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Three Genes for the Elongation Factor EF-1α in *Mucor racemosus*†

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We cloned three genes from *Mucor racemosus* coding for protein synthesis elongation factor 1α (EF-1α). A 110-base-pair (bp) EF-1α-specific cDNA clone was identified by hybrid-selected translation. The nucleotide sequence of the cDNA showed significant homology to a region of the *Saccharomyces cerevisiae* genes for EF-1α (*TEF1* and *TEF2*). The cDNA was used to isolate an 850-bp *Eco*RI genomic DNA fragment containing a portion of the EF-1α gene. Screening of a λ/*M. racemosus* genomic DNA bank with the 850-bp *Eco*RI probe resulted in the identification of three DNA fragments containing a common 850-bp *Eco*RI fragment within a short overlapping region. S1 nuclease analysis of the three EF-1α DNA fragments showed that the EF-1α transcript covered the short overlapping region in the clones. Restriction fragments purified from flanking regions in each clone were used to probe a *Hin*dIII digest of *M. racemosus* genomic DNA. Each flanking probe hybridized to one of three DNA fragments which hybridized to the 850-bp EF-1α-specific probe. Nucleotide sequence data from two random "shotgun clones" of one of the three genes show good homology to two regions of *S. cerevisiae TEF1*. The data indicate the presence of three genes for EF-1α in *M. racemosus* located at unique sites in the genome.

Elongation factor 1α (EF-1α) plays an essential role in the process of translation in eucaryotic cells. The protein directs the GTP-dependent binding of aminoacyl-tRNA to the open acceptor site on the ribosome. In the course of its activity, EF-1α interacts with nucleic acids, nucleotides, and other proteins.

As one means to obtain information about the primary structure of the EF-1α protein, several laboratories have cloned the genes coding for the protein and determined the nucleotide sequence. The two genes coding for EF-1α in *Saccharomyces cerevisiae*, *TEF1* and *TEF2*, have been cloned, and their nucleotide sequences have been determined (8, 29, 37). In *Artemia salina*, the brine shrimp, there may be as many as four genes coding for EF-1α, only one of which has been sequenced (42, 43). More progress has been made in the study of elongation factor Tu (EFTu), which functions similarly to EF-1α in procaryotic cells and in eucaryotic organelles. To date, the two *Escherichia coli* genes coding for EFTu, *tufA* and *tufB* (2, 26, 38, 46), the gene for EFTu from the *Euglena gracilis* chloroplast (27), and the EFTu gene from the mitochondria of *S. cerevisiae* (30) have all been isolated, and their nucleotide sequences have been determined. Cotrelle et al. compared the primary structure of the EF-1α and EFTu proteins from these organisms by dot matrix analysis under high- and low-stringency conditions for the comparison (9). They not only found a great deal of sequence homology when EF-1α proteins were compared, but it appeared that there were several conserved regions between EF-1α and EFTu. They speculated that these conserved regions correspond to functional domains within the protein.

During sporangiospore germination of the fungus *Mucor racemosus*, EF-1α activity was shown to increase nearly sixfold (12). At this same time, several lysine residues in the EF-1α protein are methylated to form the mono-, di-, and trimethyllysine derivatives (14). By using a cDNA probe for the *M. racemosus* EF-1α gene, it was shown that the relative abundance of EF-1α mRNA remained nearly constant during this time. A similar invariant level of EF-1α protein detected during spore germination led to the conclusion that the increase in EF-1α activity was due to the posttranslational methylations (12).

Continuing our studies on the molecular genetics of EF-1α in *M. racemosus*, we report here the presence of three genes in the *M. racemosus* genome coding for EF-1α. We cloned each of the genes, characterized them by restriction endonuclease analysis, and found that they lie in unique locations in the *M. racemosus* genome.

MATERIALS AND METHODS

Organism and culture conditions. *M. racemosus* (*M. lusitanicus*) ATCC 1216b was the source of DNA and RNA used in cloning the EF-1α genes. Sporangiospores were prepared as described previously (35) and stored at −20°C in sterile distilled water containing 20% (vol/vol) glycerol. Sporangiospores were germinated in YPG medium (2% glucose, 1% Bacto-Peptone [Difco Laboratories], 0.3% yeast extract [pH 5.5]) by shaking at 28°C in a rotatory shaker water bath. The culture was sparged with >2 volumes of sterile air per volume per min. Germlings were harvested by filtration (Whatman no. 1 filter paper) when germ tubes reached five spore diameters. The cells were frozen immediately in liquid nitrogen and stored at −70°C, or the nucleic acids were extracted immediately, as described below.

Bacterial strains and plasmids. *E. coli* LE392 (F^- *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ^-) (28) was used to propagate an *M. racemosus* gene bank in bacteriophage λ. *E. coli* JM83 [*ara* Δ(*pro-lac*) *rpsL thi* Φ80dlacZΔM15] (23) was the host strain for plasmids pUC8, pUC9 (44), and pBR322 (7), which were used in subcloning

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the EF-1α 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 by methods reported previously (6, 10). JM83 was made competent for transformation by recombinant plasmids by a $CaCl_2$ procedure previously published (18). Transformed cells were selected and maintained on LB medium supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and ampicillin (50 μg/ml, final concentration) for pUC recombinant plasmids or on LB medium supplemented with tetracycline (20 μg/ml, final concentration) for pBR322 recombinant plasmids. *E. coli* JM103 [Δ(*lac-pro*) *thi rpsL supE endA sbcC hsdR*$^-$ F′ *traD36 proAB lacI*^{q}*ZΔM15*] (24) was used as a host strain in the nucleotide sequencing of the EF-1α cDNA clone and the two *Taq*I genomic "shotgun clones." Replicative-form and single-stranded phage M13mp8 (25) were propagated and purified according to procedures published by Bethesda Research Laboratories, Inc. (5).

Construction of a limited *M. racemosus* cDNA bank. Total RNA was extracted from *M. racemosus* germlings by the procedure of Alton and Lodish (1). An aliquot of the total RNA solution containing 20 mg of RNA (450 μg of RNA per ml) was passed over an oligo(dT)cellulose column (1-ml bed volume; Bethesda Research Laboratories, Inc.) to purify the poly(A)$^+$ RNA by the procedure of Aviv and Leder (3).

cDNA copies were prepared from the poly(A)$^+$ RNA with avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories, Inc.), and homopolymer deoxycytidine monophosphate tails were added via terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Inc.) (11). The cDNA fragments were cloned into the *Pst*I site of pBR322, which had been prepared with a tract of the complementary homopolymer dGMP. The resultant cDNA recombinant plasmids were screened for EF-1α-specific sequences by means of nitrocellulose filter hybrid-selected translation (34). The poly(A)$^+$ RNA specifically bound to cDNA plasmids was translated in a rabbit reticulocyte lysate in vitro translation system by following the instructions of the manufacturer (Bethesda Research Laboratories, Inc.). Translation products were analyzed by two-dimensional polyacrylamide gel electrophoresis (31), and EF-1α sequences were identified by comparing the migration of the polypeptides translated in vitro with a pure EF-1α standard.

Construction of an *M. racemosus* gene bank in bacteriophage λ. High-molecular-weight *M. racemosus* genomic DNA was extracted from germlings by the procedure of Cihlar and Sypherd (8). The DNA preparation was fragmented by partial digestion with *Eco*RI restriction endonuclease. DNA fragments of 10 to 15 kilobases (kb) were purified on sucrose gradients (20) and ligated to the *Eco*RI arms of λgtWES · λB (16), prepared as described by Maniatis et al. (20). The recombinant DNA molecules were packaged in vitro with a kit supplied by Boehringer Mannheim Biochemicals. The phage particles were propagated in *E. coli* LE392 and screened for EF-1α sequences by the in situ plaque hybridization procedure described by Benton and Davis (4).

Restriction endonuclease analysis and agarose gel electrophoresis. Restriction endonuclease digestions of DNA were performed by the instructions of the manufacturer. DNA fragments were resolved by electrophoresis through 0.8 to 2% agarose gels (22) with a Tris acetate buffer system (50 mM Tris [pH 8.05], 20 mM sodium acetate, 2 mM EDTA [pH 8.0]). DNA in agarose gels was visualized directly by soaking gels in electrophoresis buffer containing ethidium bromide (0.5 μg/ml) and photographing the gel by transillumination with UV light (260 nm). RNA samples were resolved by electrophoresis through formaldehyde-agarose gels as described by Lehrach et al. (17).

Southern and northern blot analyses of nucleic acids. DNA resolved by electrophoresis through agarose gels was further analyzed by transfer to nitrocellulose filters by the procedure of Southern (39). The DNA transfer buffer was 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Passive transfer was allowed to proceed over a 24-h period, after which the nitrocellulose filter was baked at 80°C under vacuum for 2 h before hybridization.

The transfer of RNA from formaldehyde-agarose gels was carried out by the procedure of Thomas (41). DNA probes for hybridization were prepared from agarose-gel-purified restriction fragments (19, 45). The DNA was nick translated to high specific activity (>10^8 cpm/μg of DNA) by the incorporation of a [α-^{32}P]dGTP following the reaction conditions of Maniatis et al. (21).

Nitrocellulose filter hybridization conditions were essentially those described by Maniatis (19). Nitrocellulose filters were soaked for 3 h in prehybridization solution (50% deionized formamide, 5× Denhardt solution [1× Denhardt solution is 1% Ficoll {Pharmacia Fine Chemicals}, 1% bovine serum albumin, 1% polyvinylpyrrolidone], 5× SSPE [20× SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4 {pH 7.4}, 20 mM EDTA], 0.1% sodium dodecyl sulfate, denatured salmon testes DNA [100 μg/ml]) at room temperature. Prehybridization solution was then removed and replaced with fresh prehybridization solution containing 5 × 10^5 to 1 × 10^6 cpm of nick-translated DNA probe per ml. Hybridizations were allowed to proceed for at least 24 h at 42°C while the solution was submerged in a shaking water bath. The filters were washed twice for 15 min in 2× SSC–0.1% sodium dodecyl sulfate at room temperature and then once for 60 min in 2× SSC at 65°C. Filters were exposed to Kodak XAR-5 film (Eastman Kodak Co.) at −70°C with a Cronex Lightning-Plus intensifier screen for 4 to 24 h.

DNA sequencing. The nucleotide sequences of the cDNA EF-1α clone and the two *Taq*I genomic shotgun clones were determined by the dideoxy chain termination procedure of Sanger and Coulson (36).

S1 nuclease analysis of RNA. Total RNA (50 or 100 μg) was mixed with *M. racemosus* cloned EF-1α DNA, and the nucleic acids were coprecipitated with ethanol. The nucleic acid pellet was dissolved in 0.03 ml of S1 nuclease hybridization buffer (80% deionized formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis{2-ethanesulfonic acid}] [pH 6.4], 400 mM NaCl, 1 mM EDTA) and heated to 80°C for 5 min. The tubes were then placed in a water bath at 49°C for 3 h. After incubation, the reaction mixtures were diluted 1:10 with ice-cold S1 nuclease buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.6], 4.5 mM $ZnSO_4$), and 300 U of S1 nuclease (Boehringer Mannheim Biochemicals) was added (1,000 U/ml). The tubes were incubated at 20°C for 30 min followed by 30 min at 4°C. The DNA/RNA hybrids resistant to S1 nuclease digestion were precipitated with ethanol after the addition of 10 μg of yeast tRNA. The nucleic acids were then resolved on neutral agarose gels. Gels were further analyzed by Southern blotting as described above.

Enzymes and chemicals. The enzymes used in this analysis were purchased from Bethesda Research Laboratories, Inc., New England BioLabs, Inc., or Boehringer Mannheim Biochemicals unless specified otherwise in the text. All other

reagents were purchased from Sigma Chemical Co. or Mallinckrodt, Inc.

RESULTS

Identification of an EF-1α-specific clone. EF-1α is one of the most abundant proteins present in *M. racemosus*, constituting about 1% of the cellular protein. The protein has a molecular weight of 53,000, an isoelectric point of approximately 9.3, and is easily identified on two-dimensional polyacrylamide gels (14). Given the probability that EF-1α mRNA would be present in relatively high abundance, we chose to clone the EF-1α genes by a cDNA strategy.

Total RNA, purified from germinating sporangiospores, was fractionated by chromatography on oligo(dT) cellulose to obtain poly(A)$^+$ RNA. cDNA copies were synthesized from the poly(A)$^+$ RNA via avian myeloblastosis virus reverse transcriptase, and the cDNA was cloned into the *Pst*I site of pBR322. The resulting recombinant plasmids were introduced into *E. coli* JM83 by $CaCl_2$ transformation. Of the 400 ampicillin-sensitive, tetracycline-resistant clones, 100 were screened for the presence of EF-1α sequences by hybrid-selected translation. The clones were grown together in groups of six and the plasmid DNA was purified and then bound to nitrocellulose filters. Total *M. racemosus* RNA was hybridized to the plasmid DNA, and the specifically bound RNA fraction was translated in an optimized rabbit reticulocyte in vitro translation system. The translation products were characterized by electrophoresis on two-dimensional polyacrylamide gels. One clone, which carried a 110-base-pair (bp) cDNA fragment, was identified as a putative EF-1α clone because its hybrid-selected mRNA directed an in vitro translation product which comigrated with a purified EF-1α standard protein (12). The 110-bp *Pst*I cDNA fragment was subcloned into the *Pst*I site of the single-strand phage M13mp8, and its nucleotide sequence was determined (Fig. 1A). Open reading frames were found in three of the six possible frames. The cDNA nucleotide sequence was then compared with the complete nucleotide sequence of the *S. cerevisiae* EF-1α gene, *TEF1* (9). The stringency for the homology comparison was based on a 5-in-7-bp match (71%). The resulting dot matrix analysis plot is shown in Fig. 1B. A region of significant homology, shown by the discontinuous diagonal line, was found beginning at base 840 of the *S. cerevisiae* gene.

A

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      1
5' TGCTGTCACCACACGAAGATCAAGTTCCGTCGAAATACAC

   CACCACGAAACCCTCATTGAAGGTTTCCCGGATGACAAGC  3'
                                        80
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B

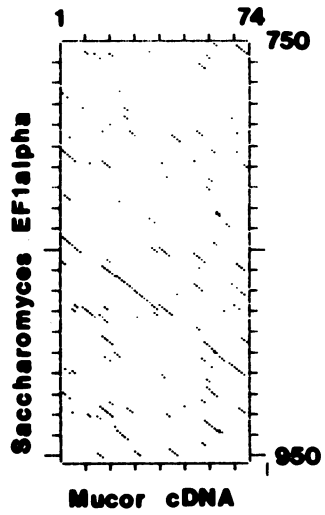

FIG. 1. Nucleotide sequence and homology analysis of the *M. racemosus* EF-1α cDNA clone. (A) The nucleotide sequence of the 80-bp *M. racemosus* EF-1α cDNA was determined by the dideoxy chain termination procedure of Sanger and Coulson (36). (B) The nucleotide sequence of the *M. racemosus* EF-1α cDNA was compared with the published sequence of the *S. cerevisiae* EF-1α gene, *TEF1* (9), by a dot matrix homology program written for the IBM PC (G. A. Gutman and B. Ward, unpublished data). *x* axis, *M. racemosus* EF-1α cDNA, bases 1 to 74; *y* axis, *S. cerevisiae TEF1*, bases 750 to 950 (numbering of *TEF1* begins with the AUG translation initiation codon).

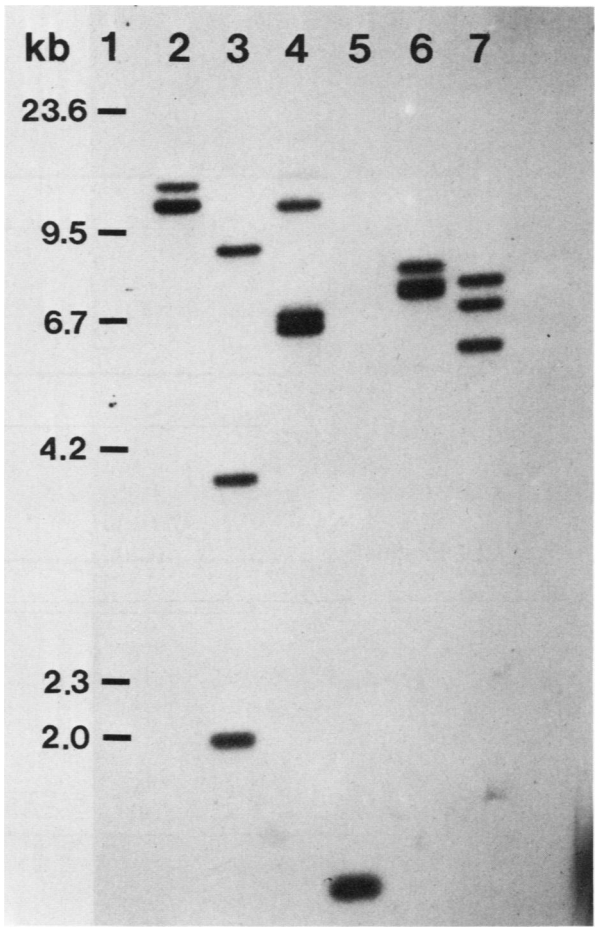

FIG. 2. Southern blot analysis of *M. racemosus* genomic DNA with a ^{32}P-labeled EF-1α probe. We fragmented 20 μg of *M. racemosus* genomic DNA with the indicated restriction endonucleases. Lanes: 1, molecular size markers (*Hin*dIII digest of λ DNA); 2, *Sal*I digest of *M. racemosus* genomic DNA; 3, *Pst*I digest; 4, *Xba*I digest; 5, *Eco*RI digest; 6, *Bam*HI digest; 7, *Hin*dIII digest.

Identification of an EF-1α-specific genomic DNA clone. The

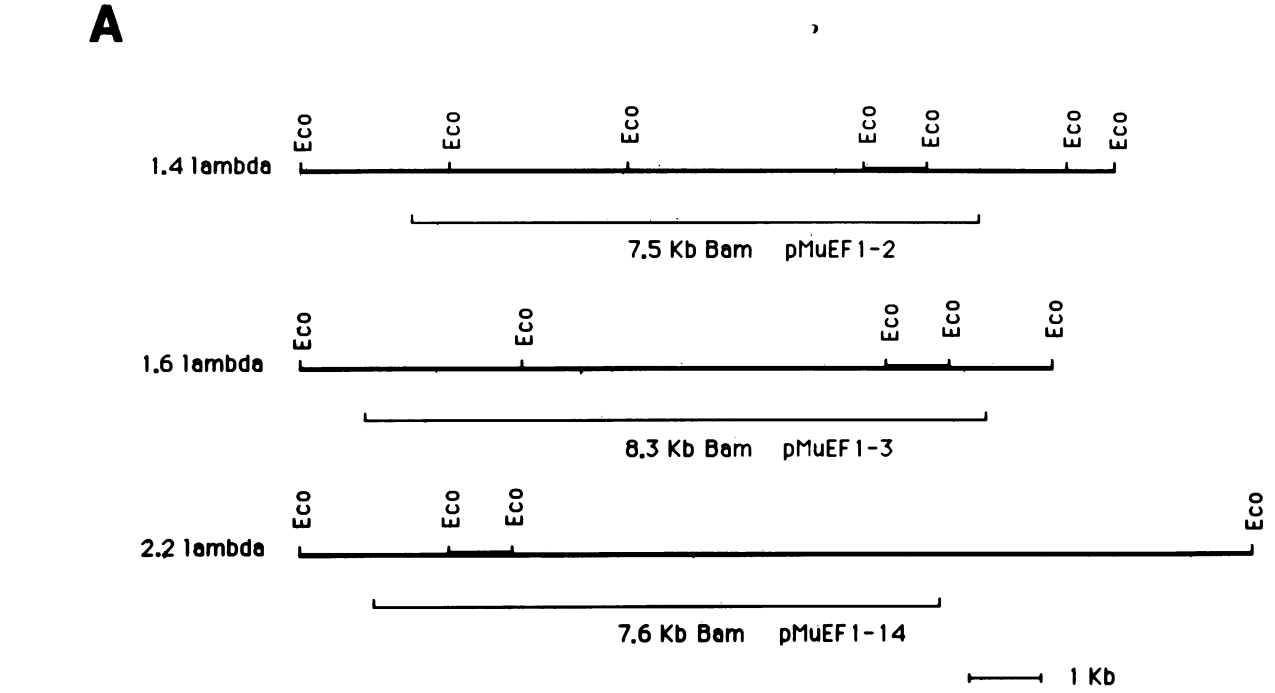

FIG. 3. Restriction endonuclease maps of *M. racemosus* EF-1α sequences from λgtWES · λB or pUC9 recombinant clones. The DNA restriction fragments were resolved by agarose gel electrophoresis, and their sizes were analyzed to generate restriction maps. Only *M. racemosus* DNA is shown in (A) and (B). (A) Representative λWES · λB clones 1.4 λ, 1.6 λ, and 2.2 λ showing the *Eco*RI restriction pattern of the *M. racemosus* DNA clones. The 850-bp *Eco*RI fragment common to all three clones is indicated by the highlighted line segment. (B) *Bam*HI restriction fragments were subcloned into pUC9 to construct the EF1 recombinant plasmids pMuEF1-2, -3, and -14. The locations of the overlapping restriction sites are indicated by the hashed lines. Bam, *Bam*HI; Eco, *Eco*RI; Hin, *Hin*dIII; Hpa, *Hpa*I; Sal, *Sal*I; and Pst, *Pst*I.

EF-1α cDNA clone was nick translated and used to probe a Southern blot of *M. racemosus* genomic DNA digested to completion with *Eco*RI or *Bam*HI to identify genomic DNA fragments which might contain the entire EF-1α gene. One 850-bp band and three *Bam*HI restriction fragments (8.3, 7.6, and 7.5 kb) hybridizing to the cDNA probe were identified on an autoradiograph of the blot. The 850-bp *Eco*RI fragment was isolated from limited genomic DNA banks generated by ligation of DNA eluted from the correct portion of an agarose gel and ligated into pBR322. The 850-bp *Eco*RI fragment was nick translated and used to probe a Southern blot of *M. racemosus* genomic DNA digested to completion with a variety of restriction endonucleases. The DNA restriction fragments were resolved by agarose gel electrophoresis and transferred to a nitrocellulose filter. The filter was incubated in the presence of the 850-bp *Eco*RI EF-1α DNA fragment which had been radioactively labeled with ^{32}P by nick translation. The filter was then washed and exposed to X-ray film. The resulting autoradiograph is shown in Fig. 2. For each restriction enzyme tested, the EF-1α probe hybridized to three bands except for the *Eco*RI digest, in which the 850-bp EF-1α probe hybridized to itself. These data suggested that there is more than one gene in the *M. racemosus* genome coding for EF-1α, because the large size of the three restriction fragments (i.e., *Bam*HI) precluded the presence of more than one restriction site in the gene.

Screening a λ*M. racemosus* DNA library for complete EF-1α genes. To obtain a complete EF-1α genomic clone, we constructed a complete library of *M. racemosus* genomic DNA in the λ vector, λgtWES · λB. This library was screened for EF-1α sequences by in situ plaque hybridization by use of the 850-bp *Eco*RI fragment as a probe. We selected 12 positive recombinant phage clones (plaques) and carried them through three rounds of single-plaque purification. DNA purified from the phage clones was digested to completion with *Eco*RI, and the restriction fragments were resolved on an agarose gel. The phage clones each contained the 850-bp *Eco*RI fragment and could be grouped into three unique *Eco*RI restriction patterns as shown in Fig. 3A. This result was further evidence suggesting the presence of more than one gene for EF-1α in the *M. racemosus* genome. A representative phage from each restriction group, designated 1.4 λ, 1.6 λ, and 2.2 λ, was digested with *Bam*HI, and the

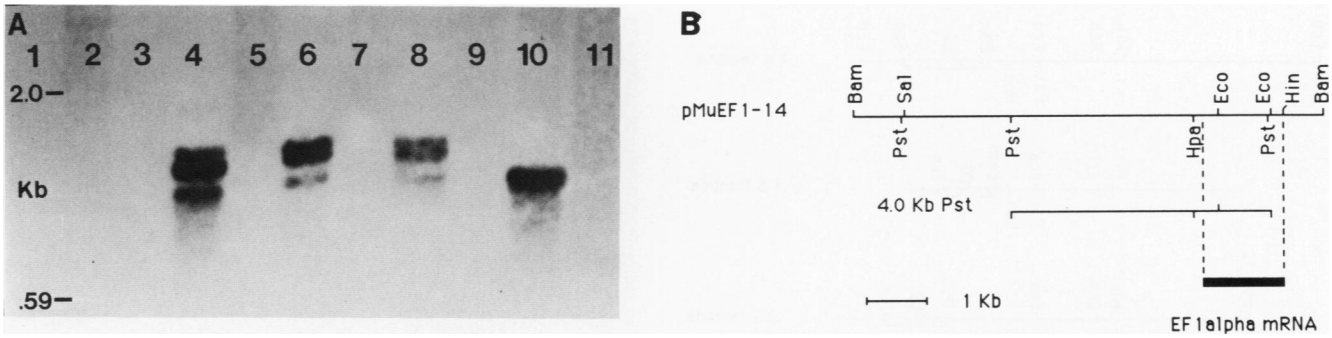

FIG. 4. S1 nuclease analysis of *M. racemosus* DNA restriction fragments containing EF-1α. (A) *Bam*HI restriction fragments from pMuEF1-2, -3, and -14, and a 4.0-kb *Pst*I fragment from pMuEF1-14 were incubated with total *M. racemosus* RNA under conditions favoring RNA/DNA hybridization. Lanes: 1, molecular size markers; 2, 7.6-kb *Bam*HI fragment from pMuEF1-14 plus yeast tRNA; 3, *M. racemosus* RNA; 4, 7.6-kb *Bam*HI fragment plus *M. racemosus* RNA; 5, 7.6-kb *Bam*HI fragment; 6, 7.5-kb *Bam*HI fragment plus *M. racemosus* RNA; 7, 7.5-kb *Bam*HI fragment from pMuEF1-2; 8, 8.3-kb *Bam*HI fragment plus *M. racemosus* RNA; 9, 8.3-kb *Bam*HI fragment from pMuEF1-3; 10, 4.0-kb *Pst*I fragment plus *M. racemosus* RNA; 11, 4.0-kb *Pst*I fragment from pMuEF1-14. (B) Position of the 4.0-kb *Pst*I fragment within the 7.6-kb *Bam*HI fragment from pMuEF-14. The location of the EF-1α transcript relative to these restriction fragments is shown by the hashed lines.

restriction fragments were cloned into pUC9. The recombinant plasmids were transformed into *E. coli* JM83. We identified subclones containing EF-1α sequences by screening transformants by colony hybridization with the 850-bp *Eco*RI restriction fragment. Three recombinant plasmids, pMuEF1-2, -3, and -14, contained a single subcloned *Bam*HI fragment which comigrated with one of the three genomic bands seen on Southern blots.

pMuEF1-2, -3, and -14 were digested with several restriction endonucleases singly and in combination to produce the restriction map shown in Fig. 3B. Two *Eco*RI sites which mark the boundaries of the 850-bp genomic DNA fragment were found to be common to all three subclones. In addition, common *Pst*I and *Hin*dIII restriction sites were identified in the same relative location in all three subclones. All other restriction sites appear to be unique to each of the clones.

The three subcloned *Bam*HI fragments were purified by agarose gel electrophoresis and analyzed further by S1 nuclease mapping to locate the ends of the EF-1α transcript in the subcloned restriction fragments. Total *M. racemosus* RNA was hybridized to each subclone under conditions which favor RNA/DNA hybridization. These hybrids were treated with 1,000 U of S1 nuclease (Boehringer-Mannheim Biochemicals) per ml. The double-stranded hybrids resistant to digestion were precipitated with ethanol, redissolved in buffer, and resolved by agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose filters, which were then incubated with the ^{32}P-labeled, 850-bp *Eco*RI EF-1α-specific probe, washed, and exposed to X-ray film. EF-1α RNA protected two major DNA fragments of 1.45 and 1.50 kb (Fig. 4A) and a minor fragment at 1.2 kb. When a 4.0-kb *Pst*I fragment from pMuEF1-14 (Fig. 4B) was subjected to S1 nuclease mapping (Fig. 4A, lanes 10 and 11), a single DNA fragment of 1.2 kb was protected, showing that part of the transcribed portion of the gene was missing in the restriction fragment. Northern blot analysis of *M. racemosus* RNA, performed as part of a previous study, detected a single EF-1α RNA transcript of 1.5 kb (12). These data show that the transcribed portion of the EF-1α gene in clone 2.2 λ is nearly centered around the 850-bp *Eco*RI fragment with approximately 350 bp of the transcript extending beyond each end of this fragment (Fig. 4B).

One final experiment was carried out to determine whether the three subclones represented three unique EF-1α genes. DNA restriction fragments isolated from flanking regions (relative to the EF-1α genes as shown in the S1 nuclease mapping experiment) of the three phage clones were purified by agarose gel electrophoresis (Fig. 5A). These three DNA restriction fragments and the 850-bp *Eco*RI fragment were ^{32}P labeled to high specific activity by nick translation and used to probe a Southern blot of *M. racemosus* genomic DNA (80 μg) digested to completion with *Hin*dIII. The restriction fragments were resolved by agarose gel electrophoresis (20 μg of DNA per lane) and transferred to a nitrocellulose filter. Individual lanes were then incubated with the DNA probes (Fig. 5A). The filters were washed and exposed to X-ray film. Each of the flanking probes hybridized to a unique *Hin*dIII fragment (Fig. 5B, lanes 3 to 5) corresponding to one of the bands which hybridized to the 850-bp *Eco*RI EF-1α probe (Fig. 5B, lane 2). This experiment shows that the flanking regions of the three subcloned genes are not homologous and that the three EF-1α genes reside at unique locations in the *M. racemosus* genome.

Partial DNA sequence analysis of one EF-1α gene. A 3.4-kb *Bam*HI/*Sal*I fragment from the subcloned 7.5-kb *Bam*HI fragment (Fig. 3B) was purified by agarose gel electrophoresis. The DNA fragment was digested to completion with *Taq*I, and the resulting restriction fragments were ligated into the *Acc*I site of M13mp10 (J. Messing and J. Viera, unpublished data). We have determined the nucleotide sequence of a small number of these *Taq*I shotgun clones by the procedure of Sanger and Coulson (36). The nucleotide sequences of two clones, *Taq*37 and *Taq*87, were compared with the nucleotide sequence of *S. cerevisiae TEF1* by dot matrix analysis (Fig. 6) by using a 4-in-5-bp match (80%). These two *M. racemosus* clones showed extensive homology to two separate regions of the *S. cerevisiae* gene, confirming our earlier conclusion that the subcloned *M. racemosus* genomic DNA fragments contained the gene for EF-1α from *M. racemosus*.

DISCUSSION

The cloning of the three EF-1α genes from *M. racemosus* was accomplished by the initial identification of a cDNA clone which hybridized specifically to mRNA, which programmed the synthesis of EF-1α protein when translated in vitro. The nucleotide sequence of the cDNA clone was

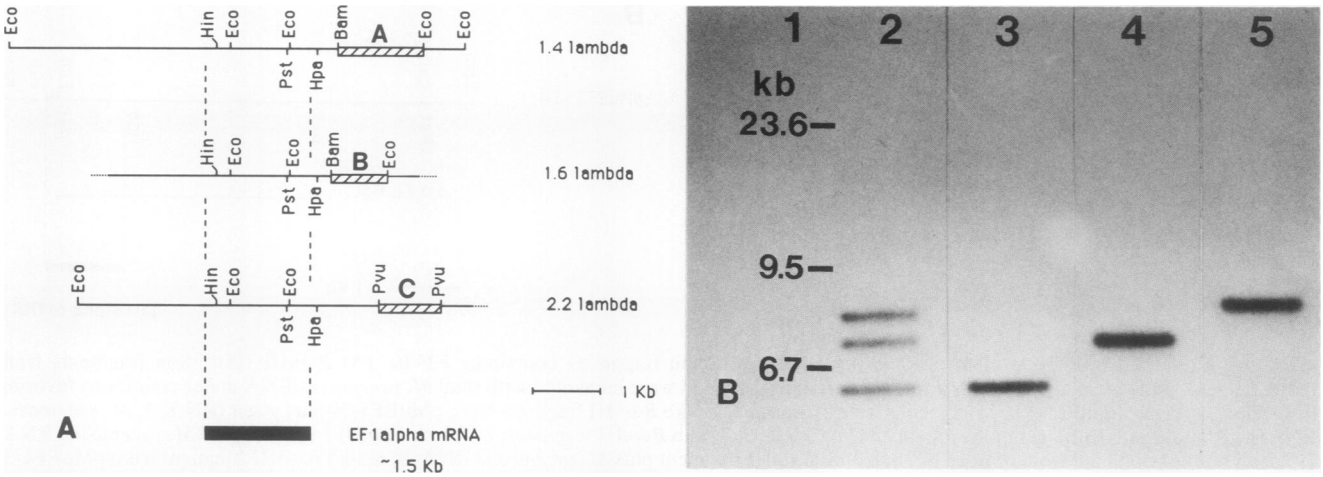

FIG. 5. Southern blot analysis of *M. racemosus* genomic DNA with ^{32}P-labeled DNA probes from regions flanking the EF-1α genes. (A) DNA restriction fragments indicated by A, B, and C were purified by agarose gel electrophoresis. The location of these restriction fragments relative to the EF-1α transcript is shown by the hashed lines. (B) Lanes: 1, molecular size markers; 2, *M. racemosus* DNA hybridized to the 850-bp *Eco*RI fragment; 3, *M. racemosus* DNA hybridized to flanking probe B; 4, *M. racemosus* DNA hybridized to probe A; 5, *M. racemosus* DNA hybridized to probe C. Bam, *Bam*HI; Eco, *Eco*RI; Hin, *Hin*dIII; Hpa, *Hpa*I; Pst, *Pst*I; Pvu, *Pvu*II.

determined and compared with that of the *S. cerevisiae* EF-1α gene, *TEF1*, by dot matrix analysis. A significant region of homology was found beginning at position 840 in the *TEF1* gene. From these combined data, we conclude that the *M. racemosus* cDNA is indeed part of the structural gene for EF-1α. The cDNA clone was used as a probe to identify a genomic clone (850-bp *Eco*RI fragment). With the nick-translated genomic fragment, we screened a λgtWES · λB gene library and identified three groups of λ/*M. racemosus* clones based on their restriction endonuclease fragment patterns with *Eco*RI. *Bam*HI restriction fragments corresponding to the *Bam*HI genomic fragments seen on Southern blots were subcloned into pUC9, and restriction endonuclease maps were generated. Two *Eco*RI sites marking the ends of the 850-bp genomic fragment and *Pst*I and *Hin*dIII restriction sites were found to be common to all three clones. The other restriction sites in the three clones did not overlap, suggesting that they are located in flanking sequences. This result, plus the results of Southern blots of genomic DNA showing three bands, provided evidence for the presence of

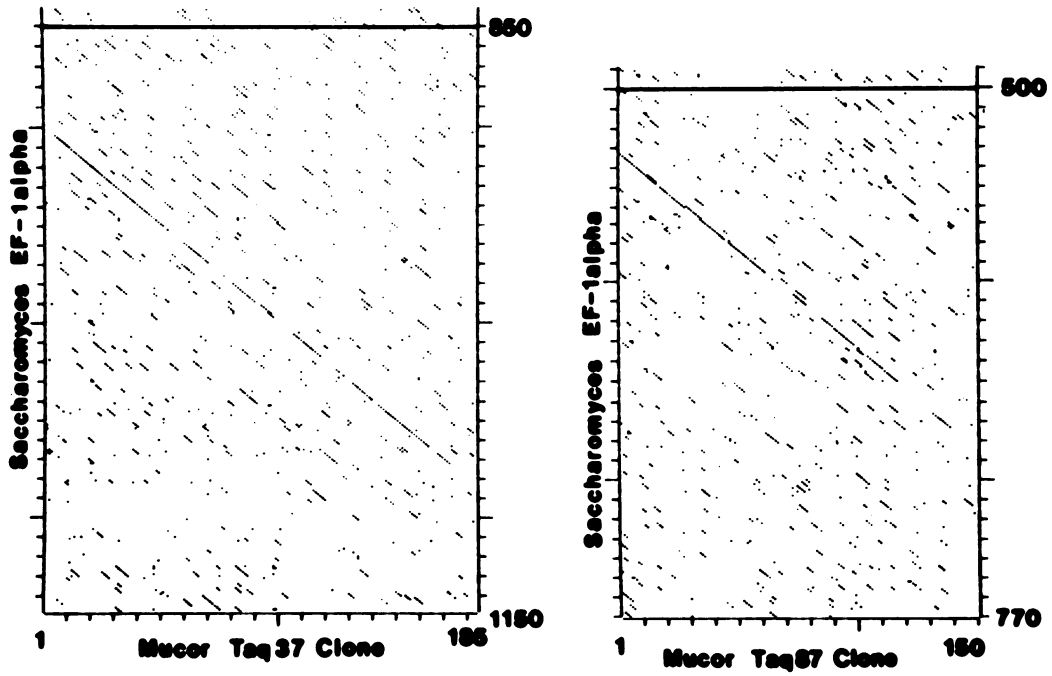

FIG. 6. The nucleotide sequences of *Taq*37 and *Taq*87 clones were determined by the procedure of Sanger and Coulson (36) and compared with that of *S. cerevisiae TEF1* by dot matrix analysis as described in Fig. 1. *x* axis, *M. racemosus Taq*37 and *Taq*87 clones; *y* axis, *S. cerevisiae TEF1* (numbering of *TEF1* begins with the AUG translation initiation codon).

three genes at unique locations in the *M. racemosus* genome. To lend further support for this notion, we probed a *Hin*dIII digest of *M. racemosus* genomic DNA with nick-translated restriction fragments from similarly located flanking regions of the three clones (relative to the EF-1α coding region). Each flanking probe hybridized to only one of the three individual *Hin*dIII fragments, while an EF-1α probe (850-bp *Eco*RI fragment) hybridized to all three genomic bands.

The role of multiple genes coding for EF-1α in eucaryotic systems is not clear. In *S. cerevisiae*, *TEF1* and *TEF2* are very closely related genes with only two bp differences between the genes in the entire open reading frame for the deduced protein sequence. Neither of these differences changes the primary structure of the protein (37). When *TEF1* is inactivated by deletion (37) or *TEF2* is inactivated by insertion of foreign DNA (9), haploid cells are able to grow, indicating that both genes are actively transcribed, but that only one gene is needed to supply the necessary protein for cell growth. It is possible that each of the genes is regulated independently such that changes in the requirements for EF-1α determine the degree to which these genes are active. This may provide a crude mechanism to regulate the EF-1α protein levels in the cell.

The actin genes in *Drosophila* represent one well-documented example of a gene family in which the individual genes are developmentally regulated (15, 40). There are six genetic loci for actin in *Drosophila* (5C, 42A, 57B, 79B, 87E, and 88F) coding for six related but different forms of this contractile protein. By using gene-specific probes, Fryberg et al. discovered that the actin genes are expressed in a tissue-specific manner (13). They also reported that no two genes displayed identical temporal patterns of expression during development of the organism to maturity. Two of the actin genes, 5C and 42A (nonmuscle actins), were found to be relatively more abundant during embryogenesis and pupal development but less abundant during muscle development. The expression of actins 57B and 87E was found mainly during larval muscle development, while the remaining two actins, 88F and 79B, were expressed almost exclusively during adult muscle development. The EF-1α genes in *M. racemosus* could represent this kind of gene family in which the proteins, though closely related, could function at different times in the *M. racemosus* life cycle. It has been reported that the rate of protein synthesis in *M. racemosus* is subject to regulation during cellular morphogenesis (32, 33). One could envision that differential synthesis of proteins with different specific activities brings about the changes in translation rate observed during morphogenesis.

Now, we do not know the number of EF-1α genes which are actually expressed in *M. racemosus*. S1 nuclease mapping of the three subcloned genes with total *M. racemosus* RNA revealed the presence of two major protected DNA fragments (1.5 and 1.45 kb) and at least one minor protected fragment (1.2 kb). The presence of multiple bands in the S1 nuclease analysis indicated the presence of more than one EF-1α transcript in the RNA population. These results may suggest that more than one EF-1α gene is being transcribed in *M. racemosus*, yielding two or more different RNA molecules with short regions of nonhomology. However, the results do not rule out the possibility that only one gene with multiple promoters or terminator sequences (or a combination) leads to the accumulation of several transcripts with different lengths. It is possible that these different-length transcripts were resolved in the S1 nuclease analysis but could not be resolved in Northern blot analysis because of the heterogeneity in length of poly(A)$^+$ tracts on the EF-1α transcripts. When a similar S1 nuclease experiment was carried out on the two *S. cerevisiae* EF-1α genes, two DNA fragments at the 3′ end of the gene, differing in length by 100 bp, were protected by the EF-1α mRNA. Only one DNA fragment was protected when the 5′ end of the transcript was mapped (37). Since the *S. cerevisiae* EF-1α genes are essentially identical throughout the open reading frame and are only 40% homologous in the nontranslated regions (5′ and 3′), it was concluded that the two mRNA transcripts are identical up to the protein synthesis termination codon but diverge sufficiently beyond this point to account for the two bands on the S1 nuclease analysis. Perhaps a similar situation exists in *M. racemosus* with its three EF-1α genes. We are pursuing this line of research with the aim of isolating a unique probe for each of the three EF-1α genes to analyze gene expression at the transcriptional level during morphogenesis.

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LITERATURE CITED

1. **Alton, T. H., and H. F. Lodish.** 1977. Developmental changes in messenger RNAs and protein synthesis in *Dictyostelium discoideum*. Dev. Biol. **60**:180–206.
2. **An, G., and J. D. Friesen.** 1980. The nucleotide sequence of *tufB* and four nearby tRNA structural genes of *Escherichia coli*. Gene **12**:33–39.
3. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
4. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180–182.
5. **Bethesda Research Laboratories, Inc.** 1985. M13 cloning/dideoxy sequencing. Instruction manual. Bethesda Research Laboratories, Inc., Bethesda, Md.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
7. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falcow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–98.
8. **Cihlar, R., and P. S. Sypherd.** 1980. The organization of the ribosomal RNA genes in the fungus *Mucor*. Nucleic Acids Res. **8**:793–804.
9. **Cotrelle, P., D. Thiele, V. Price, S. Memet, J. Micoun, C. Marck, J. Buhler, A. Sentnac, and P. Fromageot.** 1985. Cloning, nucleotide sequence and expression of one of the two genes coding for yeast elongation factor 1α. J. Biol. Chem. **260**:3090–3096.
10. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics, p. 116–119. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
11. **Efstratiadis, A., F. C. Kafatos, A. M. Maxam, and T. Maniatis.** 1976. Enzymatic *in vitro* synthesis of globin genes. Cell **7**:279–288.
12. **Fonzi, W. A., C. Katayama, T. Leathers, and P. S. Sypherd.** 1985. Regulation of protein synthesis factor EF-1α in *M. racemosus*. Mol. Cell. Biol. **5**:1100–1103.
13. **Fryberg, E. A., J. W. Mahaffey, B. J. Bond, and N. Davidson.** 1983. Transcripts of the six *Drosophila* actin genes accumulate in stage- and tissue-specific manner. Cell **33**:115–123.
14. **Hiatt, W. R., R. Garcia, W. C. Merrick, and P. S. Sypherd.**

1982. Methylation of elongation factor 1α from the fungus *Mucor*. Proc. Natl. Acad. Sci. USA **79**:3433–3437.
15. **Kindle, K. L., and R. A. Firtel.** 1978. Identification and analysis of *Dictyostelium* actin genes. A family of moderately repeated genes. Cell **15**:763–778.
16. **Leder, P., D. Tiemeier, and L. Enquist.** 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms. The λgt Wes system. Science **196**:175–177.
17. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4750.
18. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, D. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eukaryotic DNA. Cell **15**:687–701.
21. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. USA **72**:1184–1188.
22. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
23. **Messing, J.** 1979. A multipurpose cloning system based on the single stranded bacteriophage M13, p. 43–48. Recombinant DNA technical bulletin, 79–99, 2, no. 2. National Institutes of Health, Bethesda, Md.
24. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun sequencing. Nucleic Acids Res. **9**:309–321.
25. **Messing, J., and J. Viera.** 1982. A new pair of M13 vectors for selecting either strand of double digest restriction fragments. Gene **19**:269–276.
26. **Miyajima, A., M. Shibuya, and Y. Kaziro.** 1979. Construction and characterization of two hybrid ColE1 plasmids carrying *Escherichia coli tufB* gene. FEBS Lett. **102**:207–210.
27. **Montandon, P., and E. Stutz.** 1983. Nucleotide sequence of a *Euglena gracilis* chloroplast genome region coding for the elongation factor Tu: evidence for a spliced RNA. Nucleic Acids Res. **11**:5877–5892.
28. **Murray, N. E., W. J. Brammer, and K. Murray.** 1977. Lamboid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150**:53–60.
29. **Nagata, S., K. Nagashima, Y. Tsunetsugu-Yokota, K. Fujimura, M. Miyazaki, and Y. Kaziro.** 1984. Polypeptide chain elongation factor 1α (EF-1α) from yeast: nucleotide sequence of one of the two genes for EF-1α from *Saccharomyces cerevisiae*. EMBO J. **3**:1825–1830.
30. **Nagata, S., Y. Tsunetsugu-Yokota, A. Natio, and Y. Kaziro.** 1983. Molecular cloning and sequence determination of the nuclear gene coding for mitochondrial elongation factor Tu of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80**:6192–6196.
31. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12**:1133–1142.
32. **Orlowski, M.** 1981. Growth-rate-dependent adjustment of ribosome function in *Mucor racemosus*. Biochem. J. **196**:403–410.
33. **Orlowski, M., and P. S. Sypherd.** 1978. Regulation of translation rate during morphogenesis in the fungus *Mucor*. Biochemistry **17**:569–579.
34. **Parnes, J. R., B. Velan, A. Felsenfeld, L. Ramanathan, U. Ferrini, E. Apella, and J. G. Sidman.** 1981. Mouse β2 microglobulin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. Proc. Natl. Acad. Sci. USA **78**:2253–2257.
35. **Paznokas, J. L., and P. S. Sypherd.** 1975. Respiratory capacity, cyclic adenosine 3′, 5′-monophosphate, and morphogenesis of *Mucor racemosus*. J. Bacteriol. **124**:134–139.
36. **Sanger, F., and A. R. Coulson.** 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. **94**:441–448.
37. **Schirmaier, F., and P. Phillipsen.** 1984. Identification of two genes for the translation elongation factor EF-1α of *S. cerevisiae*. EMBO J. **3**:3311–3315.
38. **Shibuya, M., H. Nashimoto, and Y. Kaziro.** 1979. Cloning of an *Eco*RI fragment carrying *E. coli tufA* gene. Mol. Gen. Genet. **170**:231–234.
39. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
40. **Spudlich, J. A.** 1974. Biochemical and structural studies ofactomyosin-like proteins from nonmuscle cells. J. Biol. Chem. **249**:6013–6020.
41. **Thomas, P.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose. Methods Enzymol. **100**:255–260.
42. **van Hemert, F. J., R. Amons, W. J. M. Pluijms, H. van Ormondt, and W. Moller.** 1984. The primary structure of elongation factor EF-1α from the brine shrimp *Artemia*. EMBO J. **3**:1109–1113.
43. **van Hemert, F. J., J. A. Lenstra, and W. Moller.** 1983. Genes for elongation factor EF-1α in the brine shrimp *Artemia*. FEBS Lett. **157**:295–298.
44. **Viera, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
45. **Weislander, L.** 1979. A simple method to recover high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. **98**:305–309.
46. **Yokota, T., H. Sugisaki, M. Takanami, and Y. Kaziro.** 1980. The nucleotide sequence of the cloned *tufA* gene of *Escherichia coli*. Gene **12**:25–31.