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Expression of Three Genes for Elongation Factor 1α during Morphogenesis of *Mucor racemosus*†

JOHN E. LINZ‡ AND PAUL S. SYPHERD*

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717

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Three genes, *TEF-1*, *-2*, and *-3*, encode elongation factor 1α in *Mucor racemosus*. Neutral and alkaline S1 nuclease analyses revealed that the genetic organization is unique for each of the genes. The number and size of the intervening sequences vary in these closely related genes, which suggests that complex genetic rearrangements gave rise to the elongation factor 1α gene family. Nucleotide sequence data from restriction fragments isolated from the 5′ and 3′ ends of *TEF-2* and *-3* confirmed the presence of a second intervening sequence in these genes. These data along with S1 nuclease mapping revealed a region at the 3′ end of the three genes which was predicted to be transcribed but untranslated. Unique oligonucleotides containing 19 bases were synthesized to hybridize to this unique trailer region in the elongation factor 1α transcripts. These oligonucleotides were used as probes in standard Northern analysis of RNA purified from *M. racemosus* cells of several morphological types. It was determined that all three genes were expressed in the cell morphological types studied. However, the accumulated level of transcript derived from each gene varied considerably, with *TEF-1* mRNA present in approximately twofold greater quantity than the *TEF-3* transcript and up to sixfold greater quantity than *TEF-2*. The level of *TEF-1* and *-2* mRNA varied little among the cell morphological types studied, whereas *TEF-3* mRNA was present in twofold greater quantity in sporangiospores than in either germlings or yeast cells which had been induced to undergo morphogenesis to hyphae. These data suggest that there is differential expression of the genes encloding elongation factor 1α in *M. racemosus*. At least one gene, *TEF-3*, shows a morphology-specific pattern of transcript accumulation.

Elongation factor 1α (EF-1α) plays an essential role in protein synthesis in eucaryotic cells. The protein transfers charged tRNAs into the unoccupied acceptor site of the ribosome in a step that requires GTP. Recent data collected on EF-Tu, the analogous elongation factor in procaryotic cells and eucaryotic organelles, suggest that this protein is partly responsible for proofreading the interaction of codon and anticodon, suggesting that it plays a direct role in the fidelity of translation (5). In addition, evidence has accumulated which indicates that the expression and activity of EF-Tu and EF-1α are subject to regulation at several levels (5, 7, 8). It seemed reasonable to us that regulation of the intracellular level, the activity, or the specificity of these related elongation factors could have a profound quantitative and qualitative effect on protein synthesis.

We have been studying the regulation of expression of EF-1α in *Mucor racemosus* in an effort to understand the general regulation of protein synthesis in this fungus. During sporangiospore germination in *M. racemosus*, the relative rate of protein synthesis was observed to increase markedly (19, 20). The specific activity of EF-1α was found to increase nearly sixfold during this period, whereas the relative levels of EF-1α protein and mRNA remained nearly constant (8). Several lysine residues in the protein are methylated during the course of spore germination, which led us to conclude that this posttranslational modification was responsible for the increase in protein activity (8). To pursue our studies on the regulation of EF-1α activity and to establish its relationship (if any) to the increased level of protein synthesis, we have cloned the genes for EF-1α (12). The nucleotide sequence of *TEF-1*, one of the three genes encoding EF-1α in *M. racemosus*, revealed that the protein shares extensive homology with EF-1α proteins from other eucaryotic cells in which it has been studied (13). Secondary structure predictions of the deduced amino acid sequence of EF-1α revealed a striking conservation of alpha-helix and beta-sheet structure elements with EF-Tu from *Escherichia coli* (13). This study enabled us to predict the location of functional domains for GTP binding and hydrolysis and tRNA binding in the EF-1α protein.

The present study was undertaken to determine whether all three genes of EF-1α are transcribed and, if so, whether there is a morphology-specific pattern to the expression of each gene. These analyses were made possible by the existence of unique sequences in the 3′ transcribed but untranslated region of each gene, for which we prepared specific oligonucleotide hybridization probes.

MATERIALS AND METHODS

Organism and culture conditions. *M. racemosus* (*M. lusitanicus*) ATCC 1216B was the organism used in these studies. Sporangiospores were prepared and stored as described previously (21). Sporangiospores were germinated in YPG medium (2% glucose, 1% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 0.3% yeast extract, pH 4.5) by shaking at 28°C on a rotatory shaker water bath. The culture was sparged with >2 volumes of sterile air per volume of

* Corresponding author.

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‡ Present address: Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824.

culture per min. Germlings were harvested by filtration (Whatman no. 1 filter) when germ tubes were 5 spore diameters in length. Yeast cells were produced from sporangiospores which were germinated in YPG and grown to the mid-log phase by shaking at 28°C under an atmosphere of CO_2 (0.5 volume of sterile CO_2 per volume of culture per min). Samples of yeast cells were induced to undergo morphogenesis by transferring them to an atmosphere of air and continuing to shake the culture for 3 h. At this time nearly 80% of the cells had grown germ tubes. Cells were harvested by filtration and frozen immediately in liquid nitrogen, or the nucleic acids were extracted immediately as described below.

Bacterial strains and plasmids. *E. coli* JM83 (17) was the host strain for plasmids pUC8 and pUC9 (31), which were used in subcloning DNA restriction fragments from the original EF-1α clones in phage lambda described in an earlier study (12). Plasmid DNA was grown and purified by published procedures (3, 7a, 14). Phage M13mp10 and M13mp11 (18) were propagated, and replicative-form and single-stranded DNAs were purified as recommended by Bethesda Research Laboratories, Inc., Gaithersburg, Md. (2).

Restriction endonuclease analysis and agarose gel electrophoresis. Restriction endonuclease digestions of DNA were performed according to the manufacturer's instructions. DNA fragments were resolved by electrophoresis through 0.8 to 2% agarose gels 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). Samples of RNA were separated on denaturing formaldehyde-agarose gels by the procedure of Maniatis et al. (15).

Southern and Northern analyses of nucleic acids. DNA resolved by electrophoresis through agarose gels was further analyzed by transfer to nitrocellulose filters by the procedure of Southern (24). 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 for 24 h, and then the nitrocellulose filter was baked at 80°C under vacuum for 2 h before hybridization. RNA for Northern analysis was transferred to nitrocellulose filters by the procedure of Thomas (25). Samples of 20 μg of total *Mucor* RNA were analyzed per gel lane. RNA samples to be compared for EF-1α transcript were resolved on the same gel and transferred to a nitrocellulose filter at the same time.

DNA probes for hybridization were prepared from agarose gel-purified restriction fragments (15, 32). The DNA was nick translated to high specific activity (>10^8 cpm/μg of DNA) by incorporation of [α-^{32}P]dGTP as described by Maniatis et al. (15).

Nitrocellulose filter hybridization conditions were essentially those of Maniatis et al. (15). Nitrocellulose filters were soaked for 3 h in prehybridization solution (50% deionized formamide, 5× Denhardt solution [1× Denhardt solution is 0.1% Ficoll–0.1% bovine serum albumin–0.1% 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, and 100 μg of denaturated salmon testis DNA per 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 submerged in a shaking water bath. The filters were washed twice for 15 min each 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 at −70°C with a Cronex Lightning-Plus intensifier screen for 4 to 24 h.

Synthetic oligonucleotide probes. Gene-specific 19-base oligonucleotide probes were prepared by Louise Schmidt of the University of California at San Diego. The oligonucleotides were purified by electrophoresis on a denaturing 20% polyacrylamide gel. The proper DNA band was identified and cut from the gel, and the nucleic acid was electroeluted with an apparatus from International Biotechnologies Inc. The DNA was dialyzed against 0.1× TE buffer (1× TE is 10 mM Tris hydrochloride–0.1 mM EDTA, pH 8.0) and quantified by the absorbance at 260 nm. A 50-ng sample of purified oligonucleotide was end labeled with [γ-^{32}P]ATP and polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). To make sure that probes had the same specific activity, 2 μl of each sample was resolved on a denaturing 20% polyacrylamide gel. After electrophoresis, the gels were wrapped in Saran Wrap and exposed to Kodak XAR-5 film. The bands on the autoradiograph were quantified by scanning with a laser densitometer (UltroScan, XL; LKB Instruments, Inc., Rockville, Md.).

Nitrocellulose filters for Northern hybridization to these oligonucleotide probes were prepared as above. Prehybridization was carried out at 37°C in a solution of 6× SSC, 0.05 mM sodium pyrophosphate, 100 μg of heat-denatured salmon testis DNA per ml, and 1× Denhardt solution. Hybridization was carried out for 16 to 24 h under the same conditions, except that 100 μg of yeast tRNA per ml was included (instead of the salmon testis DNA) and 2 × 10^7 cpm of each end-labeled oligonucleotide probe (same specific activity) was added to separate filters with RNA samples which had been run in triplicate. Filters were washed three times for 20 min in 6× SCC–0.05% sodium pyrophosphate at room temperature and then for 15 min at 37°C in the same buffer. Filters were exposed to Kodak XAR-5 film for 4 to 24 h at −70°C with an intensifying screen. Bands on autoradiographs were quantified with an LKB laser densitometer. Quantification was nearly linear for the exposure times used in the experiment. All analyses were performed multiple times to control for any variability of RNA transfer during passive transfer. Under these conditions, band intensities on autoradiographs reflected the amount of *TEF* mRNA present in each sample. The results shown are representative of the overall results obtained.

DNA sequencing. The nucleotide sequence of restriction fragments isolated from *TEF-1*, *-2*, and *-3* EF-1α clones was determined by the dideoxy chain termination procedure of Sanger and Coulson (22).

Purification of RNA from *M. racemosus* cells. RNA for S1 nuclease analysis of the EF-1α genes was purified from *M. racemosus* cells by the hot phenol procedure of Maramatsu (16) as modified by Horst Domdey (9; personal communication).

S1 nuclease analysis of RNA. Total RNA (50 μg) was mixed with cloned *M. racemosus* DNA, and the nucleic acids were coprecipitated with ethanol. The nucleic acid pellet was dissolved in 0.03 ml of S1 hybridization buffer [80% deionized formamide, 40 mM 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 mixes were diluted 1:10 with ice-cold nuclease S1 buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.6], and 4.5 mM $ZnSO_4$), and 1,000 U of S1 nuclease (Boehringer) per ml was added. The

tubes were incubated at 37°C for 30 min followed by 15 min at 4°C for alkaline S1 analysis or at room temperature and then 15 min at 4°C for neutral analysis. 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 alkaline agarose gels as described by Maniatis et al. (15) or on neutral agarose gels as described above.

Computer analysis of EF-1α nucleotide sequence. The analysis of the nucleotide sequences presented here was carried out on an IBM personal computer with programs written by Brian Ward and George Gutman, Department of Microbiology and Molecular Genetics, University of California, Irvine.

Enzymes and chemicals. The enzymes used in this analysis were purchased from Bethesda Research Laboratories, New England BioLabs, Inc., or Boehringer Mannheim Biochemicals unless specified otherwise in the text. All other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Mallinckrodt, Inc. (St. Louis, Mo.).

RESULTS

S1 nuclease analysis of *TEF-1*, *-2* and *-3*. In a previous study DNA restriction fragments carrying the cloned genes for EF-1α, *TEF-1*, *-2*, and *-3*, were subjected to neutral S1 nuclease analysis (12). The data from this experiment suggested that there was a region of heterogeneity at the 3′ end of the EF-1α transcripts. To confirm the presence of this region of heterogeneity and to gain further insight into the genetic organization and expression of the *TEF* genes, we have continued our S1 nuclease mapping of the EF-1α transcripts.

The DNA restriction fragments used in this analysis are shown in Fig. 1. *Bam*HI restriction fragments carrying the entire *TEF-1*, *-2*, and *-3* genes were subjected to alkaline S1 nuclease analysis to mark the numbers and positions of intervening sequences in the *TEF* genes (Fig. 2). In alkaline S1 analysis the RNA transcript is hydrolyzed by alkali before electrophoresis. This results in single-stranded DNA fragments corresponding in size to the exons in a particular gene. The presence of three protected DNA fragments of approximately 1,160, 320, and 240 base pairs (bp) in each preparation (the EF-1α transcript is ~1,500 bp long) indicated that at least one intron was present in each of the three genes. From alkaline S1 analysis and nucleotide sequence analysis of *TEF-1* performed previously (13), we knew that this gene contained one intron located 240 bp upstream from the protein synthesis termination codon. The sizes of the protected DNA fragments (Fig. 2) suggested that the other *TEF* genes contained an intervening sequence at the same location. The 320-bp protected fragment resulted from a region of transcript heterogeneity and is discussed in more detail below. A careful inspection of the size of the largest protected fragment in each lane (on a short-exposure autoradiograph) revealed that this fragment in *TEF-2* and *-3* was about 70 bp shorter than the protected fragment in *TEF-1*. This result could be explained by the presence of a second intron in these two genes or by a shorter mRNA transcript. This question prompted us to carry out a more detailed S1 analysis of the 5′ end of the genes.

*Eco*RI restriction fragments were isolated from the 5′ end of each *TEF* gene (Fig. 1) and subjected to neutral and alkaline S1 analyses. The presence of a protected fragment of 390 bp in neutral analysis (Fig. 3A) revealed that the initiation site of the EF-1α transcript in all three genes was in the same approximate position relative to the common 5′ *Eco*RI restriction site. In neutral S1 analysis a double-stranded RNA-DNA hybrid results which corresponds in

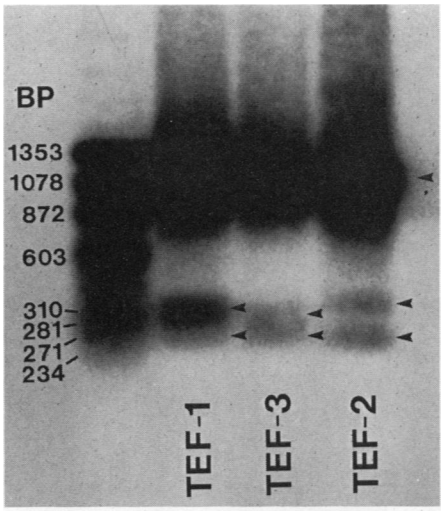

FIG. 2. Alkaline S1 nuclease analysis of *TEF-1*, *-2*, and *-3*. *Bam*HI restriction fragments carrying *TEF-1*, *-2*, and *-3* (Fig. 1) were hybridized to *M. racemosus* germling RNA under conditions which favor RNA-DNA hybrids. The protected DNA fragments were resolved on a 1.5% alkaline agarose gel and transferred to a nitrocellulose filter. A *Bam*HI-*Hin*dIII restriction fragment isolated from *TEF-1* (probe 1, Fig. 1) was used as a probe in standard Southern analysis. The arrows indicate the position of the protected DNA fragments in each preparation. DNA fragments of approximately 1,100 bp (topmost arrow), 320 to 340 bp (middle arrow), and 240 bp (bottom arrow) were protected by the EF-1α transcript. Molecular sizes were determined by a *Hae*III digest of φX174 replicative form, which generates restriction fragments ranging from 1,353 to 72 bp.

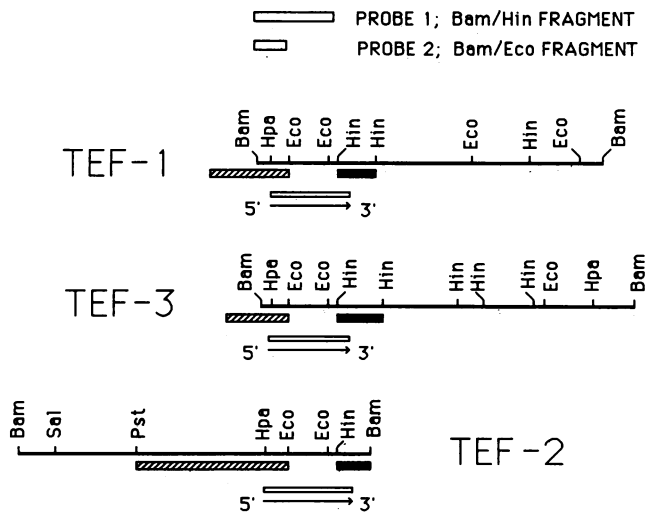

FIG. 1. Restriction fragments and DNA probes used in S1 nuclease mapping. Restriction endonuclease maps are shown for each of the genes encoding EF-1α, *TEF-1*, *-2*, and *-3* (12). Gel-purified DNA restriction fragments were used to analyze the 5′ end (▨) or 3′ end (■) of the EF-1α transcripts. Protected fragments were detected by Southern analysis with probes 1 and 2 (Fig. 2 and 3). Also shown are the approximate size (1,500 bp; □) and direction (→) of transcription in these genes. Abbreviations: Bam, *Bam*HI; Eco, *Eco*RI; *Hin*, *Hin*dIII; Hpa, *Hpa*I.

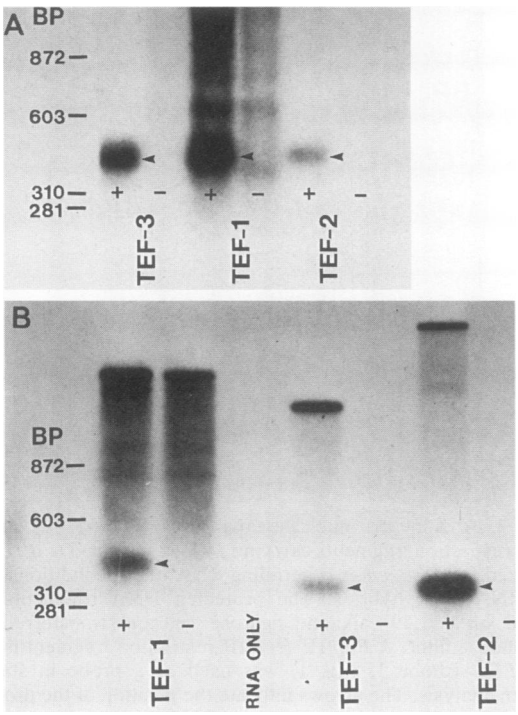

FIG. 3. S1 nuclease analysis of the 5′ end of *TEF-1*, *-2*, and *-3*. *Eco*RI or *Eco*RI-*Pst*I (*TEF-2*) restriction fragments were hybridized to *M. racemosus* germling RNA (Fig. 1). The resulting hybrids were treated with S1 nuclease, and the protected DNA fragments were resolved on 1.5% agarose gels run under neutral or alkaline conditions. DNA fragments protected from S1 nuclease by the EF-1α transcript were detected by standard Southern analysis with a *Bam*HI-*Eco*RI restriction fragment isolated from *TEF-1* (probe 2, Fig. 1) as the probe. (A) Neutral analysis. A single protected DNA fragment (arrows, approximately 390 bp) was detected only in lanes containing both *M. racemosus* RNA and DNA restriction fragments (+). Lanes marked (−) contained DNA only. (B) Alkaline analysis. A single protected fragment (arrows, approximately 390 bp in *TEF-1* and 310 bp in *TEF-2* and *-3*) was detected in this exposure of the autoradiograph (4 h). A much longer exposure (48 h) revealed the presence of a second fragment of 75 to 80 bp in the *TEF-2* and *-3* preparations (not shown).

size to the distance from the reference restriction endonuclease site (*Eco*RI) to the start or end of the transcript, depending on which end is being mapped. The presence of an intervening sequence will not affect this analysis, since only the single-strand DNA loop of the intron will be digested by nuclease S1, leaving the opposite RNA strand intact. The alkaline analysis (Fig. 3B) indicated that *TEF-2* and -3 contained a second intron located about 70 bp downstream from the initiation site of transcription since the protected fragments in *TEF-2* and *-3* were reduced in size from 390 to 320 bp. This places the position of the start site of transcription about 20 bp upstream from the initiation ATG codon for *TEF-1* and probably for *TEF-2* and -3 also, based on gene homology.

*Hin*dIII restriction fragments were isolated by gel purification from the 3′ end of the *TEF* genes to further characterize the region of transcript heterogeneity. Each of these restriction fragments was radioactively labeled with [α-^{32}P]GTP and the Klenow fragment of DNA polymerase I. These end-labeled fragments were subjected to neutral S1 analysis (Fig. 4). A protected fragment of 140 bp was found common to each of three preparations. A second protected fragment that varied in size from 225 to 250 bp was found in each preparation except in *TEF-3*, in which two larger protected fragments were seen (see below). Since the *Hin*dIII site is located 144 bp from the TAA ochre termination codon in *TEF-1* we concluded that the larger fragment of variable size contained the 80- to 110-bp region of transcript heterogeneity. The presence of a unique protected fragment with each gene also suggested, but did not prove, that all three genes were expressed in germlings of *M. racemosus*.

DNA sequence analysis, 3′ end, and 5′ end of the EF-1α genes. The *Hin*dIII restriction fragments from the previous experiment were cloned into the single-stranded phage M13 to determine the nucleotide sequence. A nucleotide sequence comparison of the 3′ end of the three genes is shown in Fig. 5. The nucleotide sequence within the coding portion of the three genes is highly conserved, with only a single base pair change seen in *TEF-2*. This base pair change results in no change in the amino acid sequence. Downstream from the ochre termination codon the sequence homology falls to less than 40%. Putative polyadenylation sequences (AATAAA) were found in each of the three genes between 65 and 90 bp from the ochre termination codon. Since polyadenylation of mRNA begins about 10 to 30 nucleotides beyond this poly(A) site (11), the length of the predicted trailer regions agrees very closely with our S1 nuclease analysis (Fig. 4). The origin of the second large protected fragment in the S1 analysis of the 3′ end of *TEF-3* is uncertain, since there is no second putative poly(A) site seen in the sequence for this gene.

We cloned restriction fragments from the 5′ end of each of

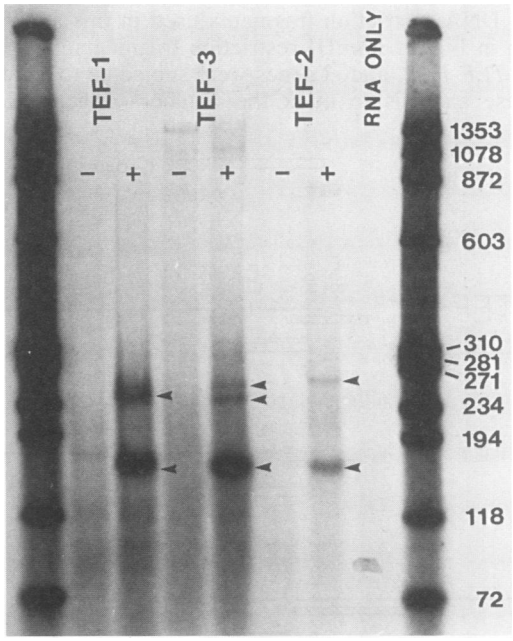

FIG. 4. S1 nuclease analysis of the 3′ end of *TEF-1*, *-2*, and *-3*. *Hin*dIII or *Hin*dIII-*Bam*HI (*TEF-2*) restriction fragments (Fig. 1) were radioactively labeled at the 3′ end with [α-^{32}P]dGTP and the Klenow fragment and subjected to S1 analysis. Protected DNA fragments (arrows) were resolved on a 5% polyacrylamide gel, which was dried and exposed directly to X-ray film. The size of the smallest DNA fragment in each preparation is approximately 144 bp. The largest fragment in each preparation is 230 bp for *TEF-1*, 250 bp for *TEF-2*, and 225 and 245 bp for the two large bands in *TEF-3*. The molecular size markers are the same as in Fig. 2.

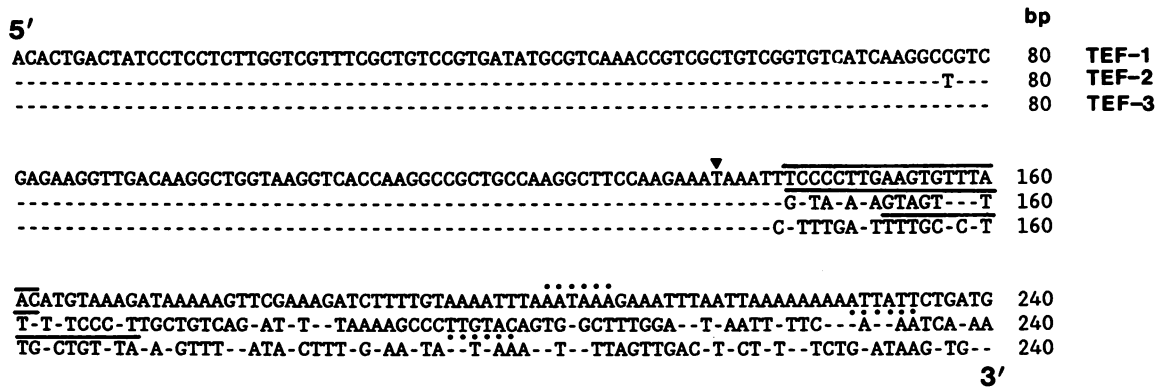

FIG. 5. DNA sequence comparison of the 3′ end of *TEF-1*, *-2*, and *-3*. The *Hin*dIII and *Hin*dIII-*Bam*HI restriction fragments in Fig. 4 were cloned into M13, and their nucleotide sequences were determined starting at the common *Hin*dIII site. The resulting sequences were aligned by a computer program written for the IBM personal computer by George Gutman and Brian Ward (unpublished). A dash in the place of a residue marks a position of exact homology to the sequence for *TEF-1*. A common ochre termination codon, TAA (▼), the position of the 19-base oligonucleotide probes (residues with a line directly above), and a putative polyadenylation site, AATAAA (residues with dots directly over them) are marked in each sequence.

the three *TEF* genes into M13 for DNA sequence analysis to confirm the presence of a second intervening sequence in *TEF-2* and *-3* and to identify any structural features which might play a role in the regulation of transcription for the genes. A representation of this analysis and the restriction fragments which were sequenced are shown in Fig. 6. The initiation site for transcription for each of the genes was S1 mapped at approximately 20 bp upstream from the ATG protein synthesis initiation codon in *TEF-1*. The nucleotide sequence in this 5′ flanking region shows little conservation among the three genes, except for the features shown in Fig. 6. In *TEF-1* and *-3* we found a conserved tetranucleotide tandemly repeated adjacent to (about 10 bp upstream) the proposed initiation site of transcription. The repeated elements are based on a TCCA motif and show a modestly conserved general spacing relative to the start site of transcription. It is possible that this sequence is part of the promoter element for these genes. Within the coding region of the three genes the nucleotide sequence showed a high degree of homology among the three genes. However, the sequences of *TEF-2* and *-3* did show 6- and 7-bp changes respectively, from *TEF-1* over this limited sequence. All of these changes were in the wobble position and did not result in a change in the amino acid sequence. After the first 18 amino acids of the EF-1α protein, an intervening sequence was found in *TEF-2* and *-3*, marked by putative splice

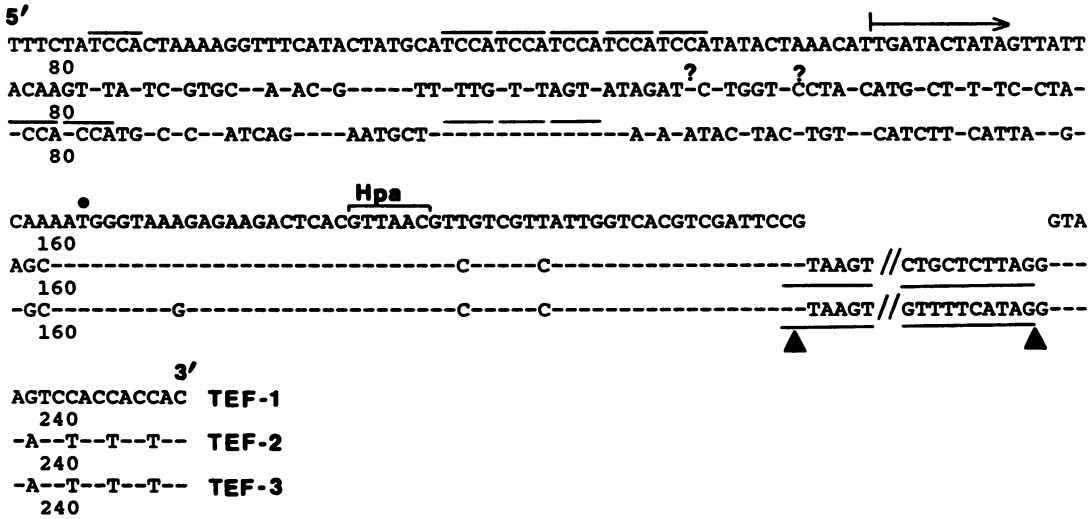

FIG. 6. DNA sequence analysis of the 5′ end of *TEF-1*, *-2*, and *-3*. DNA sequence data for *TEF-1* are taken from a previous study (13). *Hpa*I-*Bam*HI and *Hpa*I-*Eco*RI restriction fragments from *TEF-2* and *Hpa*I-*Bam*HI-*Eco*RI restriction fragments from *TEF-3* (Fig. 1) were cloned into phage M13 for sequencing by the dideoxy chain termination procedure (22). The homology comparison was carried out after first aligning the DNA sequences in the coding portion of the sequences. A dash in place of a letter represents a position of homology with the *TEF-1* sequence. The positions of a common *Hpa*I restriction site (Hpa), the ATG translation initiation codon (●), and the putative left and right splice junctions in *TEF-2* and *TEF-3* (underlined residues, ▲) are marked. Gaps (//) of 112 and 42 bp were introduced in the introns to allow maximum alignment of the three genes. The space left in the *TEF-1* sequence does not indicate an intervening sequence but was inserted to maximize alignment with *TEF-2* and *TEF-3*. The actual sequence is continuous in this region. Exact repeats of the tetranucleotide TCCA are shown with a line directly over them. In addition, the approximate initiation point and direction for transcription in each gene are shown by the arrow. The two question marks in the *TEF-2* sequence indicate positions of ambiguous nucleotide sequence.

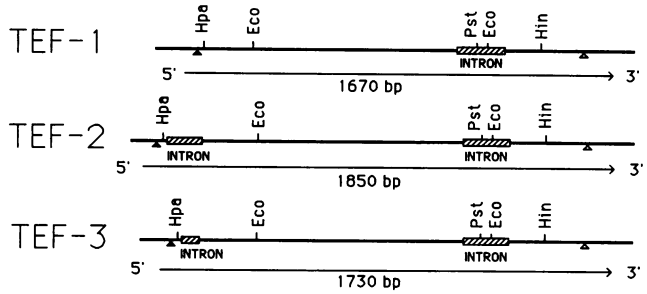

FIG. 7. Genetic organization of *TEF-1*, *-2*, and *-3*. Restriction endonuclease maps of *TEF-1*, *-2*, and *-3* were generated as part of a previous study (12). The positions of protein synthesis initiation (▲) and termination (△) codons and intervening sequences (▨) are marked for each gene. The direction and length of transcription are indicated by the arrows directly beneath the restriction maps. The length of the processed transcript in each gene is approximately 1,500 bp.

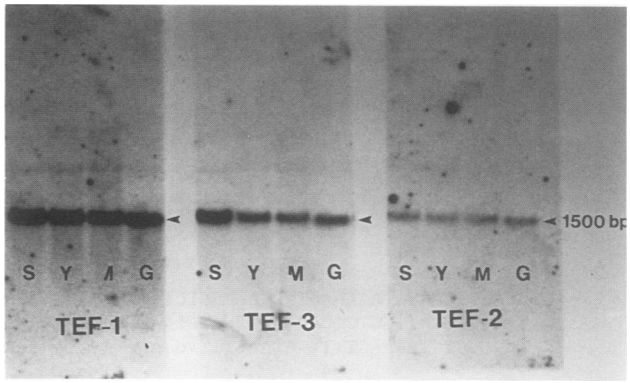

FIG. 8. Northern analysis of *M. racemosus* RNA with gene-specific oligonucleotide probes. Total RNA was purified from sporangiospores (S), yeast cells (Y), germlings (G), and yeast cells which were induced to undergo morphogenesis (M). The RNA was resolved on formaldehyde-agarose gels and analyzed by Northern blotting with end-labeled 19-base oligonucleotides as probes. The position of the EF-1α mRNA (approximately 1,500 bp) is indicated by the arrows.

junctions and the absence of an open reading frame. The position of this intron in the *TEF-2* and *-3* genes agrees closely with our predictions based on S1 analysis. The intron in *TEF-3* is 55 bp in length, whereas in *TEF-2* the intron is 128 bp long. It is interesting to note that a leftward splice junction exists at the same location in *TEF-1* where the intron is located in *TEF-2* and *-3*. Apparently this site is not recognized in the absence of a rightward splice junction, which is present in the other two genes. The nucleotide sequence in the introns of the 5′ end of the genes did not appear to be conserved.

The overall gene organization for *TEF-1*, *-2*, and *-3* as determined from these analyses is diagrammed in Fig. 7. *TEF-1* is the least complex of the genes, with a single intron located at about 320 bp from the 3′ end of the transcript. An intron is also present in *TEF-2* and *-3* at the same location and is approximately the same size (188 bp), as determined from the conservation of *Eco*RI and *Pst*I restriction sites within the intervening sequences and from the sizes of DNA fragments in restriction analysis (12). *TEF-2* and -3 in addition have a second intron located approximately 70 bp downstream from the 5′ end of the transcripts. The relative location of these introns is the same in *TEF-2* and -3, although the size and nucleotide sequence are not well conserved.

Northern analysis of *M. racemosus* RNA with gene-specific probes. Unique 19-base oligonucleotide probes were synthesized for each gene such that they would hybridize to the unique 3′ trailer region of RNA transcripts from the *TEF* genes. The position of these oligonucleotides is shown in Fig. 5 (underlined residues). The oligonucleotides were purified by gel electrophoresis and end labeled to high specific activity with [γ-^{32}P]ATP and polynucleotide kinase. The probes were used in standard Southern blot analysis of *Bam*HI restriction fragments containing *TEF-1*, *-2*, and *-3*. Each probe hybridized specifically to the gene fragment for which it was synthesized (data not shown). These gene-specific probes were used to analyze the accumulated levels of EF-1α mRNA from several morphological types of *M. racemosus* cells to determine whether there was a morphology-specific pattern of gene expression for EF-1α. RNA was purified from sporangiospores, germlings, yeasts, and yeast cells which had been induced to undergo morphogenesis to hyphae. The RNA was resolved on formaldehyde-agarose gels and analyzed by Northern analysis (Fig. 8). The actual densitometry value averaged for two separate autoradiographs are shown in Table 1; *TEF-1*, *-2*, and *-3* are transcribed in all morphological types tested. However, the accumulated level of transcript derived from each gene differs markedly. The level of *TEF-1* mRNA is up to 2.5 times as high as that of *TEF-3* and up to 5 to 6 times the level of *TEF-2*. The levels of *TEF-1* and *-2* mRNAs vary less than 30% when looking at the different cell morphologies. However, the level of *TEF-3*-specific mRNA varies more than twofold (decrease) when comparing sporangiospores to either germlings or cells undergoing morphogenesis. When an 850-bp *Eco*RI fragment, which is common to all three genes, was used as the probe there was less than a 40% change in the total EF-1α mRNA levels from the four morphological types (data not shown). These data suggest that there is differential expression of the three genes encoding EF-1α in *M. racemosus* and that at least one gene, *TEF-3*, shows a morphology-specific pattern of transcript accumulation.

DISCUSSION

Organization of the genes encoding EF-1α in *M. racemosus*. The three genes encoding EF-1α in *M. racemosus* are located at unique positions in the genome (12). The DNA rearrangements which lead to the formation of this relatively complex gene family are open to speculation. A logical sequence of events could entail a single predecessor gene for EF-1α containing two introns at the positions which they are now found. A tandem duplication event would lead to the generation of the predecessors to *TEF-2* and *-3*. *TEF-1* may

TABLE 1. Densitometry values at various stages of growth

Gene	Densitometry values[a]			
	Spore	Yeast	Cells undergoing morphogenesis	Germlings
TEF-1	4.94	5.22	3.66	5.05
TEF-2	1.05	1.01	1.31	1.17
TEF-3	4.48	2.73	2.02	2.17

[a] Values are given in absorbance units times the width of the bank in millimeters. The values are the averages of values obtained from scanning autoradiographs from two separate experiments.

have arisen by reverse transcription of an intermediate in transcript splicing from either *TEF-2* or *-3* or their common predecessor, followed by integration into the chromosome. This spliced intermediate could have lost the first intervening sequence but not the second. Partial DNA sequence analysis of coding sequences at the 5′ end of *TEF-2* and -3 indicates that these genes are more closely related to each other than to *TEF-1*, because their nucleotide sequences differ at only 1 bp, whereas there are 6- to 7-bp differences with *TEF-1*. These data would support the idea that the generation of the predecessor to *TEF-1* occurred before the tandem duplication event leading to modern *TEF-2* and *-3*. Nucleotide sequences in the 3′ and 5′ flanking regions of the three genes are not well conserved, except for the TCCA tandem repeats referred to in the results. There appears to be a correlation between the number of exact repeats of TCCA and the accumulated level of EF-1α transcript. The presence of this conserved sequence and the absence of Hogness box or CAAT box elements (11) adjacent to the initiation site of transcription (the neartest TATA element is located at −200 bp from the initiation site of transcription) lend credence to the notion that the TCCA repeat may be involved in the regulation of transcription of the EF-1α genes. Alternatively different levels of EF-1α transcript could arise from different mRNA stabilities. A search for a similar sequence in the *TEF* genes from *Saccharomyces cerevisiae* (6, 23) did not turn up any homology. However, the published sequence for *Artermia salina* EF-1α did reveal a sequence TCCATTCCA 22 bp upstream from the ATG initiation codon. The functional significance of this sequence is not clear at this time. A more complete study of the intervening sequences, splice junctions, codon usage, and amino acid sequence homology awaits completion of the nucleotide sequence of *TEF-2* and *-3*.

Expression and regulation of EF-1α and EF-Tu. The presence of multiple copies of the gene encoding EF-1α has been demonstrated in several organisms, although as yet the function of more than one gene is unknown (6, 23, 30). In *S. cerevisiae* either of the two genes encoding EF-1α, *TEF-1* or *-2*, can be inactivated with no apparent effect on growth of the organism (6, 23). Both genes are transcribed at high levels, yielding proteins which have identical primary structure (23). A similar case exists in the expression of EF-Tu in *E. coli* in which two genes exist, *tufA* and *tufB*, encoding this elongation factor (1, 33). The proteins resulting from these genes are nearly identical, with only a single amino acid difference at the carboxy terminus of the protein.

There is a large body of evidence to show that the expression of EF-1 in eucaryotes and EF-Tu in procaryotes and eucaryotic organelles is regulated at several levels. Several laboratories have demonstrated that EF-1α and EF-Tu proteins are posttranslationally modified either through methylation or phosphorylation (10, 26, 28, 29). In the case of EF-1α in rabbit reticulocytes, reversible autophosphorylation of the protein subunit enhances binding of tRNA to the ribosome (28). In *M. racemosus*, several lysine residues on the EF-1α subunit become methylated during sporangiospore germination (8). The level of methylation has been correlated to a large increase in the specific activity of the protein (3). The expression of the *tufB* gene in *E. coli* is subject to translational regulation (5). It has been proposed that EF-Tu is able to bind to a domain at the 3′ end of the *tufB* transcript, preventing its translation. Recently, development regulation of EF-1α at the level of transcription has been demonstrated in *A. salina*, the brine shrimp (7). In the present study we demonstrated that the accumulated mRNA level for each of the three genes encoding EF-1α in *M. racemosus* varies severalfold so that *TEF-1* > *TEF-3* > *TEF-2*. In addition, mRNA levels for *TEF-3* show a morphology-specific pattern of accumulation.

What is the selective advantage for cells which contain multiple genes encoding EF-1α and which are regulated at several levels during the course of cell growth? One possible explanation is suggested by research which demonstrates multiple functions for EF-Tu in *E. coli*. In addition to its function in protein synthesis, EF-Tu is an important element in the replicase for phage QB (4) and also has been implicated in the positive regulation of rRNA gene expression (27). Although it is unlikely that a single amino acid difference consigns a particular form of the protein to a specific task, in this instance it may be possible that subtle differences in the primary structure of elongation factor proteins make them subject to differential regulation at the posttranslational level. In the case of EF-1α in *M. racemosus*, one can speculate that subtle differences in the primary structure of proteins derived from *TEF-1*, *-2*, and *-3* could have a significant effect on the pattern of posttranslational methylation, leading to differences in either the specific activity of the protein or the recognition properties for groups of tRNAs. These posttranslational modifications could also alter EF-1α to an alternative functional role. Our task for future research is to demonstrate a direct link between the observed methylation of EF-1α and the large increase in the specific activity of the protein. In addition, we would like to determine the functional significance of differential transcription of the three genes encoding EF-1α.

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