Family in the Dimorphic Fungus *Mucor racemosus* Which Exhibits Striking Similarity to Human *ras* Genes

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Sporulation, spore germination, and yeast-hypha dimorphism in the filamentous fungus *Mucor racemosus* provide useful model systems to study cell development in eucaryotic cells. Three *RAS* genes (*MRAS1*, *MRAS2*, and *MRAS3*) from *M. racemosus* have been cloned, and their nucleotide sequences have been determined. The predicted amino acid sequences and the sizes of the three *MRAS* proteins exhibit a high degree of similarity with other *ras* proteins, including that encoded by H-*ras*, which have been implicated in regulation of proliferation and development in eucaryotic cells by mediating signal transduction pathways. The *MRAS* proteins show conservation of functional domains proposed for *ras* proteins, including guanine nucleotide interaction domains, an effector domain, a binding epitope for neutralizing antibody Y13-259, and the COOH-terminal CAAX box, which is a site of thiocylation and membrane attachment. Amino acid sequences unique to each *MRAS* protein occur adjacent to the CAAX box, consistent with the location of the hypervariable region in other *ras* proteins. Northern (RNA) analysis was used to study expression of the three *MRAS* genes in relation to cell development. Gene-specific probes for two of these genes, *MRAS1* and *MRAS3*, hybridized to different 1.3-kb mRNA transcripts. The accumulation of these transcripts depended on the developmental stage, and this pattern was different between the two *MRAS* genes. No transcript for *MRAS2* was detected in the developmental stages examined. The unique patterns of *MRAS* transcript accumulation suggest that individual *MRAS* genes and proteins may play distinct roles in cell growth or development.

The fungus *Mucor racemosus* is a saprophytic zygomycete that provides a useful system for studying eucaryotic cell differentiation. It is a relatively simple organism with a small genome (1.6×10^7 bp per haploid genome [34]) and an available transformation system (36) which facilitate molecular genetic investigations. *M. racemosus* is dimorphic: it exhibits filamentous (hyphal) growth in the presence of oxygen, but under anaerobic conditions with a fermentable hexose available, the organism grows as a single-celled, budding yeast (4). The fungus also undergoes morphogenesis during sporulation and germination of two asexual spore types, sporangiospores and arthroconidia, and a sexual spore called a zygospore.

Morphogenesis in *M. racemosus* is correlated with changes in cyclic AMP (cAMP) and phospholipid metabolism (4, 10, 13, 14, 21–23). *M. racemosus* yeast cells contain about four times the level of intracellular cAMP than do hyphal cells. During hypha-to-yeast morphogenesis, there is a large burst in cAMP synthesis (14). Conversely, there is a large decrease in cAMP levels during yeast-to-hypha morphogenesis. Under aerobic conditions, which normally induce yeast-to-hypha morphogenesis, exogenous cAMP or dibutyryl cAMP added to yeast cells prevents morphogenesis and many biochemical changes that accompany this process (7, 14). A similar correlation between dimorphism and cAMP metabolism is reported for *Mucor rouxii* (24). In *Mucor mucedo* and *Mucor genevensis*, spherical growth of sporangiospores at an early stage in germination is accompanied by a burst in cAMP synthesis (22). There is also a large increase in phospholipid metabolism during yeast-to-hypha morphogenesis, especially in the turnover of phosphatidylethanolamine and phosphatidylinositol (PI) (13). Cerulenin prevents phospholipid turnover and yeast-to-hypha morphogenesis under conditions in which cell growth continues at rates typical of yeast cells.

The implication of cAMP and PI as secondary-messenger molecules in signal transduction pathways involving *ras* proteins, the ubiquity of *ras* proteins in eucaryotes, and the putative role of *ras* in cell differentiation and proliferation (2, 28) suggested that *ras* proteins may play a role in regulation of cellular morphogenesis in *M. racemosus*. *ras* proteins bind guanine nucleotides, have GTPase activity, are associated with the plasma membrane, and therefore have similarity to G proteins in regard to these biochemical characteristics (11). *ras* proteins, like G proteins, may therefore function as transducers in signal transduction pathways.

Commonly studied models of *ras* function in eucaryotes have been the *Saccharomyces cerevisiae RAS* genes. The *YRAS2* protein increases cAMP levels via stimulation of adenylate cyclase (20, 31, 35). Yeast *RAS1* protein has a lesser effect on cAMP metabolism but may be more important in regulation of glucose-stimulated PI turnover. Developmental regulation of a single *ras* gene, Dd*ras*1, has been observed in another fungus, the slime mold *Dictyostelium discoideum* (26). Transcript accumulation in this organism was inducible by cAMP, suggesting that *ras* function in this organism may also be linked to the cAMP developmental cascade. Mammalian *ras* proteins are apparently not directly related to the adenylate cyclase system but more likely are associated with the PI signaling pathway (2, 28). Specific point mutations in mammalian *ras* genes can confer trans-

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forming ability, and mutated *ras* are the most frequently identified oncogenes in human cancer (2, 3).

Prompted by the similarities between biochemical correlates of morphogenesis in *M. racemosus* and the suspected functions of *ras* proteins in other cell types (2, 28), we have cloned three *RAS* genes (*MRAS1*, *MRAS2*, and *MRAS3*) from this fungus to investigate their possible involvement in cell differentiation. The *MRAS* genes in *M. racemosus* show extensive sequence similarity to members of the *ras* gene family. Two *MRAS* genes are differentially expressed in different developmental stages and during cellular morphogenesis. Because of the nucleotide sequence and size similarities between *MRAS* genes and H-*ras*, *MRAS* protein function in this simple eucaryote may provide important insights into *ras* function in mammalian cells and malfunctions in *ras* protein activity that lead to tumor development.

MATERIALS AND METHODS

Strains and plasmids. DNA was isolated from *M. racemosus* Fresenius (*Mucor circinelloides* f. *lusitanicus* van Tiegham f. Schipper) ATCC 1216b. Lambda libraries of *M. racemosus* 1216b genomic DNA were kindly provided by Paul Sypherd, University of California, Irvine (in lambda EMBL4) and John Paznokas, Washington State University (in lambda EMBL3a). pUC9, pUC18, and pUC19 were used to subclone DNA fragments for restriction endonuclease analysis and for use as probes; fragments were subcloned into M13mp18, m13mp19, and pUC19 for deoxynucleotide sequencing. *Escherichia coli* LE392 was used as the host for EMBL3a and EMBL4 libraries; *E. coli* DH5α and JM101 were hosts for pUC and M13 vectors, respectively. *S. cerevisiae RAS1* and *RAS2* genes were supplied by M. Wigler, Cold Spring Harbor Laboratory, and the human c-H-*ras*1 gene was supplied by E. Stanbridge, University of California, Irvine.

Chemicals. Restriction endonucleases and nucleic acid-modifying enzymes were purchased from Boehringer Mannheim Biochemicals. DNA sequencing was performed by using the Sequenase DNA-sequencing kit from United States Biochemical Corp. New England Nuclear was the source for [α-^{35}S]dATP for DNA sequencing and [α-^{32}P] dGTP for radiolabeled DNA probes. Other reagents were obtained from Sigma Chemical Co. and were of reagent grade or better.

Hybridization of *ras* probes to *M. racemosus* genomic DNA. DNA was extracted from *M. racemosus* germlings by the method of Cihlar and Sypherd (5). *M. racemosus* genomic DNA was digested with *Bam*HI or *Eco*RI, separated by agarose gel electrophoresis, and blotted to nitrocellulose filters (19). Radiolabeled DNA probes were prepared from *YRAS1* (1.6-kb *Hin*dIII fragment), *YRAS2* (1.2-kb *Hpa*I fragment), and c-H-*ras*1 (2.9-kb *Sac*I fragment) by random-primer extension (9). Southern hybridization analysis (19) was performed under low-stringency conditions in hybridization buffer (6× SSC [1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0], 40% formamide, 5× Denhardt solution [1× Denhardt solution is 0.1% Ficoll, 0.1% bovine serum albumin, and 0.1% polyvinylpyrolidone], 0.1% sodium dodecyl sulfate, 5 mM EDTA, salmon sperm DNA [100 μg/ml]) at 37°C for 16 h. The nitrocellulose filters were washed in 2× SSC–0.1% sodium dodecyl sulfate two times for 15 min each time at room temperature and in the same buffer at 37°C for 1 h.

Screening of *M. racemosus* genomic DNA libraries by hybridization to heterologous probes. Libraries of *M. racemosus* genomic DNA in lambda EMBL4 and lambda EMBL3a were screened with radiolabeled *RAS1* and *RAS2* probes by in situ plaque hybridization (19) under the low-stringency conditions described above.

DNA sequencing. Deoxynucleotide sequences of *MRAS1*, *MRAS2*, and *MRAS3* were determined by the dideoxynucleotide chain termination procedure (27), using overlapping restriction fragments cloned into M13mp18, M13mp19, and pUC19.

Probes for Northern (RNA) analysis. Two categories of restriction fragments from *MRAS* genes (see Fig. 5) were selected as probes for Northern analysis. Probe 1 (1,000-bp *Eco*RV-*Xba*I fragment from *MRAS1*), probe 3 (580-bp *Pvu*II-*Bam*HI fragment from *MRAS2*), and probe 5 (800-bp *Hin*dIII fragment from *MRAS3*) were gene specific and encompassed DNA encoding the hypervariable domain and 3′-flanking DNA of each of the *MRAS* genes. Probe 2 (313-bp *Kpn*I-*Eco*RV fragment from *MRAS1*), probe 4 (355-bp *Eco*RV-*Pvu*II fragment from *MRAS2*), and probe 6 (450-bp *Sac*I-*Hin*dIII fragment from *MRAS3*) contained DNA fragments located entirely within the coding region of each gene (internal probes). The internal probes shared sequence similarity among the three *MRAS* genes. A probe was also prepared from *M. racemosus TEF-1* (18, 29), a gene that encodes elongation factor 1α, to use as a control for normalizing for differences in the quantity of RNA loaded onto agarose gels (see below).

Restriction fragments used as probes in Northern analysis were radiolabeled by random-primer extension (9).

Fungal culture and isolation of poly(A)$^+$ RNA. Sporangiospores, germlings, and yeasts were obtained from *M. racemosus* as previously described (18). Germlings (12 h old) were induced to shift to yeasttype growth by replacing air with CO_2; these germling-to-yeast transition cells were harvested after 1 h. Yeast-to-hypha transition cells were induced by replacing CO_2 in a yeast culture with air and harvesting cells 3 h later. Cells were rapidly chilled in an ice water bath and immediately harvested onto Whatman no. 1 filter paper by vacuum filtration.

Sporulating fungal tissue was collected as follows. Sporangiospores (10^5) were spread on the surface of YPG agar (2% glucose, 1% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 0.3% yeast extract, 1.5% Bacto-Agar [Difco], pH 4.5) in a plastic petri dish (100 by 15 mm) and incubated at 22°C under normal laboratory lighting. After 2 days, the colony had begun to sporulate. Each plate was flooded with liquid N_2, and the frozen (brittle) aerial mycelium (which included sporangiophores, immature sporangia, and aerial hyphae) was harvested with a cold spatula, carefully avoiding mycelium growing directly on the surface of the medium. Aerial mycelium from each plate yielded approximately 160 μg of total RNA.

All harvested tissue was frozen in liquid N_2 and ground in a mortar previously chilled with liquid N_2. RNA was extracted with a hot phenol procedure as previously described (18). All glassware used to prepare RNA was baked overnight at 200°C, and solutions were treated with diethyl pyrocarbonate (1). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity chromatography (1).

Northern analysis. Approximately equal amounts of poly(A)$^+$ RNA from each of the developmental stages indicated in Fig. 6 were resolved by electrophoresis in 1% agarose-formaldehyde gels (19). Four identical sets of RNA samples were resolved on identical agarose gels, transferred to nitrocellulose filters (19), and hybridized (18) to equal quantities of one of the radiolabeled *MRAS* probes or the

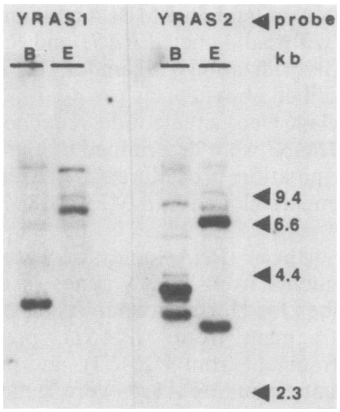

FIG. 1. Hybridization of *YRAS1* and *YRAS2* probes to *M. racemosus* genomic DNA. *M. racemosus* DNA digested with *Bam*HI (lanes B) or *Eco*RI (lanes E) was separated by electrophoresis through a 1% agarose gel; after transfer to a nitrocellulose membrane, duplicate sets of lanes were probes separately with either *YRAS1* or *YRAS2*.

TEF-1 probe. The filters were washed under high-stringency conditions (2%× SSC–0.1% sodium dodecyl sulfate twice for 15 min each time at 37°C and then for 60 min at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate) and exposed to Kodak XAR-5 film with an intensifier screen for 96 h (*MRAS* probes) or 1 h (*TEF-1* probe). Bands on autoradiographs were quantified by using a scanning densitometer.

Northern analyses were repeated for a total of three times with the 3′ gene-specific probes (probes 1, 3, and 5) and twice with the internal probes (probes 2, 4, and 6). For Northern analysis of total RNA, the concentration of RNA was quantitated by the A_{260} of an aqueous solution (1 U = 40 μg/ml). A 20-μg sample of RNA was loaded per lane on agarose formaldehyde gels and analyzed as above.

Nucleotide sequence accession number. The sequences of *MRAS1*, *MRAS2*, and *MRAS3* have been assigned the GenBank accession numbers M55175, M55176, and M55177, respectively.

RESULTS

Cloning *MRAS* genes. Several DNA fragments in restriction digests of *M. racemosus* genomic DNA hybridized to radiolabeled DNA probes from *S. cerevisiae RAS1* (*YRAS1*) and *RAS* (*YRAS2*) (Fig. 1) and human c-H-*ras*1 (data not shown). *YRAS2* and c-H-*ras*1 probes hybridized strongly to two common *M. racemosus* *Bam*HI restriction fragments (3.6 and 3.9 kb). The *YRAS2* probe hybridized weakly to a third *Bam*HI fragment (3.8 kb). The *YRAS1* probe also hybridized to different degrees with two of these same fragments (3.8 to 3.9 kb). These data suggested that the *M. racemosus* genome contains at least three genes with sequence similarity to the heterologous *ras* genes.

Screening lambda libraries of *M. racemosus* genomic DNA by in situ plaque hybridization yielded four lambda clones that hybridized to *YRAS1* and eight lambda clones that hybridized to *YRAS2* probes. On the basis of restriction endonuclease analysis, these lambda clones fell into three groups containing either a 4.2-kb *Hin*dIII fragment, a 3.8-kb *Bam*HI fragment, or a 4.3-kb *Bam*HI-*Sma*I fragment that hybridized to *YRAS* probes. These DNA fragments were subcloned into pUC9 or pUC19 from representative lambda clones from each restriction group and were called *MRAS1*, *MRAS2*, and *MRAS3*, respectively (Fig. 2).

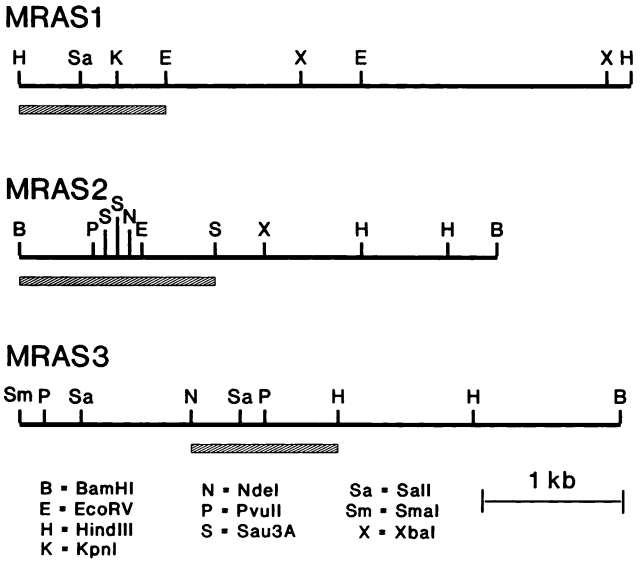

FIG. 2. Restriction maps of three subcloned DNA fragments from *M. racemosus* derived from recombinant lambda clones that hybridized strongly to a *YRAS1* or *YRAS2* probe. Shaded bars indicate regions of each subcloned fragment hybridizing to the probes.

Nucleotide sequence analyses of *MRAS* genes. Deoxynucleotide sequences of relevant regions of *MRAS1*, *MRAS2*, and *MRAS3* are shown in Fig. 3. Protein-coding regions (exons) and intervening sequences (introns) (see Fig. 5) were suggested by open reading frames, potential GT-AG splice junctions, and comparison of predicted amino acid sequences with published sequences of heterologous *ras* proteins (28) (Fig. 4). The DNA sequences predict *MRAS1*, *MRAS2*, and *MRAS3* proteins with molecular masses of 23,197, 21,723, and 23,352 D, respectively. In *MRAS2*, another ATG initiation codon occurs 20 bp upstream from the second exon (which begins at deoxynucleotide position 63; Fig. 3), with an open reading frame (coding for Met-Thr-Asn-Leu-Thr-Ile-Ser) extending to and continuous with the second exon; this alternative NH_4-terminal sequence shows no homology (DNA or amino acid) with sequences of other *ras* proteins.

Northern analyses. The *MRAS* 3′ probes (Fig. 5; probes 1, 3, and 5) did not cross-hybridize under the conditions used for Northern analysis (data not shown) and were therefore gene specific.

The gene-specific probes for *MRAS1* (probe 1) and *MRAS3* (probe 5) hybridized to different 1.3-kb transcripts in Northern blots of poly(A)$^+$ RNA (see Fig. 7) from different developmental stages of *M. racemosus* (Fig. 6). However, the amount of hybridization was dependent on the developmental stage and varied between the two probes. To more carefully quantitate the accumulated *MRAS* transcript levels, the autoradiographs in Fig. 7 were subjected to densitometric analysis (Table 1). The *TEF-1* transcript was previously shown to be present at approximately equal levels (<40% variation) in four of the developmental stages studied (sporangiospores, yeasts, germlings, and yeast-to-hypha transition cells) (18). We confirmed that the *TEF-1* transcript was present at nearly constant levels in the other developmental stages discussed herein (germinating spores, 1 h;

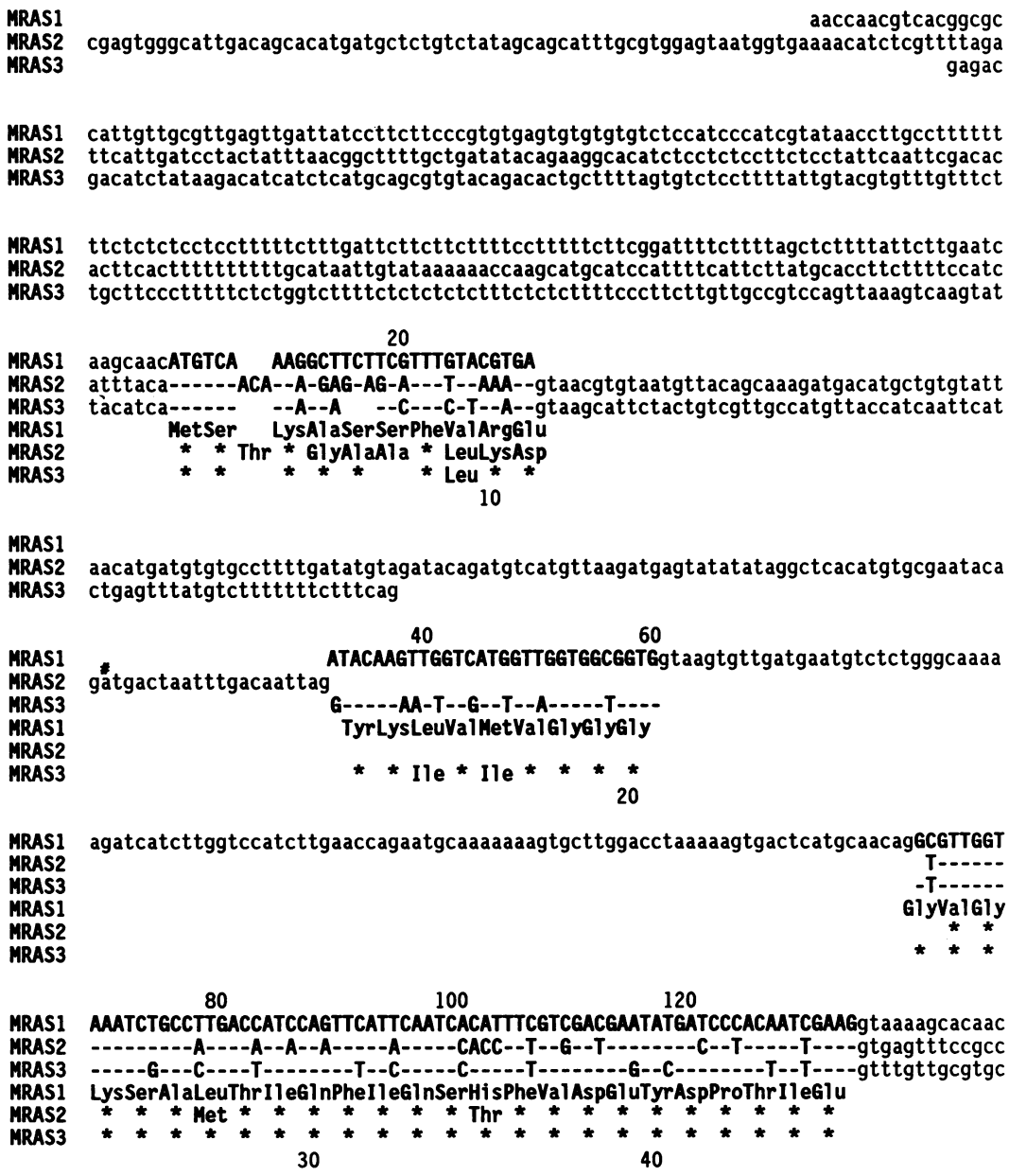

FIG. 3. Deoxynucleotide and predicted amino acid sequences for *M. racemosus MRAS1*, *MRAS2*, and *MRAS3* genes. Uppercase letters indicate open reading frames with similarity to published sequences of heterologous *ras* genes; lowercase letters indicate predicted introns and also sequences 5′ and 3′ to the putative *RAS* coding regions. Dashes or asterisks indicate deoxynucleotides or amino acids, respectively, that are the same as for *MRAS1* at that position. Spaces are included for optimal alignment of sequences; therefore, numbers for deoxynucleotides within exons (placed above sequences) and for amino acids (placed below sequences) are for reference only and may be slightly different than the actual position in each sequence. Another possible ATG start site in *MRAS2*, 20 bp upstream from exon 2, is indicated (#).

germinating spores, 4 h; and sporulating mycelium), using *TEF-1* to probe total RNA from these stages (Fig. 8). The only sample that was significantly different in terms of the *TEF-1* transcript was in germling-to-yeast transition (3 h after shift). The *TEF-1* transcript was present at fivefold-lower levels in these cells. Since the relative level of the *TEF-1* transcript in total RNA was known for each of the various developmental stages, the variation in the intensity of the signal of the 1.5-kb *TEF-1* transcript in poly(A)$^+$ RNA samples was used to generate a correlation factor for normalizing the *MRAS* hybridization data for differences in the quantity of poly(A)$^+$ RNA loaded for each morphological stage (Table 1).

The *MRAS1* probe hybridized to a 1.3-kb transcript (Fig. 7) in all developmental stages analyzed [a faint band in sporangiospore poly(A)$^+$ RNA was barely visible but was not detected above background by the densitometer]. The *MRAS1* signal increased during germination and germ tube growth (Table 1; Fig. 7, lanes 2 to 4), was present at relatively high levels during yeast-to-hypha morphogenesis (lane 8), but was most intense in sporulating mycelium (lane 9). The *MRAS3* probe hybridized strongly to a 1.3-kb band

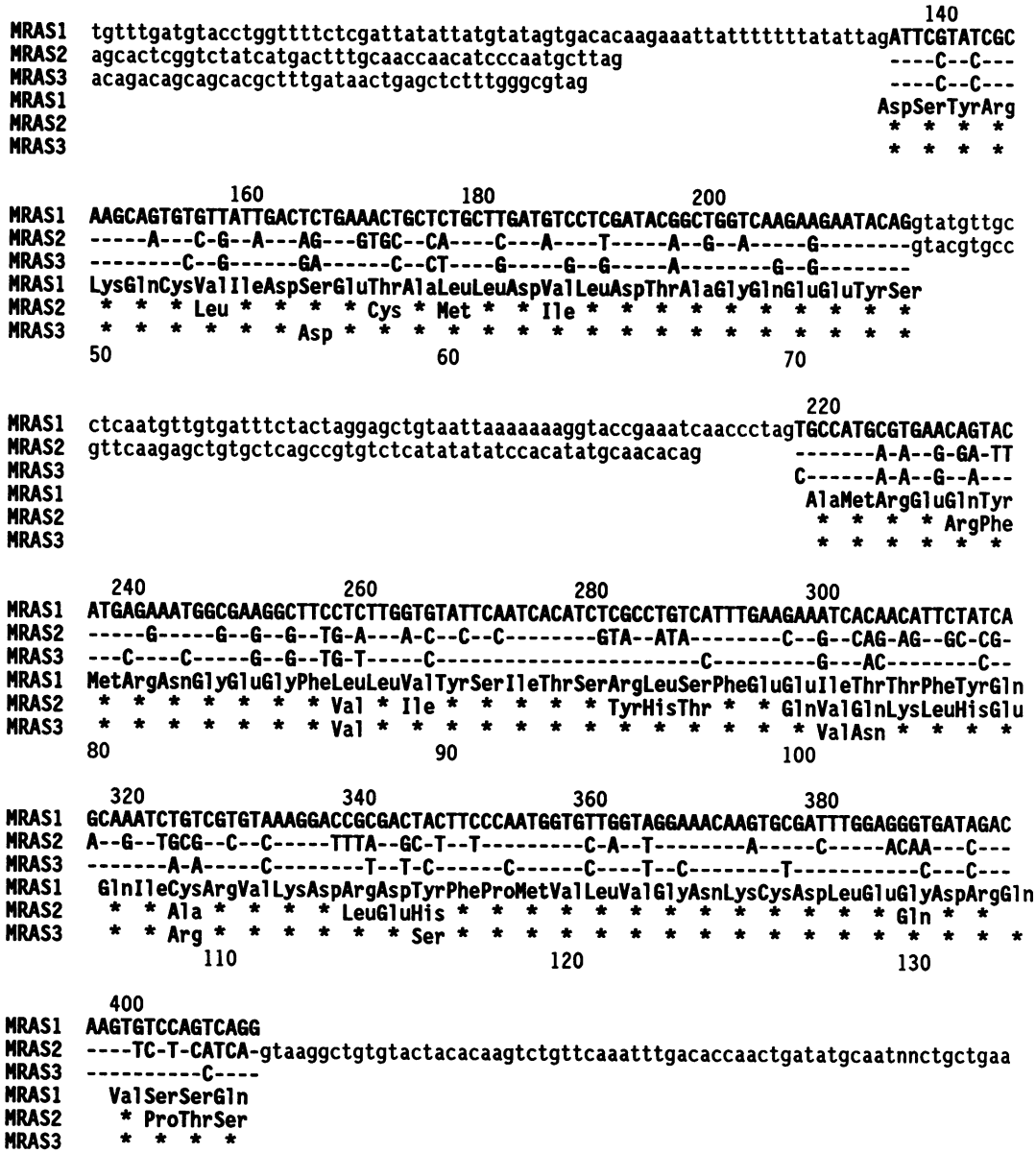

FIG. 3—*Continued.*

from sporulating mycelium (Fig. 7, lane 9), 10- and 50-fold less strongly (Table 1) to the same-size transcript from germlings (Fig. 7, lane 4) and yeasts (lane 7), respectively, but to no other developmental stage. The *MRAS3*-specific probe (probe 5) also hybridized to a 2.4-kb transcript (lanes 7 and 9, FL). Probe 6, prepared from an internal restriction fragment from *MRAS3*, did not hybridize to the 2.4-kb transcript, indicating that the hybridization of probe 5 to this band was due to 3′-flanking DNA, not *MRAS* sequences. No transcripts for *MRAS2* were detected in any developmental stage.

DISCUSSION

***MRAS* gene structure and organization.** Santos and Nebreda (28) define four domains in the primary structure of *ras* proteins on the basis of conservation of amino acid sequences among mammalian *ras* proteins: (i) the highly conserved first 80 amino acids; (ii) the next 80 amino acids, which are slightly less conserved; (iii) the nonconserved hypervariable region with sequences specific for each *ras* protein; and (iv) the COOH-terminal CAAX box (where C is Cys, A is any aliphatic amino acid, and X is any amino acid) involved in posttranslational modification and plasma membrane attachment. The first 80 amino acids are almost completely conserved among all mammalian *ras* proteins and very highly conserved (>80%) among lower eucaryotes. The predicted *MRAS1* and *MRAS3* proteins have 86 and 83% similarity, respectively, with human H-*ras*1 protein within this first domain (Fig. 4). The similarity is even greater if conservative substitutions (i.e., Glu for Asp) are accepted. *MRAS2* protein is comparable to H-*ras*1 protein; however, the *MRAS2* protein sequence suggests that a deletion occurred in the first exon, resulting in the loss of 10 amino acids within this first domain (see below). The next 80 amino acids are slightly less conserved (70 to 80% similarity

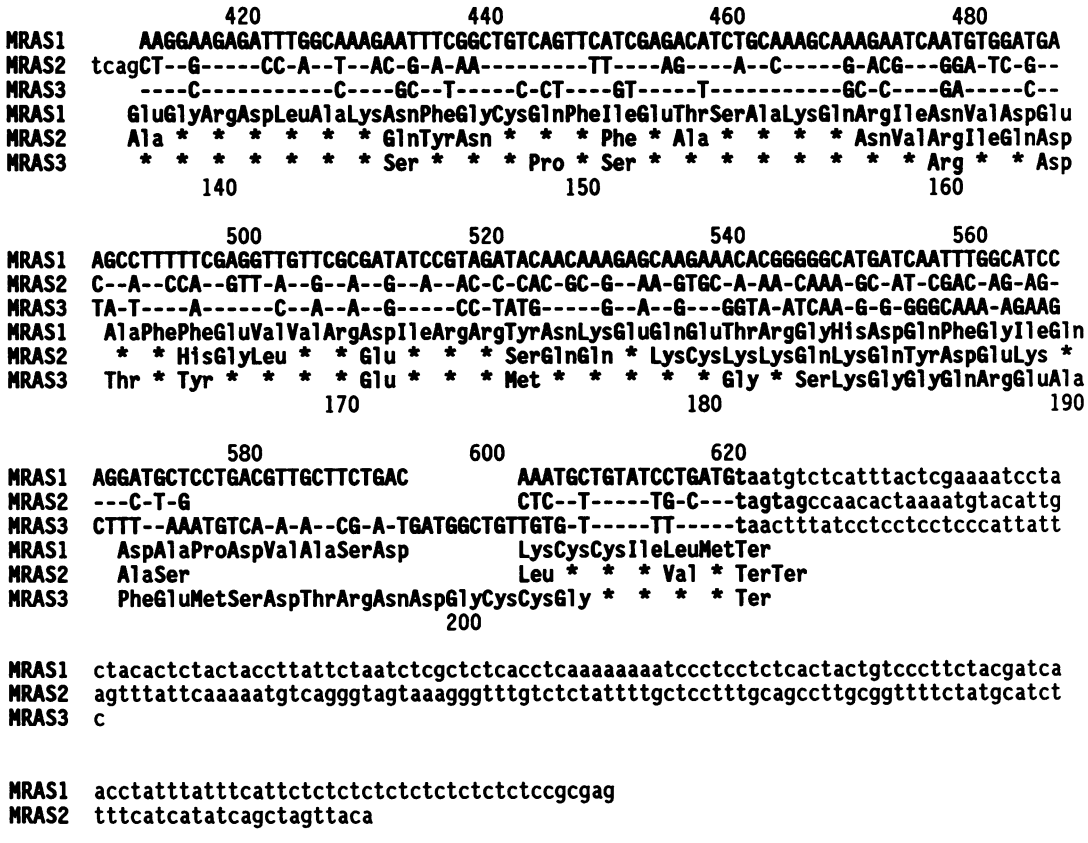

FIG. 3—*Continued.*

between pairs of vertebrate *ras* proteins): *MRAS* proteins have 53% similarity to H-*ras*1 protein within this second domain (excluding conservative substitutions), which is comparable to that of the other fungal *ras* proteins (Fig. 4). The high degree of conservation shown in these first two domains suggests some function common to all *ras* proteins.

The rest of the amino acid sequence, except for the last four amino acids, shows little or no conservation among *ras* proteins and is therefore termed the hypervariable region. The divergence of amino acid sequences of *MRAS* proteins within this region is generally consistent with the variation observed among all *ras* proteins. Notable, however, is that *MRAS1*, *MRAS3*, and *SPRAS* (*Schizosaccharomyces pombe*) proteins but not *MRAS2* protein maintain sequence similarity into the hypervariable region (Fig. 4). The hypervariable region has been suggested as providing individual *ras* proteins with specificity for function or receptor interaction. Therefore, the similarity of *MRAS1* and *MRAS3* proteins in this region may indicate interaction with the same or a similar receptor molecule. The CAAX box is present in all three *MRAS* proteins, suggesting potential localization of these proteins at the plasma membrane.

Functional domains for binding and hydrolysis of guanine nucleotides and for interactions with a putative cellular target (effector domain) have been proposed for *ras* proteins on the basis of crystallographic data and mutagenesis studies (28). Except for the absence in *MRAS2* protein of the conserved Gly-Gly-Gly-Gly within the first guanine nucleotide-binding region (which may be due to a deletion in exon I), the three *MRAS* proteins differ from H-*ras*1 protein within the proposed guanine nucleotide-interacting and effector regions only in the substitutions of Gly (in *MRAS1* and *MRAS3* proteins) for Ala-ll (H-*ras*1 protein) and Ala (in *MRAS2* protein) for Thr-144 (H-*ras*1 protein) (Fig. 4). Within these regions, *MRAS* proteins are identical to *SPRAS* proteins; *S. cerevisiae YRAS1* and *YRAS2* and *D. discoideum* Dd*ras*1 proteins have only one additional difference at Cys-118 (H-*ras*1 protein). Although *ras*-related proteins show conservation of these functional domains, similarity of the three *MRAS* proteins to other *ras* proteins extends well beyond these regions. There is also conservation of *MRAS* amino acid sequences within the epitope for binding of the neutralizing monoclonal antibody, Y13-259, that inhibits GTP-GDP exchange but not binding or hydrolysis of guanine nucleotides (12).

The extensive deoxynucleotide and predicted amino acid sequence similarity, both within and extending beyond proposed *RAS* functional domains, and the conservation of the position of introns (especially the intron between *MRAS* exons II and III, which is present in all *MRAS* genes and is also the exact position of an intron in human H-*ras*1) suggest that the three *MRAS* genes are truly homologous (i.e., evolutionarily related) with each other and probably with *ras* genes previously reported from other organisms, including mammals, birds, insects, and fungi (28).

Despite these similarities, the precise evolutionary relationships among the three *MRAS* genes are not clear from existing data. At many positions, the sequence of *MRAS3* has nucleotides in common with either *MRAS1* or *MRAS2* but not both. Therefore, *MRAS1* and *MRAS2* appear, on the basis of nucleotide sequence, more closely related to *MRAS3* than they are to each other. However, the predicted structures of the three *MRAS* genes reveal other relationships. Understanding these relationships is facilitated by

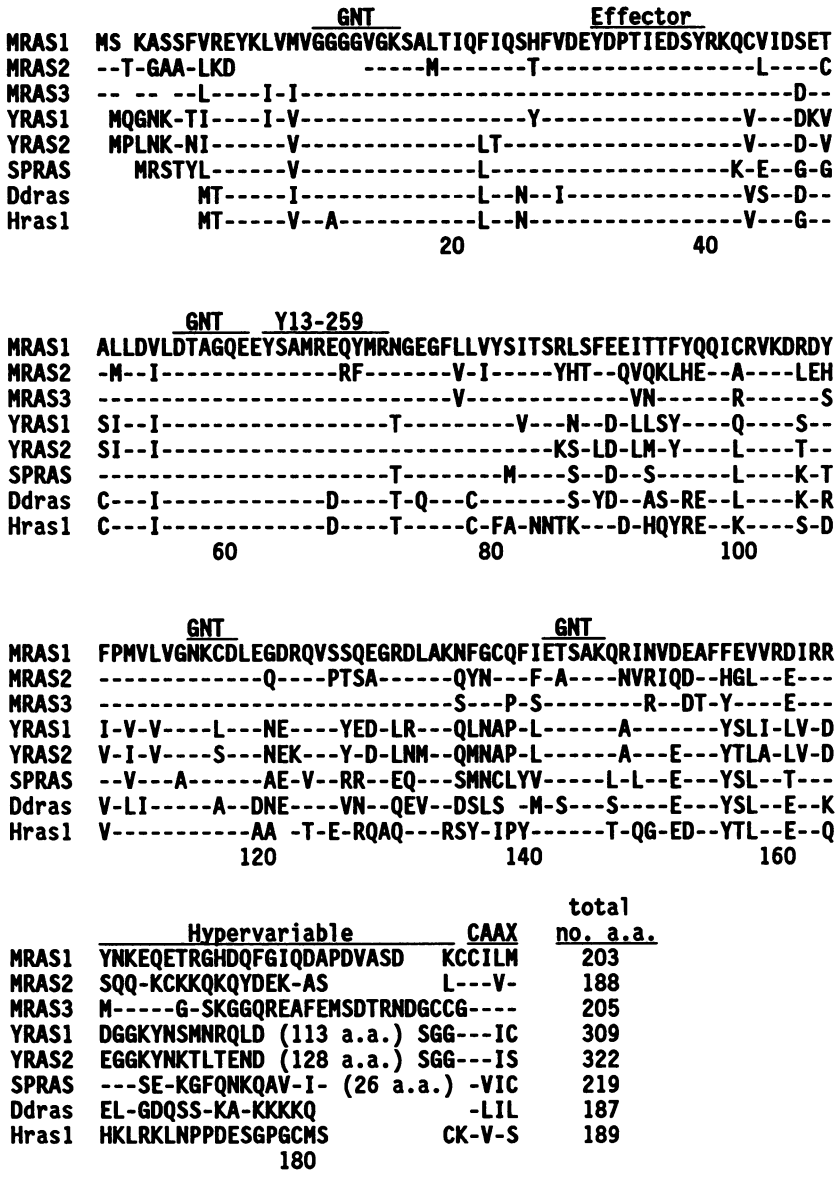

FIG. 4. Comparison of predicted amino acid sequences for *M. racemosus MRAS1*, *MRAS2*, and *MRAS3* proteins with published amino acid sequences (17) for *ras* proteins from *S. cerevisiae* (*YRAS1* and *YRAS2*), *Schizosaccharomyces pombe* (*SPRAS*), *D. discoideum* (Dd*ras*), and humans (H-*ras*1). Dashes indicate amino acids that are the same as for *MRAS1* protein at that position. Numbers below sequences refer to positions of amino acids in the H-*ras*1 protein. Bars above sequences indicate conserved regions believed to be involved in guanine nucleotide interaction (GNT), effector interactions, antibody Y13-259 binding, and posttranslational modification and membrane localization (CAAX, where C is Cys, A is any aliphatic amino acid, and X is any amino acid) and the hypervariable region (see text). a.a., Amino acids.

assuming five possible exons, although in none of the *MRAS* genes are all five complete and present as discrete exons (Fig. 5). Exons II and III are separated by introns in all three *MRAS* genes, and significantly, H-*ras*1 also contains an intron at this exact location (8). The arrangements of exons III, IV, and V are different for each of the three *MRAS* genes. In *MRAS2*, exons III, IV, and V are all separated by introns. *MRAS1*, however, lacks the intron between exons IV and V, and in *MRAS3*, exons III, IV, and V are contiguous. This pattern suggests a progression from *MRAS2* to *MRAS1* to *MRAS3*, with sequential loss of introns.

The relationships at exon I are more complex. In *MRAS1*, exon I is continuous and separated from exon II by an intron. In *MRAS3*, however, exon I is interrupted by an intron, resulting in exons Ia and Ib (Fig. 4), with exon Ib contiguous with exon II. *MRAS2* retains exon Ia (as judged by DNA sequence similarity and position of a potential splice site following deoxynucleotide 32; Fig. 3) but has apparently lost exon Ib as the result of a deletion. However, no sequence is missing from the 5′ end of exon II, which is nearly identical among the three *MRAS* genes. Although the DNA sequence of *MRAS2* indicates consensus sequences for proper RNA splicing of exon Ia to II, the presence of another open reading frame (with a potential ATG start site) upstream from exon II suggests an alternate NH_4-terminal peptide that has no similarity to other *ras* proteins. From these data, we are unable to choose among several possible rearrangements that would result in these patterns at exon I and II.

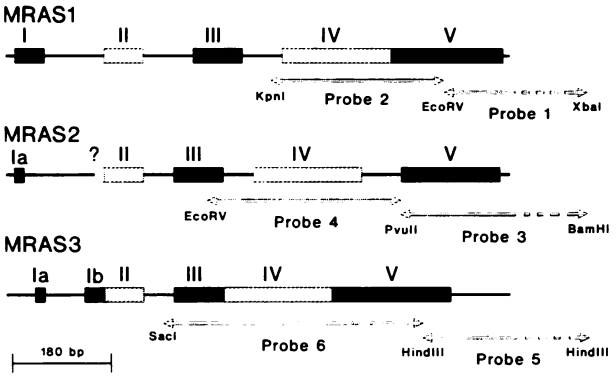

FIG. 5. Probes for Northern analysis. DNA restriction fragments used as probes for Northern analysis are shown below each *M. racemosus MRAS* gene. Exons (I to V) are indicated by solid and shaded boxes. In *MRAS2*, an alternate open reading frame (lacking homology with other *ras* genes) contiguous with exon II and containing an ATG at the 5′ end is indicated (?).

MRAS gene expression. *MRAS1* and *MRAS3* transcripts were present in greatest amounts in sporulating mycelium, although transcripts were either not detected (*MRAS3*) or barely detectable (*MRAS1*) in mature sporangiospores. Regulation of *MRAS3* expression was the more dramatic: the level of *MRAS3* transcripts in sporulation poly(A)$^+$ RNA was 10- and 50-fold higher than the level in germling and yeasts, respectively (Table 1; Fig. 7). *MRAS3* transcripts were not detected in any other developmental stage. Though this finding suggests a role for *MRAS3* in sporulation, it is also possible that *MRAS3* functions during sporangiospore germination. Although previous studies suggest that a pool of mRNA is stored in sporangiospores and translated during germination (15–17), the *MRAS3* gene may be transcribed and translated during sporulation, with the protein stored in sporangiospores and then activated during germination without de novo biosynthesis of *MRAS3* protein. In *M. mucedo* and *M. genevensis*, spherical growth of sporangiospores at an early stage in germination is accompanied by a burst in cAMP synthesis (22). Therefore, storage of *MRAS3* protein in sporangiospores and activation during germination could argue for a role in stimulation of cAMP biosynthesis during germination. In contrast, a major role seems doubtful for

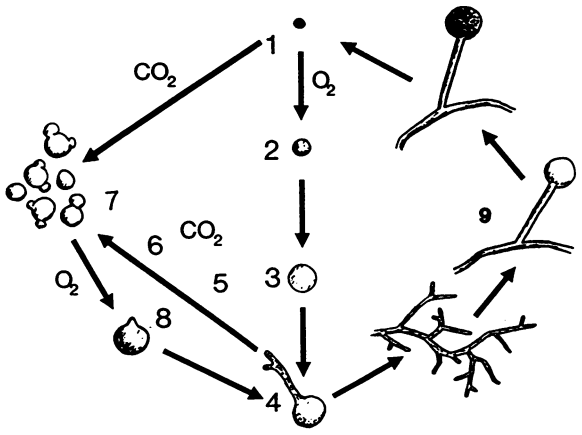

FIG. 6. Morphological stages of *M. racemosus* selected for isolation of poly(A)$^+$ RNA used in Northern analysis (Fig. 7). Stage 1, Sporangiospores; stage 2, 1-h-old germinating spores; stage 3, 4-h-old germinating spores; stage 4, 12-h-old germlings; stage 6, germling-to-yeast transition 3 h after switch to CO_2; stage 7, yeast; stage 8, yeast-to-hypha transition 3 h after switch to O_2; stage 9, immature sporangia and sporangiophores. Solid arrows indicate aerobic processes; shaded arrows indicate anaerobic processes.

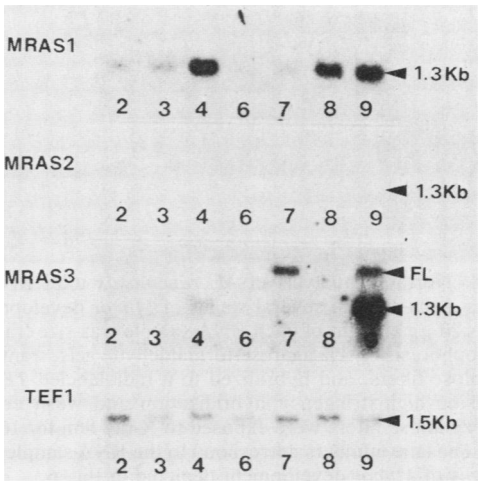

FIG. 7. Northern analysis of poly(A)$^+$ RNA from different developmental stages of *M. racemosus* probed with gene-specific radiolabeled restriction fragments from *MRAS1* (probe 1), *MRAS2* (probe 4), and *MRAS3* (probe 5). A *TEF-1* probe was used to normalize densitometry data (Table 1) to account for unequal loading of poly(A)$^+$ RNA. Lane numbers correspond to developmental stages shown in Fig. 2. FL refers to a transcript that was shown to hybridize to the 3′-flanking region in probe 5 (*MRAS3*).

TABLE 1. Densitometer measurements of the autoradiograph shown in Fig. 7

Lane	Morphology[a]	Peak wt (mg)[b]	Correction factor[c]	Corrected peak wt (% of maximum)
MRAS1-specific probe (probe 1)				
—[d]	Spore	0	5.30	0
2	GS, 1 h	40	0.65	8.8
3	GS, 4 h	64	2.32	50.5
4	Germling	238	1.05	85.0
6	G-Y, 3 h	33	0.40	4.5
7	Yeast	57	0.85	16.5
8	Y-H, 3 h	195	1.11	73.6
9	SPN	210	1.40	100.0
MRAS3-specific probe (probe 5)				
—[d]	Spore	0	5.30	0
2	GS, 1 h	0	0.65	0
3	GS, 4 h	0	2.32	0
4	Germling	45	1.05	9.4
6	G-Y, 3 h	0	0.40	0
7	Yeast	14	0.85	2.4
8	Y-H, 3 h	0	1.11	0
9	SPN	360	1.40	100.0

[a] GS, Germinating spores; G-Y, germling-to-yeast transition; Y-H, yeast-to-hypha transition; SPN, immature sporangia and sporangiospores.
[b] Peaks from densitometer tracings were cut out and weighed.
[c] Replicate Northern blots were probed with constitutively expressed *TEF-1* for normalizing data to account for unequal loading of poly(A)$^+$ RNA.
[d] Northern analysis of sporangiospore poly(A)$^+$ RNA is not shown in Fig. 7.

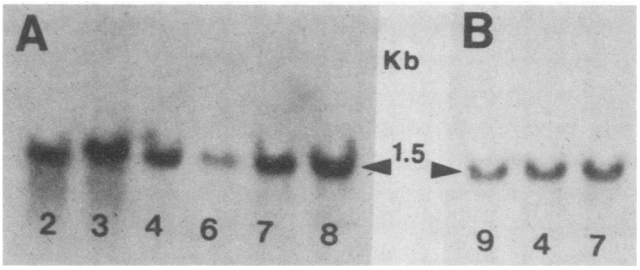

FIG. 8. Northern analysis of *M. racemosus* total RNA. Total RNA was isolated from several stages of *Mucor* development (see Fig. 6). A 20-μg amount of each RNA sample was size fractionated by electrophoresis on 1% agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized to a radiolabeled *TEF-1* gene probe, using high-stringency hybridization and wash conditions. The nitrocellulose filters were exposed to X-ray film for 16 h (A) or 24 h (B). The lane numbers correspond to the RNA samples isolated from stages of *Mucor* development depicted in Fig. 6.

MRAS3 protein in increasing cAMP synthesis during hypha-to-yeast morphogenesis (14, 24) or phospholipid (including PI) turnover in yeast-to-hypha morphogenesis (13). *S. cerevisiae YRAS2* protein increases cAMP biosynthesis by activation of adenylate cyclase (20, 31, 35), whereas *YRAS1* (31) and mammalian *ras* proteins (2, 28) may be involved in PI metabolism. Posttranscriptional activation of *MRAS* protein cannot be eliminated, however, since *MRAS3* transcripts are present at low levels in germlings and yeasts, although *MRAS3* transcripts were not detected during morphogenesis.

The pattern of *MRAS1* transcript accumulation (Table 1) suggests a function for *MRAS1* in germination and hyphal growth. Originally barely detectable in dormant sporangiospores, *MRAS1* transcripts increased throughout the early stages of germination (spherical growth of the sporangiospore) and germ tube growth to 85% of the maximum level detected in sporulating mycelium. High levels of *MRAS1* transcripts were also detected during yeast-to-hypha morphogenesis, whereas relatively low levels were detected in yeasts and during hypha-to-yeast morphogenesis. If *MRAS1* has any role in regulating cAMP metabolism, it would seem to suppress cAMP biosynthesis or increase cAMP turnover. With respect to PI metabolism, accumulation of *MRAS1* transcripts during yeast-to-hypha morphogenesis is correlated with increased rates of phospholipid (including PI) turnover (13).

MRAS2 differs from *MRAS1* and *MRAS3* in that the predicted amino acid sequence for *MRAS2* protein is missing a stretch of 10 amino acids that contains a series of four glycine residues that are highly conserved among *ras* proteins. In all other respects, *MRAS2* protein is comparable to *MRAS1*, *MRAS3*, and other *ras* proteins. Crystallographic and mutagenesis data (2, 6, 28, 33) suggest that this region is one of four guanine nucleotide-interacting domains. Point mutations resulting in the conversion of the third of these glycines (Gly12) to valine activates H-*ras*-1 in the T24 human bladder carcinoma oncogene and confers the ability to transform NIH 3T3 cells (25, 30, 32). The absence of these amino acids in *MRAS2* protein is apparently the result of a deletion removing exon Ib (Fig. 5). *MRAS2* contains another potential ATG initiation codon, however, followed by an open reading frame of 21 bp that is upstream from and contiguous with exon II (Fig. 5). This situation suggests two possible *MRAS2* proteins, neither of which contains a domain that is apparently critical for normal *ras* function. One of these possible proteins should have an NH_4 terminus similar to that of *ras* proteins but with an internal deletion. The alternative protein should have an NH_4-terminal peptide, unlike *ras* proteins. In either case, we predict that the *MRAS2* protein, if expressed, would lack or have significantly altered functions common among other *ras* proteins. In the morphologies examined, we have in fact found no transcript that hybridizes to an *MRAS2*-specific probe. *MRAS2* may be transcribed in other morphologies or under different environmental conditions, or it could be a pseudogene. We are interested in looking for transcription of *MRAS2* during other stages in the life cycle of *M. racemosus*, for example, during mating or zygospore germination.

The powerful genetic tools available for *S. cerevisiae* have led to considerable study of *YRAS1* and *YRAS2* with the hope of understanding the functions of mammalian *ras* proteins (31). However, *YRAS1* and *YRAS2* proteins are 120 and 133 amino acids larger, respectively, than mammalian *ras* proteins (189 amino acids). The sizes of the predicted *MRAS* proteins and the locations of domains within them are very similar to those of human *ras* genes. Together with evidence that expression of the different *Mucor RAS* genes depends on the stage of development, these observations argue that *M. racemosus* is a relevant system for the study of *ras* function in relation to cell development in eucaryotic cells in general. Of particular interest in this regard, the unique patterns of *MRAS* transcript accumulation suggest that individual *MRAS* genes and proteins may play distinct roles in cell growth and development.

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